Universidade de Aveiro Departamento de Biologia
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GISELA JOÃO RIBEIRO LEMOS DIONÍSIO

Effects of climate change on the physiology and photobiology of photosynthetic sea slugs

Os efeitos das alterações climáticas na fisiologia e fotobiologia das lesmas do mar fotossintéticas

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Ricardo Calado, Investigador Principal do Departamento de Biologia da Universidade de Aveiro, do Doutor Rui Rosa, Investigador Principal do Laboratório Marítimo da Guia da Faculdade de Ciências da Universidade de Lisboa e do Professor Doutor João Serôdio, Professor Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro.

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Prediction is very difficult, especially about the future. **Niels Bohr**

o júri

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Doutor Amadeu Mortágua Velho da Maia Soares
Professor Catedrático do Departamento de Biologia da Universidade de Aveiro

Doutor Mário Emanuel Campos de Sousa Diniz
Professor Auxiliar do Departamento de Química da Faculdade Ciências e Tecnologia, Universidade Nova de Lisboa

Doutor Ricardo Jorge Guerra Calado (Orientador)
Investigador Principal do CESAM-Centro de Estudos do Ambiente e do Mar da Universidade de Aveiro

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Esta tese é dedicada às novas gerações de biólogos. Espero que consigam manter um espírito naturalista numa sociedade e oceanos em alteração.

palavras chave Lesma-do-mar, alterações climáticas dos oceanos, simbiose, cleptoplastia, desenvolvimento ontogenético, cloroplasto, foto-simbiose, metabolismo, stress-oxidativo.

resumo

A vulnerabilidade das simbioses fotossintéticas marinhas face às alterações climáticas tem recebido particular atenção nos últimos anos. Porém, enquanto existe um número crescente de estudos para as espécies emblemáticas, como os corais, pouco se sabe acerca dos grupos menos carismáticos como as lesmas do mar "movidas a energia solar". Estes organismos possuem uma das particularidades mais intrigantes do reino animal: uma associação molusco-plasto, que resulta da sua capacidade de reter cloroplastos fotossinteticamente ativos (cleptoplastos) "roubados" às algas de que se alimentam. Dada a sua biologia peculiar, as lesmas do mar destacaram-se nos últimos anos como organismo "ferramenta" na investigação da fotobiologia, modelo nos estudos biomédicos e de bioprospeção de novos compostos marinhos, tornando-se ainda pretendidas para o comercio da aquariofilia marinha.

Por forma a apresentar uma visão global acerca do conhecimento destes organismos fascinantes e estabelecer critérios para a investigação sobre as alterações climáticas, as características biológicas e ecológicas da associação molusco-plasto foram revistas e ainda identificadas as condições ótimas de cultivo para diferentes fases do seu ciclo de vida.

O impacto da acidificação e o aquecimento dos oceanos foi avaliado nos estágios iniciais do desenvolvimento e nos adultos da lesma do mar temperada (*Elysia viridis*) e na tropical (*Elysia clarki*). Neste contexto, novas abordagens metodológicas foram desenvolvidas por forma a aceder de forma não invasiva à foto-fisiologia dos cleptoplastos de acordo com as futuras condições do oceano.

Os resultados mostraram que a acidificação e o aquecimento do oceano podem influenciar as características biológicas das lesmas do mar "movidas a energia solar", incluindo a sobrevivência, sucesso reprodutivo, crescimento, incidência de deformações, eficiência fotossintética dos cleptoplastos, metabolismo, e as respostas contra o choque térmico e de ação antioxidante.

Contudo, a tolerância das lesmas do mar às condições futuras do oceano revelou ser especifica de cada espécie. A lesma do mar temperada *E. viridis,* apesar da baixa sobrevivência, apresentou mecanismos eficientes contra o choque térmico, defesa antioxidante e elevadas taxas de fotossíntese e respiração quando exposta à acidificação e ao aquecimento, sugerindo um complexo molusco-cleptoplasto mais tolerante e capacidade em lidar contra os cenários futuros. Por outro lado, a lesma do mar tropical *E. clarki* mostrou ser vulnerável às condições futuras do oceano. A reduzida capacidade e ausência de mecanismos para lidar com o *stress* ambiental pode, em parte, explicar a depressão metabólica do holobionte e a reduzida eficiência fotossintética dos cleptoplastos, que levaram ao branqueamento das lesmas do mar e a reduzida sobrevivência.

Este trabalho é o primeiro a reportar a ocorrência de branqueamento em circunstâncias de alterações climáticas noutras simbioses marinhas que não as associações cnidário-dinoflagelado.

Estes resultados têm amplas implicações e podem ajudar a antecipar os possíveis impactos negativos no recrutamento das lesmas do mar "movidas a energia solar" nos oceanos de amanhã. Contudo, é importante notar que as lesmas do mar "movidas a energia solar" poderão ter tempo e oportunidades evolutivas de adaptação às condições do futuro oceano.

Keywords

Sea slug, ocean climate change, symbiosis, kleptoplasty, ontogenetic develpment, chloroplast, photosymbiosis, metabolism, oxidative stress.

Abstract

The vulnerability of marine photosynthetic symbioses to climate-driven changes has deserved particular attention in recent years. However, while there is an increasing number of studies on emblematic species such as symbiotic corals, little is known about less charismatic groups such as solar-powered sea slugs. These organisms display one of the most puzzling features observed in the animal kingdom: the mollusc-plastid association, which results from their ability to retain photosynthetically active chloroplasts (kleptoplasts) “stolen” from their algal food sources. Given their peculiar biology, sea slugs have stood out as tool organisms for academic research on photobiology, biomedical studies and bioprospecting of new marine drugs, becoming also desired critters in the marine aquarium trade. In order to provide an overview of state-of-the-art on our knowledge on these fascinating organisms, and lay down the foundations for climate change research, the biological and ecological features of the mollusc-plastid association were reviewed and optimal culture conditions for their different life stages were identified. The impact of ocean acidification and warming was evaluated on early stages and adults of temperate (*Elysia viridis*) and tropical (*Elysia clarki*) sea slugs. In this context, new methodological approaches were developed to non-invasively assess the photophysiology of kleptoplasts under future ocean conditions. Our results have shown that acidification and warming may impact several biological features of solar-powered sea slugs, including survival, reproductive success, growth, incidence of deformities, kleptoplasts photosynthetic efficiency, metabolism, heat shock and antioxidant responses. However, sea slug tolerance to future ocean conditions was shown to be species-specific. The temperate sea slug *E. viridis*, in spite of their low survival, presented efficient heat shock and antioxidant defence mechanisms and high rates of photosynthesis and respiration when exposed to acidification and warming, suggesting the existence of a more tolerant mollusc-kleptoplast complex and capacity to cope with future scenarios. In contrast, the tropical sea slug *E. clarki* showed to be quite vulnerable to future ocean conditions. The reduced capacity or lack of mechanisms to deal with environmental stress may, in part, explain the metabolic depression of the holobiont and the reduced photosynthetic efficiency of kleptoplasts, leading to bleaching and a lower survival. This work is the first reporting the occurrence of bleaching under climate change in other photosynthetic symbiosis than the cnidarian-dinoflagellate association. These results have broad implications and may help us to anticipate potential negative impacts on the recruitment of solar-powered sea slugs in the oceans of tomorrow. However, it is worth noting that solar-powered sea slugs may have time and evolutionary opportunities to adapt to future ocean conditions.

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Chapter 1 Introduction

1.1 General Introduction

1.1.1 Global changes

Human activities are having profound and diverse consequences for marine ecosystems. The accumulation of carbon dioxide (CO_2) in the atmosphere rose dramatically during the 20th century (Fig. 1.1A). Since the industrial revolution fossil fuel combustion and industrial processes have released tons of carbon into the atmosphere and, at present, this value surpasses six billion metric tons per year (IPCC, 2013). As a consequence, atmospheric CO_2 concentrations have greatly increased from 280 µatm at pre-industrial levels to 398 µatm (IPCC, 2013), at a rate of ~1% to 3.4% per year (Le Quéré *et al*., 2009). Climate experts predict that future levels may reach 1000 µatm by the end of the century (Caldeira and Wickett 2003; IPCC, 2013) if anthropogenic emissions remain within the same rates. The impact of climate change is one of the most significant environmental challenges facing the world today and is primarily driven by the increase in CO_2 concentration (IPCC 2013). As a result of enhanced greenhouse effects, global sea surface temperature (SST) has risen 0.09-0.13 °C (per decade) between 1971–2010 (Rhein *et al.*, 2013). Assessed projections have estimated global average surface temperature to increase by an additional 1.1-6.4 °C and that the frequency of heat waves will also increase and become more extreme by the end of the century (Fig. 1.1B) (IPCC, 2013). There is an overwhelming scientific consensus that there will be a global warming during the current century and in the years to come (IPCC, 2013, 2014).

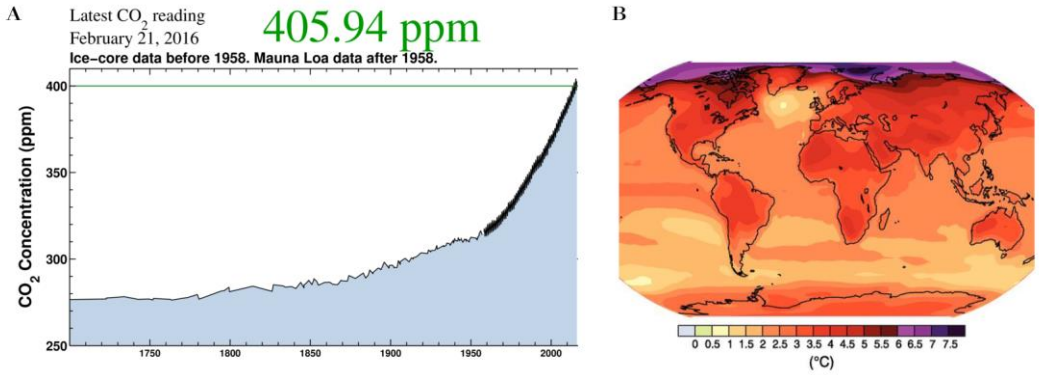

Fig. 1.1 A) Carbon dioxide concentration levels since 1700 until February 21st 2016 (Graph from NOAA and data from Mauna Loa Observatory); and B) Projected surface temperature changes for the late 21st century (Figure SPM.6 from IPCC 2007 report); temperatures are relative to the period 1980-1999).

1.1.2 Oceans

The oceans play a key role in the mitigation of climatic changes, sequestering heat, and carbon from the atmosphere. In the last decades, oceans stored more than 90% of the atmosphere heat content (IPPC, 2013). However, the heat-uptake by the oceans may decrease over time, allowing the atmosphere to warm-up.

Fossil fuel use, cement manufacture, and land-use changes are the primary sources of anthropogenic CO_2 to the atmosphere, with the oceans absorbing from 20 to 30 % of all anthropogenic CO_2 emissions (Sabine *et al*., 2004; Bates *et al*., 2012). Additionally, the continuous uptake of CO_2 by the oceans will unequivocally change seawater chemistry. When combined with seawater, CO_2 reacts and forms carbonic acid (H_2CO_3), which dissociates to form bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) ions and releases hydrogen ions (H^+). The increase of H^+ levels will raise the partial pressure of CO_2 (P_{CO_2}), a process known as hypercapnia and will thereby reduce oceans pH (Caldeira & Wickett, 2003). Moreover, CO_3^{2-} concentration and saturation states of calcium carbonate (CaCO3), aragonite (Ωar) and calcite (Ωca) will decrease on ocean surface waters during this process (Feely *et al*., 2008).

Since pre-industrial times, ocean's pH has already dropped an average of 0.1 units (Meehl *et al*., 2007), representing a 30% increase in H^+ ions (Caldeira and Wickett, 2003). Additionally, P_{CO_2} at the surface ocean is increasing in line with rising atmospheric CO_2 (Doney, 2010). Based on current and projected future CO_2

emissions, ocean pH could may drop a further 0.4–0.5 units (Caldeira and Wickett 2005) (Fig. 1.2) and P_{CO_2} levels may exceed 900 µatms by the end of this century (Meinshausen *et al*., 2011). Although global warming is recognized to affect the ecological structure and functioning of marine ecosystems, the consequences of ocean acidification for these ecosystems are only starting to be unravelled.

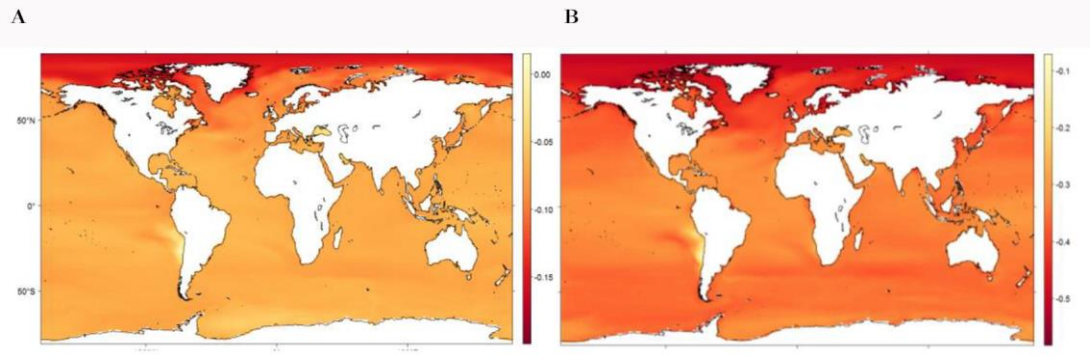

Fig. 1.2 A) Changes in ocean surface pH (1976-2005 to 2071-2100) for the IPCC ARS, RCP 2.6 scenario; B) RCP 8.5 scenario, graphs are a courtesy from Joana Boavida-Portugal.

1.2 Climate change effects on marine ecosystems

Marine ecosystems are maintained by the flow of energy from primary producers at the base of food webs through intermediate consumers and top predators and then back again through decomposition and detrital pathways (Doney *et al*., 2012). The success of these biological networks depends on the success of the species and directly or indirectly through various biological interactions (e.g., competition, mutualism, symbiosis) (Doney *et al*., 2012). Direct effects of changes in ocean temperature and chemistry may alter the physiological functioning, behaviour and demographic traits of organisms, leading to shifts in the size structure, spatial range, and seasonal abundance of populations (Brierley & Kingsford, 2009). These shifts, in turn, can modify species interactions and trophic pathways, with climate signals thereby propagating over ecosystems through both bottom-up and top-down processes (Weatherdon *et al.*, 2016). Changes in community structure and ecosystems function may result from disruptions in biological interactions. Consequently, investigating the responses of individual species to single forcing

factors, although essential, provides an incomplete story and highlights the need for more comprehensive, multifactor and multispecies level analyses (Doney *et al.*, 2012).

1.2.1 Ocean acidification

Predicted levels of ocean acidification have already revealed to negatively influence a diversity of marine organisms and ecosystems, from deep sea to coastal areas (Fabry *et al.*, 2008, Feely *et al.*, 2010, Duarte *et al*., 2013). There is an urgent need to better understand how projected changes in carbonate chemistry will affect marine species, communities, and ecosystems (Gattuso & Hansson, 2011, Kroeker *et al.*, 2013b). The rapidly growing body of literature and experimental research on the biological impacts of ocean acidification reveals a broad range of species responses (Kroeker *et al.*, 2010). By lowering carbonate ion levels and increasing carbonate solubility, oceans could experience major impacts on biogenic habitats (e.g., coral reefs), food webs (e.g. pteropods and other molluscs), and geochemical cycles (e.g., pelagic coccolithophore algae) (Doney *et al.*, 2009, Kroeker *et al.*, 2013b). For calcifying organisms (e.g., corals, bivalves, crustaceans), changes in water chemistry poses a major threat, as these organisms combine calcium with carbonate ions from surrounding seawater to produce their shells and skeletons, and a decline in carbonate may directly impact the ability of these organisms to produce biogenic carbonate (Anthony *et al.*, 2008, Byrne *et al.*, 2011). Additionally, increased CO_2 levels may be responsible for deleterious effects on survival, growth, development, behaviour, reproduction, metabolism and physiology of several marine species (e.g., molluscs, corals and fishes) (Byrne *et al.*, 2011, Fabry *et al.*, 2008, Gazeau *et al.*, 2010, Gazeau *et al.*, 2011, Lischka *et al.*, 2011, Melzner *et al.*, 2009, Munday *et al.*, 2012, Ou M *et al.*, 2015, Rosa *et al.*, 2014c, Widdicombe & Spicer, 2008). For carbon-limited autotrophs (comprising seagrasses and some phytoplankton species), increased CO_2 promotes photosynthesis, whereas for others (e.g., calcifying taxa), photosynthesis may be either reduced or not impacted (Dupont *et al.*, 2012, Koch *et al.*, 2013).

The combined effects of acidification and other environmental factors, such as warming, may further drive organisms outside their physiological tolerance boundaries, compromising their overall fitness and survival. Recently, several authors have focused on the effect of these synergies (Byrne & Przeslawski, 2013, Flynn *et al.*, 2015, Gibson *et al.*, 2011, Lischka & Riebesell, 2012, Pimentel *et al.*, 2014, Przeslawski *et al.*, 2005) and those studies (that combined field and laboratory experiments) demonstrated that the adverse effects of global warming is sometimes exacerbated when high-temperature scenarios overlap with acidification (Byrne & Przeslawski, 2013, Kroeker *et al.*, 2013b, Rodolfo-Metalpa *et al.*, 2011).

1.2.2 Global warming

Climate changes due to global warming have been long recognized to exert both direct and indirect biological consequences on marine ecosystems. Marine biota respond to warming through shifts in their distribution and abundance (Alabia *et al.*, 2015, Hiddink *et al.*, 2015, Rutterford *et al.*, 2015), phenology (Adrian *et al.*, 2006, Edwards & Richardson, 2004) and body size (Cheung *et al.*, 2013), which overall impact the community structure (Dulvy *et al.*, 2008, Meerhoff *et al.*, 2007) and interactions (Harley, 2011, Rosa *et al.*, 2014b, Sampayo *et al.*, 2008). The extent of its impact is extremely variable and depends on the species thermal range and development stage (Dionísio *et al.*, 2016). Thermal stress imposed by future ocean warming is expected to especially favor those organisms that do not live close to their thermal limits and are consequently more capable to tolerate temperature changes (Calosi *et al.*, 2013, Donelson *et al.*, 2011, Helmuth *et al.*, 2006, Leal, 2014, Pecorino *et al.*, 2013, Stillman & Somero, 2000). Species living in more thermally variable environments, such as those found in back reefs, reef flats or temperate intertidal zones, are often found to be more resistant to temperature stress compared to species from thermally more stable environments (Oliver & Palumbi, 2011, Palumbi *et al.*, 2014, Rosa *et al.*, 2014b, Somero, 2010, Stillman & Somero, 2000).

Recent findings suggest that transgenerational acclimation that involves phenotypic plasticity (e.g. responses in physiology, morphology or behaviour) can modify the impact of climate change and allow populations to persist across their current thermal range (Guillaume *et al.*, 2015, Munday *et al.*, 2013, Muñoz *et al.*, 2015, Sunday *et al.*, 2014). Many of these processes are optimal within a narrow thermal tolerance window, which contributes to established performance levels (Pörtner, 2010). The limits at which organisms performance start to diminish are known as *pejus* temperatures. Within these limits, organisms are still capable to perform compensatory mechanisms (Pörtner, 2008). However, when temperatures drive organisms outside their thermal optimum, their aerobic scope and performance might be reduced and constrained by a limited capacity of oxygen supply mechanisms (Pörtner *et al.*, 2006). If this critical threshold is approached or exceeded ("critical temperatures") several biological functions, such as growth, behaviour, feeding, reproduction, biochemical processes, and metabolisms, are negatively affected (Pörtner & Farrell, 2008). The drop of the aerobic scope will lead to an anaerobic mode of energy production, a compensatory mechanism that only supports survival during short periods (Pörtner & Farrell, 2008). Beyond this point, warming may lead to a loss of integrity of molecular structures, which progressively activates antioxidant defence (e.g. glutathione-S-transferase, GST) and heat-shock response (e.g. heat shock protein, HSP) that can contribute to extend the period of passive tolerance to thermal stress (Lesser, 2006, Pörtner, 2010).

1.2.3 Photosynthetic symbiosis under climate change

It has long been known that photosynthesis takes place in chlorophyll-containing plants, algae, and some bacteria. Photosynthesis serves to provide oxygen and energy in the form of biomass to support heterotrophic life. Some animals within the Cnidaria (Hexa- and Octocorallia, some Scyphozoa such as *Cassiopea*), Mollusca: e.g., Bivalvia of genera *Tridacna* and *Hippopus*; Nudibranchia: *Melibe* spp. (Burghardt *et al.*, 2008, Yellowlees *et al.*, 2008); Acoelomorpha (Burghardt *et al.*, 2008, Burghardt & Wägele, 2014, Kempf, 1984, Kempf, 1991); Porifera

(Barneah *et al.*, 2007, Muscatine *et al.*, 1974) and Foraminifera (Steindler *et al.*, 2002) have evolved mechanisms to capture photosynthetic products through symbiotic associations with intact unicellular algae or cyanobacteria. In these cases, the photobiont (algae or cyanobacteria) acts as an autonomous photosynthetic factory, providing reduced carbon as a source of energy to the heterotroph, often receiving nutrients in return (Yellowlees *et al.*, 2008).

The ability of animals to acquire photobionts appears limited to aquatic environments and to the few phyla mentioned above. The morphology of these multicellular organisms is an important factor leading to such associations. Photosynthetic animals often exhibit simple body plans coupled with a large body surface:volume ratio to host the symbionts (Venn et al., 2008). Adaptive modifications are seen in morphologically advanced molluscs (bivalves, nudibranchs, and sacoglossans) (Rumpho *et al.*, 2011b). Symbionts are restricted to regions of the body cavity that can enable them to efficiently capture light. Frequently, morphological adaptations by the host facilitate symbiont light capture, a trade-off that appears to compensate algal symbionts for their loss of motility (Trench *et al.*, 1973).

Photosynthetic animals were generally assumed to be more vulnerable to future ocean conditions than other marine organisms (e.g. fishes) (Przeslawski *et al*., 2008). Impacts of climate change have been shown in a variety of organisms living in association with photosynthetic organisms (Anthony *et al.*, 2008, Reusch, 2014, Rodolfo-Metalpa *et al.*, 2011). Photosynthetic symbiosis are regulated by intrinsic (e.g. signalling molecules) and extrinsic parameters (e.g. environmental stress) and can break down under certain conditions of light, increased P_{CO_2} and temperature (Weiss, 2008, Brownlee, 2009, Fitt *et al.*, 2001, Hoogenboom *et al.*, 2012, Muller-Parker *et al.*, 2015).

Under increasing P_{CO_2} and temperature, the association between animals and photosynthetic symbionts was shown to break down, a phenomenon known as bleaching. Bleaching, defined as the loss or degradation of microalgae or their associated pigments, has been documented in several photosynthetic symbiotic associations, such as those feature by corals (Lowe *et al.*, 2016, Plass-Johnson *et*

al., 2015, Schmidt, 2015, Yellowlees *et al.*, 2008), sea anemones (Anthony *et al.*, 2008, Carpenter *et al.*, 2008, Fitt *et al.*, 2001, Hoegh-Guldberg, 1999, Sampayo *et al.*, 2008, Wild *et al.*, 2011), sponges (Dunn, 2002, Dunn *et al.*, 2004, Perez *et al.*, 2001), giant clams (Addessi, 2008), Foraminifera (Fromont & Garson, 1999, López-Legentil *et al.*, 2008, McMurray *et al.*, 2011) and recently also in nudibranchs (Schmidt *et al.*, 2014, Sinutok *et al.*, 2014). The photosynthetic efficiency, survival and growth of dinoflagellate photosynthetic symbionts was also shown to be impacted by climate shifts (Watson, 2015, Watson *et al.*, 2012). Nevertheless, some photosymbiotic organisms have displayed a high resistance to future climate change, including corals harbouring dinoflagellates (Palumbi *et al.*, 2014) and acoel worms harbouring green microalgae (Dupont *et al.*, 2012). Genetic diversity in hosts and symbionts leads to a diversity of responses to mild temperature increases and pH, but severe temperature and pH anomalies almost always lead to widespread bleaching and death (Doney *et al.*, 2012, Hume *et al*., 2016). As an example, reefs are likely to become dominated in the future by symbiotic associations with warm/pH-tolerant zooxanthellae, allowing some corals to survive moderate temperature and P_{CO_2} increases (Doney *et al.*, 2012).

Most research on biological climate-related impacts has been conducted on multicellular organisms and their symbiotic relationship with single-celled dinoflagellates (*Symbiodinium*), and until now little information is available for other organisms/taxa that might be less charismatic, such as metazoans harbouring chloroplasts (see section 1.2.3.1 Kleptoplasty and sacoglossan sea slugs). This is unfortunate, as the comparison of different symbiotic associations, including the more vulnerable and more tolerant physiotypes, could provide us with those physiological traits that are crucial for ecological success in the oceans of tomorrow (Melzner *et al.*, 2009).

1.2.3.1. Kleptoplasty and sacoglossan sea slugs

A rare adaptation that rests somewhere between endosymbiosis and predation is the retention of functional chloroplasts within the bodies of some metazoans (Händeler *et al.*, 2009), known as kleptoplasty. This phenomenon is documented in several marine protists including foraminifers (Lee, 2006), dinoflagellates (Gast

et al., 2007) and ciliates (Johnson *et al.*, 2007). However, chloroplast retention in the Metazoa is known only in the gastropod taxon Sacoglossa, a group of sea slugs that feeds mainly on green algae (Clark *et al.*, 1990, Curtis *et al.*, 2005, Händeler & Wägele, 2007, Jensen, 1997).

Sea slugs that contained chlorophyll-pigmented granules similar to those of plants were described one hundred and forty years ago (de Negri & de Negri, 1876). While we now understand that these “green granules” are plastids that slugs sequester from algae upon which they feed, little is known about the mechanisms of interaction between the foreign organelle and its host animal cell (de Vries *et al.*, 2014b).

Sacoglossan sea slugs are widespread, from tropical to temperate waters. They are specialized gastropod molluscs with economic and ecological value (Dionísio *et al.*, 2013). Some species (e.g. from genus *Elysia* spp., Fig. 1.3) feed by slicing or puncturing siphonaceous algal cells and sucking out its contents. Most cell contents, including the algal nucleus, are digested, whereas chloroplasts are retained as functional organelles within the cells of their new host – the sea slug (Rumpho et al., 2000).

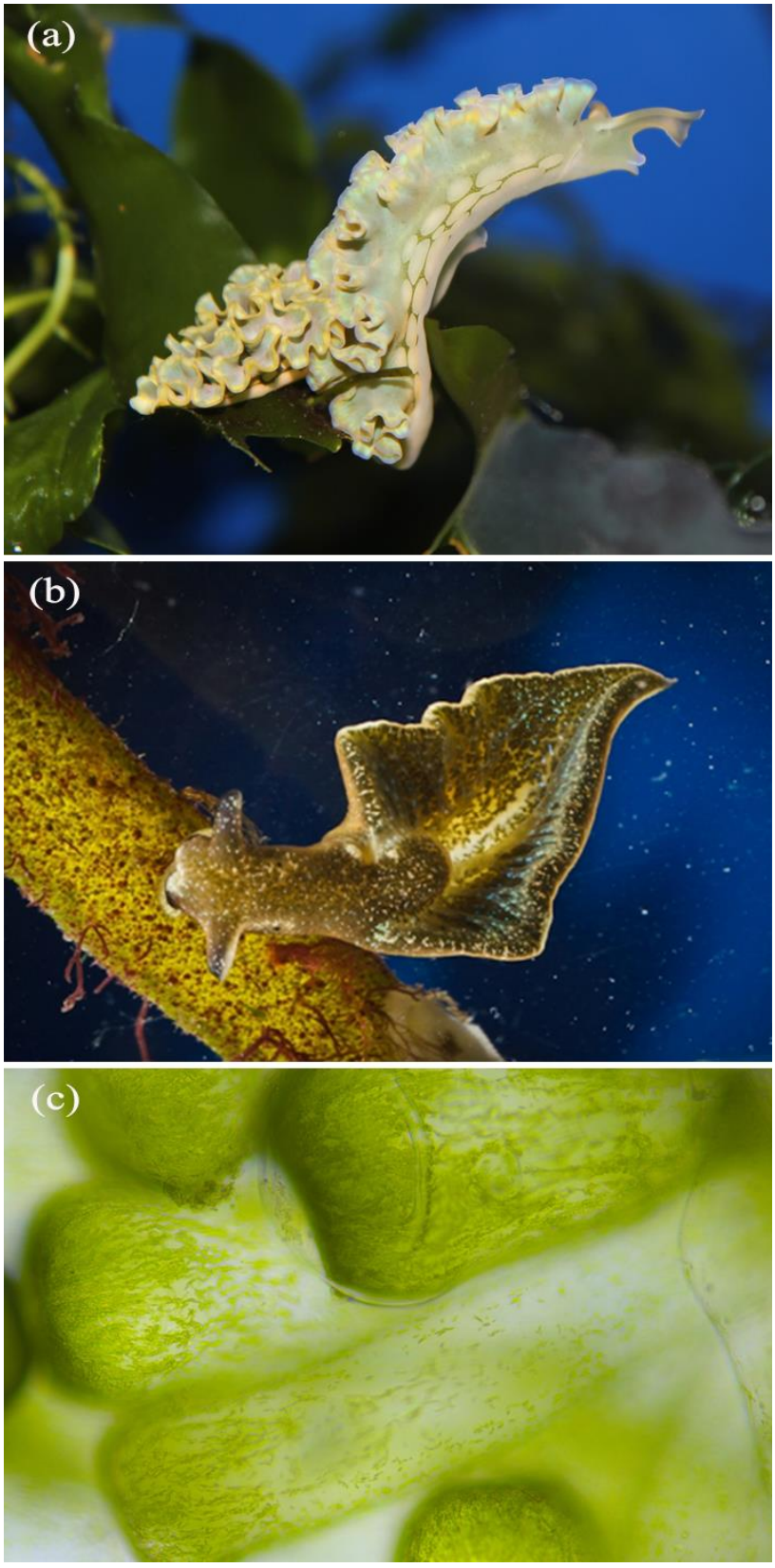

Fig. 1.3 Model organisms. (a) Tropical *Elysia clarki*, (b) temperate *Elysia viridis*, and (c) *Codium tomentosum* as an example of a siphonaceous algae, the dietary prey of some sacoglossans.

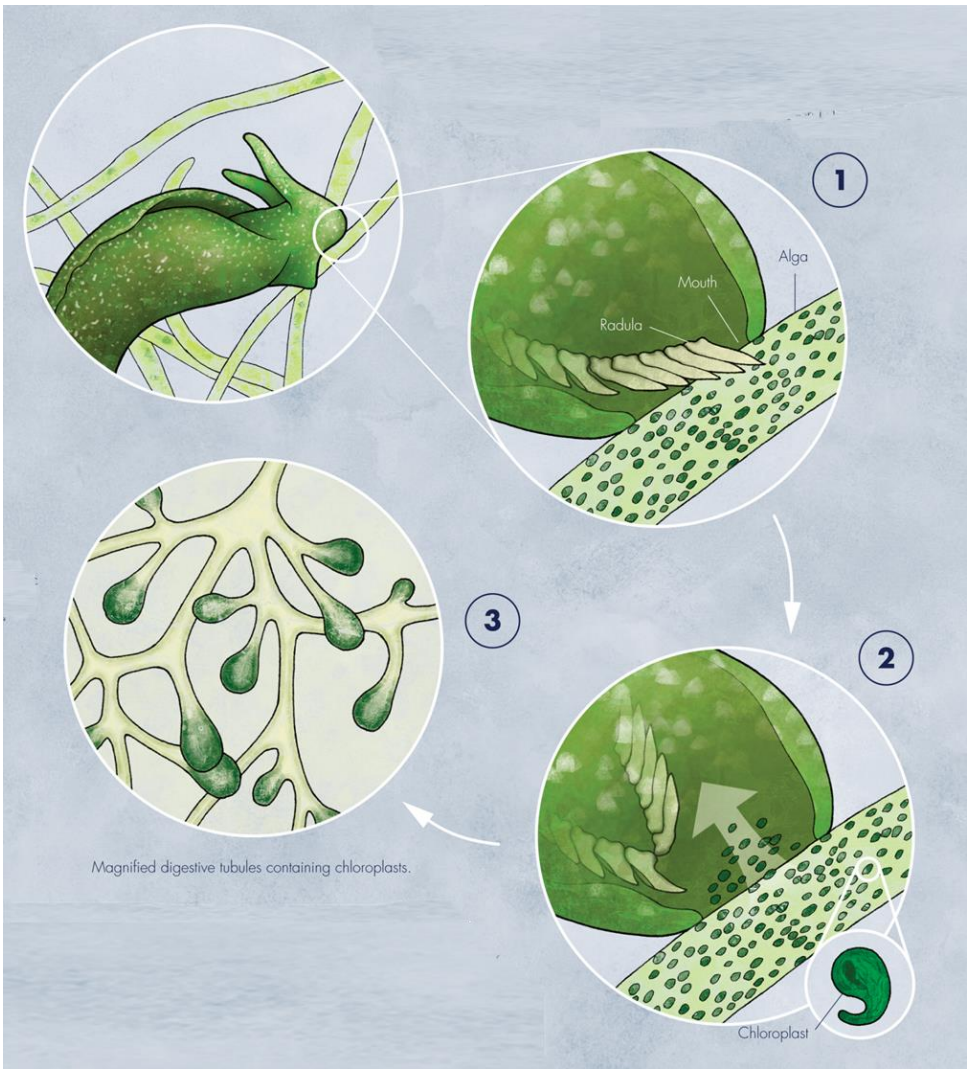

Fig. 1.4 Scientific illustration of sea slug chloroplasts acquisition - kleptoplasty. 1) The slug does not feed on the entire alga, but rather use the radula's tooth to penetrate the cell wall of siphonaceous algae; 2) they suck out the entire cytosolic content of the algae including the chloroplasts and all other compartments. 3) The kleptoplasts (acquired chloroplasts) are found in cells lining the extensive tubules of the digestive diverticula, which ramify throughout the body. The Illustration is a a courtesy from Robin K. Herman.

This puzzling behaviour has been termed chloroplast symbiosis, plastid sequestration or kleptoplasty (Fig. 1.4) (Johnson, 2011, Pierce *et al.*, 2006). Several levels of kleptoplasty have been recorded in a number of sacoglossan genera. Using a Pulse Amplitude Modulated (PAM) Fluorometer, Wägele and Johnson (2001) and Rumpho et al. (2000) demonstrated the existence of photosynthetic activity in species of genus *Elysia*, *Costasiella*, *Alderia, Oxynoe, Thuridilla* and *Plakobranchus*, although not all sacoglossans display the ability to retain functional chloroplasts (e.g., *Placida dendritica, E. catulus, Acobulla ulla,*

Cyerce nigricans). Using the same methods over a longer period, Evertsen *et al*. (2007) demonstrated longer retention of chloroplasts in *P. ocellatus* and *E. timida*, whereas other *Elysia* spp. and *T. hopei* lost photosynthetic activity within a few days. The record for chloroplast retention is 14 months for *E. chlorotica* (Rumpho *et al.*, 2006). It is also known that light exposure affects the chloroplasts retention in the sacoglossan mollusc *E. viridis* (Vieira *et al*., 2009).

Maintenance of photosynthetic activity in the absence of an algal nucleus is particularly intriguing considering the fact that the chloroplast genome is expected to encode only a small fraction of the proteins that are considered necessary for photosynthesis (Eberhard *et al.*, 2008). It was suggested that horizontal gene transfer, from the algal nucleus to the slug, with nucleus-encoded transcription factors and/or regulatory molecules being retargeted to the kleptoplasts, could explain the long-term survival and functioning of plastids in sea slugs (Curtis, 2006, Pierce *et al.*, 2012, Rumpho *et al.*, 2008). However, recent research do not support this hypothesis and argued that functional plastids are robust *per si* (Bhattacharya *et al.*, 2013, Rumpho *et al.*, 2011b) and/or result from a combination of physical and molecular mechanisms not yet determined (de Vries *et al.*, 2014a, de Vries *et al.*, 2013, Green *et al.*, 2005). Beside the efforts conducted in the last years to study this unique symbiosis, we currently lack the comprehensive knowledge on the environmental factors and mechanisms that maintain this association. Although, we do know that in environments where algae are scarce, seasonal, or highly variable, long-term kleptoplastic associations (such as the case of *E. clarki* and *E. viridis*), provide slugs with an advantage over organisms that must feed continuously (Rumpho *et al.*, 2011a). For sacoglossans with short-term functional kleptoplasty photosynthesis may function as a supplement for sustaining growth when food is available (Akimoto *et al.*, 2014, Curtis, 2006), as previously been demonstrated in kleptoplastic protists (Baumgartner *et al.*, 2015). Moreover, photosynthetic performance of kleptoplasts-bearing hosts revealed to be higher than corresponding chloroplasts within their original algal cells (Middlebrooks *et al.*, 2012). This has led to the assumption that non-photosynthetic hosts are able to enhance trophic energy transfer and benefit from the functioning of sequestered plastids (Costa *et al.*, 2012, Evertsen & Johnsen, 2009), as already postulated by

other authors (Serôdio *et al.*, 2014). As kleptoplasty is assumed to have evolved once near the base of sacoglossans (Hansen, 2011, Johnson, 2011), with long-term chloroplasts retention having evolved only in a limited number of lineages (Maeda *et al.*, 2012), such regular supplementary use (i.e. to sustain growth) may be the original function of kleptoplasty. Different hypothesis have been proposed to explain the advantages of kleptoplasty, but for some species it may certainly represent a mechanism to overcome periods when algal food is absent (e.g. *Codium* spp., the primary food algae of *E. viridis*, can be absent during winter months), or during periods when the algal food are calcifying (e.g. in the case of *Acetabularia acetabulum*, the food source of *E. timida*, (Giménez Casalduero & Muniain, 2008). In this context, photosynthetic plastids have been linked to a source of "junk food", which meets the intermediate energetic demands and other carbon-based requirements of the host (Venn *et al.*, 2008). Concomitantly, symbiosis would be valuable to the animal in environments where other sources of nutrients are in short supply (Venn *et al.*, 2008) and/or benefit the host in scenarios of environmental variations, such as pH and temperature variations.

The field of marine photosynthetic symbiosis research is still in a relatively early stage of development for mollusc-kleptoplasts association when compared with other animal–alga symbioses, namely corals. Recent research has focused on the molecular basis explaining plastid long-term maintenance in the absence of the algal nuclei (mostly using the association *E. chlorotica*/*Vaucheria litorea* as a biological model (Bhattacharya et al., 2013).

It is known that acidification and warming can have simple additive effects (both significant, but no significant interaction) or have complex interactive effects with synergistic effects (increased stress greater than the sum of the effects of the individual stressors) or antagonistic effects (decreased stress) in marine photosymbionts biological processes (e.g. Schmidt et al. 2014; Sinutok et al., 2014; Harvey et al., 2013; Rudolpho-Metalpa et al., 2011). Contrarily, the individual and/or combined effects of P_{CO_2} and temperature remain unknown for this unique mollusc-plastid association. Moreover, the integration of P_{CO_2} effects and temperature across different Sacoglossan sea slugs life-history stages, as

these organisms are born non-photosynthetic and acquire their plastids at a juvenile or adult stage, it is still unknown.

Summing up, it is of paramount importance to determine how ocean acidification and increasing water temperature will impact the symbiotic association displayed by the “crawling leaves”, in tropical as well as temperate regions. Under this scenario, such symbiotic associations present in the marine environment may be facing one of the biggest challenges in their life history, as environmental stress imposed to both members of the symbiosis may ultimately result in the disruption of the association.

1.3 General aim and research questions

Based on the important gaps of knowledge outlined above, the aim of this thesis was to increase the understanding of the impacts of acidification and warming of the marine environment on the ecophysiology and photobiology of sacoglossan sea slugs associated with functional chloroplasts, along with the ecological implications of these effects.

In this way, the present thesis aims to answer the following questions:

1. How can we maximize the production of the tool organism *Elysia* spp. to study sea slug-kleptoplast association?(**Chapter 2)**
2. How can we improve the non-invasive and non-destructive survey of the photophysiological performance of kleptoplasts in motile organisms such as sacoglossan sea slugs? (**Chapter 3**)
3. Will future ocean conditions impact the fitness of tropical photosynthetic sacoglossan sea slugs over different life stages? Will chloroplast acquisition, the first step required for kleptoplasty, be at risk under future ocean climate change scenarios? (**Chapter 4**)
4. How does acidification and warming affect the physiology and photobiology of tropical and temperate photosynthetic sacoglossan sea slugs? (**Chapter 5.1 and 5.2**)

1.4 Thesis outline

This thesis is composed by a general introduction (**Chapter 1**), four research chapters (**Chapter 2-5**) and a general discussion with final considerations (**Chapter 6**). **Chapter 2** summarizes the major issues impairing the culture of sea slugs and presents relevant biological and ecological data that can assist in the development of suitable culture protocols. Information on the most suitable husbandry, larviculture and grow-out techniques are critically discussed, with emphasis on their application on some of the most relevant groups of sea slugs, from an academic and commercial point of view. The knowledge acquired in this chapter allowed to produce large numbers of individuals that were crucial for the experiments described in chapters 4 and 5. Moreover, a novel methodological technique was developed to improve the study of phototrophy in motile sea slugs-bearing functional chloroplasts (**Chapter 3**). More specifically, the effect of two anaesthetics, eugenol and MS-222, on the photosynthetic activity of kleptoplasts was investigated, as well as on the behaviour (immobilization) of sea slugs. **Chapter 4** describes a factorial experiment to assess the combined effect of future acidification and warming (expected for the end of the century), on the early ontogenetic development of the tropical sea slug *Elysia clarki*. This chapter combines quantitative and qualitative measurements on sea slug reproductive output and chloroplast acquisition by juveniles. **Chapter 5** reports two climate change-related experiments designed to study, for the first time, the effect of climate change on the morphology and photobiology of adult sacoglossan sea slugs. The first experiment comprised a preliminary trial (short term exposure) that aimed to investigate the impact of environmental hypercapnia (acidification) on the morphology of *E. viridis* digestive diverticula and associated kleptoplasts. The second experiment comprised a factorial design testing the combined effect of acidification and warming (long-term exposure) on the holobiont fitness, metabolism and kleptoplasts photobiological activity in both tropical (*E. clarki*) and temperate (*E. viridis*) adult sacoglossan sea slugs. The potential effect of ocean acidification and warming on oxidative stress-related physiology (heat shock response and antioxidant enzyme activities) of sacoglossan sea slug-kleptoplast

association was also investigated. Finally, the General Discussion and Conclusions (including future research lines) are presented in **Chapter 6**.

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Chapter 2 Current status of sea slugs culture

Abstract

Research on sea slugs production has steadily increased in the last decades as a result of their use as model organisms for biomedical studies and bioprospecting for new marine drugs. Additionally, these organisms have also experienced a growing demand for academic research and the marine aquarium trade. However, standardized methods for culturing sea slugs are still limited to a reduced number of species. The main bottlenecks impairing sea slugs aquaculture are the lack of knowledge on suitable larval diets and settlement cues that can induce metamorphosis in competent larvae. Additionally, the stenophagous feeding regime displayed by several species requires the collection and/or culture of their prey, which commonly impairs large-scale production. Nevertheless, significant breakthroughs have been achieved in recent years through the development of innovative culture techniques. The present review summarizes the major issues impairing the culture of sea slugs and presents relevant biological and ecological data that can assist in the development of suitable culture protocols. Available information on current husbandry, larviculture, and grow-out techniques are critically discussed, with emphasis on their application, on the culture of some of the most important groups of sea slugs, from an academic and commercial point of view: sea hares (*Aplysia* spp.), nudibranchs (e.g., the marine ornamental species *Aeolidiella stephanieae*) and "solar powered" sacoglossans (e.g., *Elysia* spp.).

Keywords

Aeolidiella; Aplysia; Elysia; Husbandry; Larviculture; Grow-out

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2.1 Introduction

Sea slugs are delicate, colored and “sludgy” gastropod molluscs commonly referred to as the underwater version of the butterfly and the caterpillar combined (Debelius and Kuiter, 2007). Outwardly defenceless and often displaying a bizarre silhouette, these gastropods have always been fascinating subjects for marine biologists. Several scientific works already investigated and discussed their morphology (Mikkelsen, 2002), life cycle (Clark, 1975; Harris, 1975; Avila et al., 1997), ecology (Carefoot, 1987; Angeloni and Bradbury, 1999), feeding habits (Aboul-Ela, 1959; Ritson-Williams et al., 2003; Hoover et al., 2012) and systematics (Bouchet and Rocroi, 2005; Jörger et al., 2010; Schrödl et al., 2011).

Sea slugs are currently considered members of the Heterobranchia (according with the most recent classification proposed by Jörger et al., 2010) (Fig. 2.1), a highly diversified and successful group of marine gastropods presenting a global distribution and occupying a wide range of ecological niches. Sea hares *Aplysia* spp. (Anaspidea) (Fig. 2.1) are probably one of the most well-studied groups of sea slugs because of their key role in medical research (Sattelle and Buckingham, 2006). Their popular use as model organisms, particularly in neurobiological sciences, prompted researchers to develop suitable culture protocols to allow their mass production under controlled conditions (Capo et al., 2009). Nudibranchs (Nudipleura) (Fig. 2.1) have been of interest to researchers in biotechnology due to their potential for the bioprospecting of new marine natural products (e.g., *Felimida* spp., formerly known as *Chromodoris*) (Leal et al., 2012a).

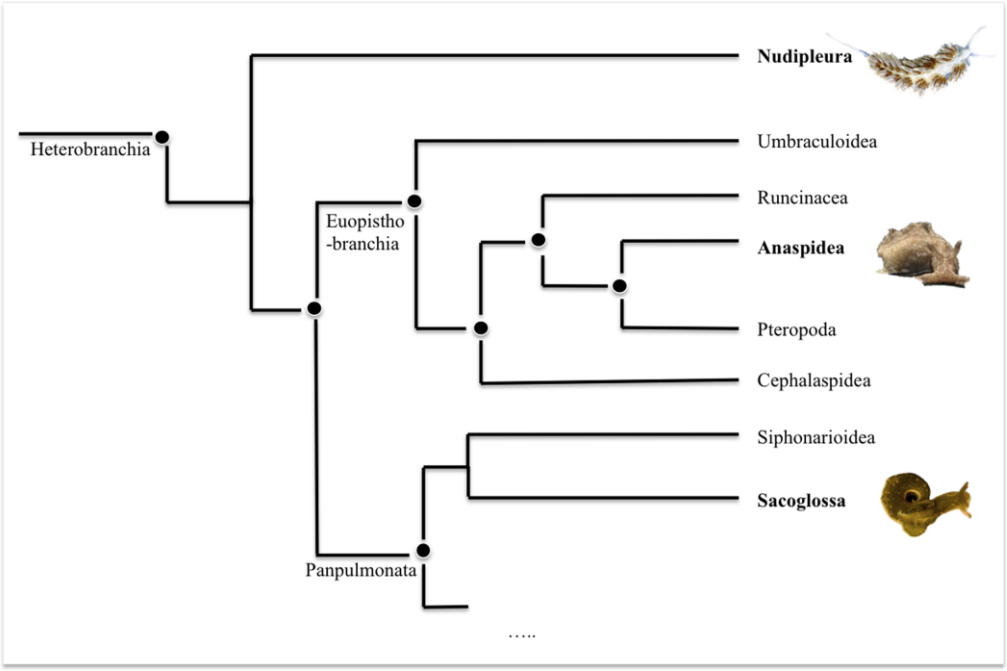

Fig. 2.1 Simplified classification of the most commonly cultured groups of sea slugs (i.e. kleptoplasts may provide carbon substrates to the host, Stirts & Clark, 1980, Thompson & Jarman, 1989, Trench *et al.*, 1973). The differences between branches reflect a measure of divergence time. Black nodes show significant support. Nudipleura are represented by *Aeolidiella stephanieae*, Anaspidae by *Aplysia* sp. and Sacoglossa by *Elysia viridis.* (Note: the group Panpulmunata is not represented in the figure).

Additionally, nudibranchs are also widely used as biological tools for scientific research (e.g., *Aeolidiella stephanieae* and *Spurilla nepolitana*), particularly to study their chemical ecology and photosymbiotic associations (Carroll and Kempf, 1990; Cimino and Ghiselin, 2009; Greenwood, 2009). The most dazzling coloured nudibranchs are also highly popular among marine aquarium hobbyists, with a number of these ornamental species (e.g., *A. stephanieae*) already being produced in captivity and reaching high retail values in the marine aquarium trade (Olivotto et al., ornamental trade (e.g., *Elysia crispata*) because of their ability to control the growth of nuisance algae (Sprung, 2002). Nonetheless, the culture of sacoglossans (e.g., *Elysia chlorotica*, *E. timida* and *E. viridis*) has mostly provided biological material to researchers addressing one of the most puzzling features displayed by marine invertebrates – the ability to keep functional algal chloroplasts within their animal cells (Johnson, 2011). This remarkable feature has provided these sacoglossans the nickname of “solar-powered sea slugs” or “photosynthetic animals” (Rumpho et al., 2000, 2011).

The present review provides a comprehensive overview of the most significant breakthroughs on sea slugs aquaculture, as well as current bottlenecks impairing their large-scale production. Special emphasis is given to species playing an important role for biomedical and academic research, for bioprospecting of new drugs and for the marine aquarium trade. The husbandry requirements of reproductive broodstock are addressed, along with the most relevant aspects of sea slugs reproduction from an aquaculture perspective. Larviculture protocols for the most commonly cultured sea slugs are reviewed, with emphasis on culture systems, diets, and cues known to trigger metamorphosis. Current methodologies available for the grow-out of juvenile sea slugs are presented, with a particular focus on the challenging task of providing suitable diets to stenophagous species.

2.2 Why culture sea slugs?

2.2.1 Sea slugs as biological models and tools

Sea slugs are often used as a “biological model” or “biological tool”. The term “model” describes non-human biological systems that are used to better understand human disorders. A biological tool is used in research studies that are not related to human disorders (Sive, 2011). Species within genus *Aplysia* are good examples of biological models that have been successfully used on a broad range of experimental studies addressing biomedical topics (see Capo et al., 2009). In contrast, “solar-powered” sea slugs (e.g. *Elysia* spp.) are biological tools used to study endosymbiotic associations between metazoan cells and functional chloroplasts (Pelletreau et al., 2011; Rumpho et al., 2011). Fig. 2.2 provides a schematic overview of different research topics where sea slugs culture is performed for commercial or academic purposes.

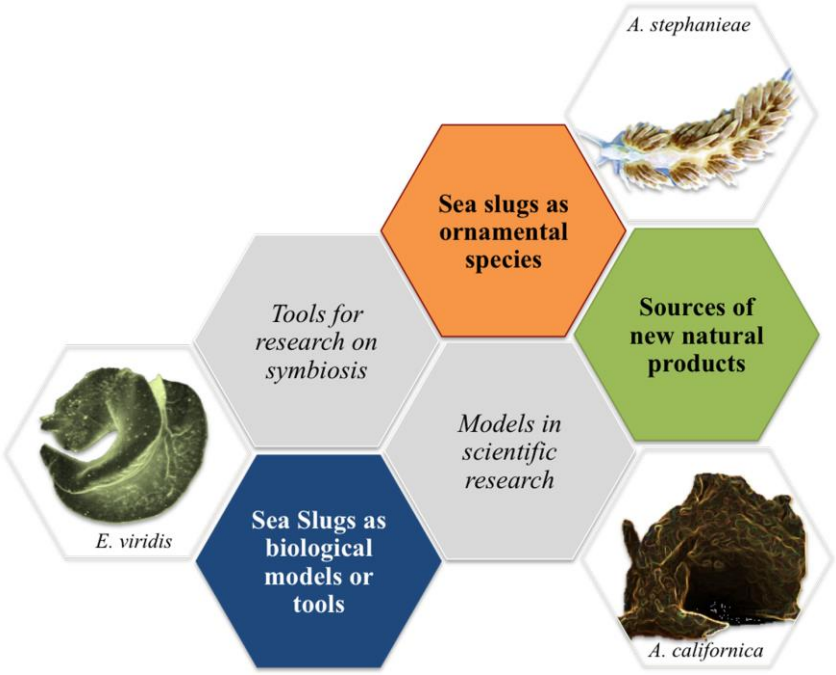

Fig. 2.2 Schematic overview of different research topics where sea slugs culture is performed for commercial or academic purposes. Sea slugs as biological models and tools (blue hexagon) divided in two major themes: tools for research in symbiosis and models in scientific research (grey hexagons) (e.g., biological model— *Aplysia californica*; biological tool — *Elysia viridis*), as ornamental species (orange hexagon) (e.g., *Aeolidiella stephanieae*), and as sources of new marine natural products (green hexagon) (e.g., *Aplysia* spp.).

2.2.1.2 Sea hares *Aplysia* spp. as models in scientific research (the beasts)

Aplysia species are acknowledged as one of the most important invertebrate model organisms in biomedical studies (Kandel, 2001). Species within genus *Aplysia*, namely *Aplysia californica*, have relatively simple biological systems. The use of these organisms in scientific research allowed significant breakthroughs on the clarification of molecular mechanisms involved in all phases of implicit memory and cellular basis of behaviour (Hawkins et al., 2006; Kandel, 1982). *Aplysia* species are also important models for research on neural control of hormone secretion (Wayne, 1995), aging (Bailey et al., 1983) and Alzheimer's disease (Shemesh and Spira, 2010).

2.2.1.3 "Solar-powered" sea slugs as tools for research on symbiosis between metazoan cells and functional chloroplasts

The popularity of "solar-powered" sacoglossan sea slugs (e.g., *Elysia* spp.) has grown among researchers since the 1960s. These organisms are able to "steal" functional chloroplasts from their algal prey and keep them functional in

animal tissue, somehow continuing to photosynthesize without the support of the whole native algal cell (Pierce and Curtis, 2012; Rumpho et al., 2011). These highly specialized organisms feed by slicing or puncturing siphonaceous algal cells and sucking out their contents. Most cell contents, including the algal nucleus, are digested, whereas chloroplasts are retained as functional organelles within the cells of its new host — the sea slug (Rumpho et al., 2000). This puzzling behaviour has been termed chloroplast symbiosis, plastid sequestration or kleptoplasty (Johnson, 2011; Pelletreau et al., 2011). Several levels of kleptoplasty (i.e., different retention abilities of non-functional and functional chloroplasts) have been recorded among genera of sacoglossans (e.g., *Alderia*, *Bosellia*, *Caliphylla*, *Elysia*, *Hermaea*, *Limapontia*, *Mourgona*, *Oxynoe*, *Plakobranchus*, *Tridachia*, and *Thuridilla*), although not all sacoglossan species display the ability to retain functional chloroplasts (e.g., *Placida dendritica*, *E. catulus*, *Acobulla ulla*) (Evertsen et al., 2007; Rumpho et al., 2011). The mechanisms of interaction between the foreign organelle (the stolen chloroplast or kleptoplast) and its host animal cell have just started to be unravelled by the scientific community (Händeler et al., 2009; Pelletreau et al., 2011; Pierce et al., 2012; Rumpho et al., 2008; Vieira et al., 2009; Wägele et al., 2011). Different strategies to retain functional chloroplast within animal cells have been recorded among sacoglossan: species retaining non-functional chloroplasts for some hours, and species retaining functional chloroplasts for days, weeks or even months. Such differences may provide a unique opportunity for researchers to witness an ongoing evolutionary process of endosymbiosis. Most efforts to culture “solar-powered” sea slugs in the laboratory have targeted *Costasiella lilianae*, *E. chlorotica, Elysia clarki, E. timida* and *E. viridis* due to their rapid embryonic development and the ease of rearing their larvae and juveniles (Curtis et al., 2006; Rumpho et al., 2008; Trowbridge, 2000).

2.2.1.4 Sea slugs as sources of new natural products

A large number of natural products with remarkable bioactivities have been discovered during the last few decades from marine invertebrates (Blunt et al.,

2012). The Heterobranchia follows the same trend and several new marine natural products have been extracted from this group of organisms (Leal et al., 2012a), particularly amino acids and peptides (Leal et al., 2012b). While not all molecules discovered from sea slugs have interesting biomedical applications, some of them have been the starting point for a number of potential drug candidates (Barsby, 2006; Putz et al., 2010). In some cases, the origin of natural products in sea slugs has been attributed to bio-accumulation or biotransformation of molecules acquired through the ingestion of their prey (Avila, 1995). However, sea slugs may also synthesize their own molecules de novo, which is one of the most striking aspects of the ecology of the Heterobranchia (Cimino and Ghiselin, 2009). While significant breakthroughs have been achieved in the field of organic synthesis in the recent years, several molecules recorded from marine organisms, such as sea slugs, exhibit structural peculiarities that are still difficult to recreate through chemical synthesis (Baran et al., 2007). Another restrictive step in the development of new marine drugs from sea slugs, as for other marine organisms, is the frequent lack of an adequate and consistent supply of raw material for standard screening assays, especially for rare and/or small-sized species. The paucity of cost-effective techniques for molecular isolation, identification and synthesis are additional bottlenecks that impair the development of new marine drugs from sea slugs. Under this scenario, aquaculture may provide a potential solution to some of these constraints as it can supply the required biomass throughout the drug discovery pipeline (Leal et al., 2012a). Under a controlled environment, the aquaculture of sea slugs in the laboratory may be fine-tuned to maximize the production of particular molecules, namely by shifting biotic and/or abiotic factors that can favour certain metabolic pathways. Aquaculture may also be advantageous for those species that acquire their natural products through their prey as the long-term husbandry of sea slugs may allow the maximization of the concentration of target molecules. The rationale for this claim is that sea slugs commonly intensify their chemical weaponry through the ingestion of their prey (Molinski et al., 2009).

From all sea slugs screened so far for new products, members of genus *Aplysia* are unquestionably the ones yielding the highest number of compounds over the last two decades (58 new compounds representing 10.2% of new natural products obtained from marine molluscs) (Leal et al., 2012a). Some of these molecules identified from *Aplysia*, as well as from other sea slugs, have been shown to be feeding-deterrents and to display cytotoxic and ichthyotoxic properties (Avila et al., 2006). Table 2.1 summarizes the most relevant molecules yielded so far from sea slugs, as well as their bioactivities.

Table2.1 List of molecules and respective bioactivities isolated from marine sea slugs that have been determinant for drug development.

Source organism	Compounds	Bioactivities	References
Aplysia dactylomela (A)	Escapin	Antimicrobial	1
Aplysia kurodai (A)	Pericosine A and B	Anti-tumour	2
Bursatella leachii (A)	bursatellanin-P	Anti-HIV	3
Dolabella auricularia (A)	Dolastatin 10, Dolastin 15 and Synthadotin	Anti-cancer	4
Chromodoris aspersa (N)	Various sesquiterpenes	Antimicrobial, antifungical	5
Dendrodoris carbunculosa (N)	Various dendocarbins	Anti-cancer	6
Doris kerguelenensis (N)	Palmadorin A, Labdane, Austrodorin	Antibacterial, anti-foulant, among others	7
Leminda millecra (N)	Toluquinone	Anti-cancer	8
Elysia rufescens (S)	Kahalalide F and Kahalalide A	Anti-tumour and anti-tuberculosis (respect.)	9-10

A - Anaspidea, N - Nudipleura, S – Sacoglossa; 1) Yang, et al. 2005; 2) Numata, et al. 1997; 3) Rajaganapathi, et al., 2002; 4) Pettit, et al. 1987, 1989; 5) Gunthorpe and Cameron, 1987; 6) Sakio, et al. 2001; 7) McClintock, et al 2010; 8) Whibley, et al. 2007; 9) Hamann and Scheuer, 1993; 10) Hamann, et al. (1996).

2.2.1.5 Sea slugs as ornamental species (the beauties)

In the last decade, several studies have addressed the aquaculture potential of marine invertebrates commonly traded for marine aquariums (Olivotto et al., 2011). Nevertheless, little information is currently available on the commercial relevance of sea slugs in this trade. Most sea slugs traded as ornamental species are dazzling coloured (e.g., *Felimida* spp.), but commonly have very low chances of surviving in captivity and invariably starve to death. The most common reason for such poor husbandry success is their stenophagous feeding regime. These organisms only accept one type (or at the most a limited range) of prey as food, such as cnidarians, bryozoans, tunicates, sponges or even other nudibranchs (Calfo and Fenner, 2003; Sprung, 2001).

The nudibranch *A. stephanieae*, formerly known as *Berghia verrucicornis*, is a good example of this rule, as it preys exclusively upon one of the most feared pests by marine aquarium hobbyists — glass anemones of genus *Aiptasia* (Carroll and Kempf, 1990; Leal et al., 2012c). Commonly employed as a biological weapon to control the outbreaks of these sea anemones in reef aquariums, *A. stephanieae* easily thrives and reproduces in the presence of large populations of *Aiptasia* (Banger, 2011). Due to its popularity in the marine aquarium trade, *A. stephanieae* commonly fetches high market values (up to 25 € per specimen – retail values for 2012) being a highly prized species for hobbyists breeding marine ornamentals. Another group of sea slugs commonly traded for marine aquariums are those that eradicate nuisance algae. The presence of certain sea slugs, such as *E. crispata*, slows down the growth rate of undesired algae in aquarium reefs, such as the green-hair like algae *Bryopsis* (Sprung, 2002). It is important to highlight that many Heterobranchia are relatively short-lived, thriving for only a couple of months to one year under optimal husbandry conditions (Calfo and Fenner, 2003). From a commercial point of view, this may be an advantage for breeders, as species employed to control nuisance organisms (e.g. glass anemones and undesirable algae), may have to be regularly replaced by new specimens, thus ensuring a continuous demand for these organisms.

2.3. Bibliometric analysis of sea slugs culture

Sea slug's research has experienced a considerable increase over the last decades, as a result of the above mentioned drivers. In the early 20th century, the main research topics on sea slug were their biology and taxonomy. At present, most studies performed with sea slugs have focused on neurobiology. As bibliometric analysis is a powerful tool to evaluate research priorities across entire disciplines (Neff and Corley, 2009), the published scientific literature on sea slugs over the last half-century (1958–2012) was surveyed and all published papers listed on the online database Web of Knowledge published by Thompson Reuters (available at http://apps.webofknowledge.com, and consulted the 24th of August 2012) were retrieved. The following search

factors were used in the field “topic” as search request for referenced publications until August 2012: “Opisthobranchia” (for the general search) and “Opisthobranchia” AND “culture” (for the restricted search on sea slugs culture). While the term “Opisthobranchia” is no longer used (changed to Heterobranchia in 2010; see the Introduction section and Fig. 2.1), its use allowed a complete survey of all previous works published on sea slugs. The search performed retrieved 948 works referring to Opisthobranchia from 1958 to August 2012. Overall publication activity was characterized by an increase in the number of published articles per year. Most articles on this topic have been published only in the last 12 years (2000–2012) (418 articles, representing 44% of total publications on this topic) (Fig. 2.3). Only 30 of the retrieved publications specifically address the culture of sea slugs. Apart from recent efforts targeting culture, given their importance for mainstream scientific areas such as neurobiology, sea slug production at a large scale has been poorly investigated. This trend in publications on sea slug's culture reflects how limited our scientific knowledge on this topic is and the need for further research to help our understanding of this important group of organisms.

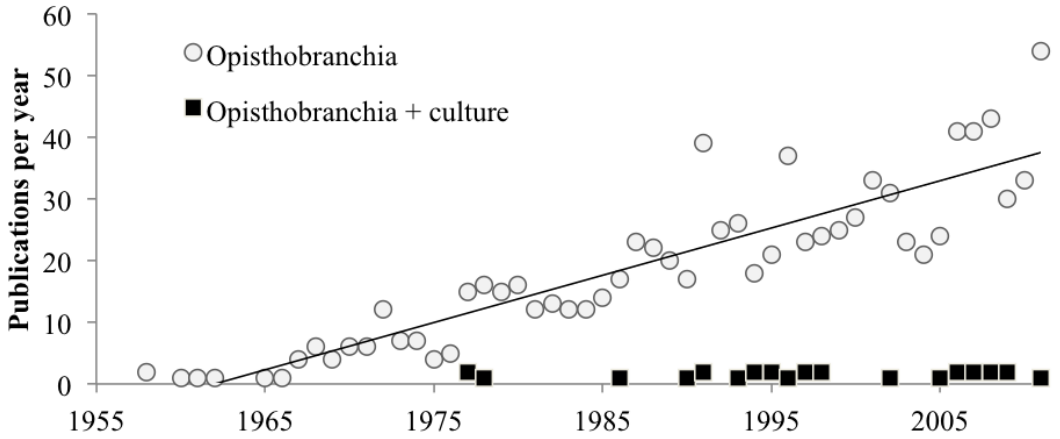

Fig. 2.3 Number of scientific articles published between 1958 and 2012, according to Web of Knowledge online database (on 2th August 2012), using the search terms “Opisthobranchia” and “Opisthobranchia AND Culture”.

2.4. Broodstock husbandry and reproduction

2.4.1 Collecting broodstock

Sea slugs usually occur in intertidal and coastal areas, with large sized species (e.g*., Aplysia* spp.) being easily detected even by inexperienced collectors. However, the detection of most sea slugs in the wild requires a careful inspection, mostly due to the remarkable mimetic ability displayed by some species (Debelius and Kuiter, 2007). Sea slugs can either be manually collected after visual detection, or researchers may employ underwater suction devices to collect specimens that were brushed from a surface commonly covered by the prey species of the sea slug (Bleakney, 1969; Clark, 1971; Franz, 1975). When employing SCUBA diving equipment, collecting the most frequent dietary prey of the target sea slug species (e.g. a sponge or a coral) is often a good option to harvest small-sized or highly mimetic animals (the limited air supply of the collector may impair a detailed inspection in situ). Harvested samples can later be easily inspected in the laboratory (Franz, 1975), where the use of flexible tweezers is recommended for manipulating sea slugs. Collected sea slugs can be shipped in aerated containers for short distances, or placed in round-bottomed plastic bags with one-third of their volume filled with seawater and the two-thirds with oxygen for long distances (as described by Wabnitz et al., 2003 for marine ornamental species).

Seasonal variability in the abundance of Heterobranchia is probably most pronounced in temperate regions, such as north-eastern Atlantic coasts, where significant seasonal shifts in water temperature occur (Franz, 1970). Knowledge on the zoogeography and reproductive ecology of target species will certainly be helpful to maximize collection efficiency. Local restrictions and collecting permits should be assessed prior to collecting in order to avoid any illegal actions that may involve fines or other legal sanctions.

2.4.2 Husbandry

The husbandry of sea slugs broodstock strongly depends on the availability of adequate food to keep breeding pairs under proper nutritional conditions and allow stocked animals to produce large numbers of high-quality embryos. The rule of thumb in sea slugs husbandry is that the more intense the animal's color, the healthier it is. Nonetheless, stocking breeding pairs in captivity under optimal conditions is far from an easy task for some species, particularly when researchers ignore their feeding regimes. Additionally, potential environmental or nutritional stressing events that may have affected sea slugs prior to the collection can also negatively affect the success of their husbandry, regardless of employing optimal stocking procedures. The life history of field collected-specimens, such as age, parental lineage and the reproductive state may also significantly influence their breeding performance in captivity (e.g., *A. californica*, *E. viridis*).

The optimal type of system used to stock and breed sea slugs mostly depends on the target species and the purpose of its production, i.e., research scale vs. semi-commercial or commercial scale. So far, the only sea slug being commercially produced at a semi-industrial scale is *A. californica* (University of Miami/NIH, USA), with an overall production of 30,000 animals per year (Capo et al., 2009). While the ornamental sea slug *A. stephanieae* is currently only commercially cultured at a small scale (Olivotto et al., 2011), the work by Banger (2011) reports that semi-industrial scale production of this species can be achieved by using an innovative breeding system (see the Section Larviculture techniques for a detailed description).

Different broodstock systems have already been successfully employed for sea slugs production, from flow-through systems (Capo et al., 2002), such as the one used by the National Resource of *Aplysia* (University of Miami/NIH), to recirculated systems (Banger, 2011; Peretz and Adkins, 1982), that are often employed for academic research and/or small scale production of ornamental sea slugs. Recirculated systems operating with synthetic seawater have already allowed the culture of large numbers of sea slugs in a small space,

with biological, chemical and mechanical filtration assuring high water quality and little system maintenance (Banger, 2011).

While no data is currently available to reliably compare the success of different broodstock systems for sea slugs, the location of the breeding facility (coastal areas vs. inland) and the availability of natural seawater with a suitable quality seems to rule the choice between flow-through and recirculated systems. As sea slugs have natural seawater (Banger, 2011; Capo et al., 2002; Carroll and Kempf, 1990), it seems evident that the production of these organisms can be successfully achieved at inland facilities with no access to natural seawater. Sea slugs may be housed in glass, fiberglass, or plastic tanks, with some interesting examples of sea slug broodstock systems being provided by Capo et al. (2002) and Banger (2011) (Fig. 2.4A and B).

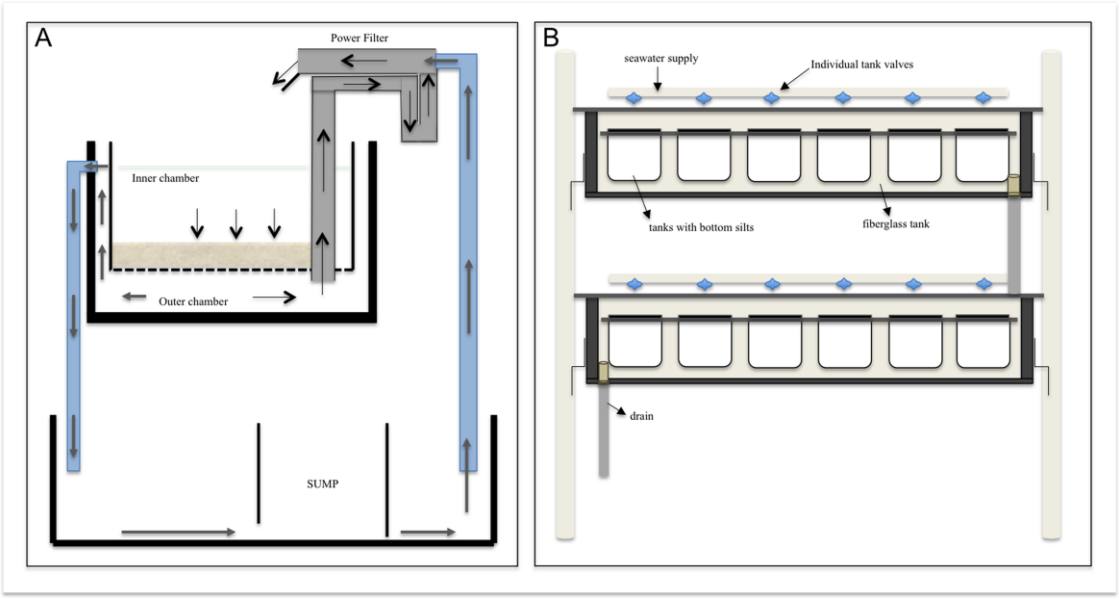

Fig. 2.4 Illustrations of two different systems employed for commercial sale production of sea slugs: A) Recirculated "breeding chamber" culture system for *Aeolidiella stephanieae* (adapted from Banger, 2011). Filtered seawater enters the inner chamber from the sump, passes through the substrate, and exits the breeding chamber via a drain located on the side of the outer chamber and returns to the sump for filtration; broodstock, larvae, and juveniles remain inside the inner chamber. B) Flow-through culture system for *Aplysia californica* (adapted from Capo et al., 2009). Polycarbonate chambers hold in a large fiberglass tank are continuously supplied with chilled seawater; each chamber presents an open top to facilitate the unidirectional flow of seawater supplied through individual valves: supplied seawater passes through the slits at the bottom of the chamber to the fiberglass tank and is discharged.

Sea slugs generally require seawater at a salinity of 30–35 and a pH of 8.0–8.2, with water temperature being dependent on the temperate or tropical

origin of the sea slug species being cultured. As temperature is an important factor for gonadal maturation, it is possible to induce breeding throughout the year in captivity through the manipulation of water temperature (Kriegstein et al., 1974). Furthermore, it is vital to adjust this parameter to optimal values when stocking species with symbiotic associations, as it is known that abnormal temperatures may disrupt the association between animal hosts and endosymbiotic photosynthetic dinoflagellates (e.g., *Symbiodinium*) (Venn et al., 2008). Light is also an important factor to consider when stocking sea slugs with photosynthetic endosymbionts (see the Section "Solar-powered" sea slugs as tools for research on the symbiosis between metazoan cells and functional chloroplasts). Successful husbandry has been achieved with fluorescent lamps (e.g., Vieira et al., 2009), light emitting diodes (LEDs) (e.g., Cruz et al., 2012), or natural sunlight (e.g., Schmitt et al., 2007). Table 2.2 summarizes some of the useful conditions for the successful husbandry of the most relevant sea slugs for academic research. The use of inadequate physico-chemical water parameters (such as temperature), along with nutritionally unbalanced diets, may result in poor egg quality, abnormal egg loss during incubation, and ultimately on the death of reproductive breeding pairs (Schlesinger et al., 2009).

Table2.2 Stocking conditions employed for the successful husbandry of marine sea slugs.

Species	Temperature (ºC)	Diet	References
Aeolidiella stephanieae (N)	24	*Aiptasia pallida*	1
Aplysia californica (A)	13-18	*Gracilaria ferox*; *Agardhiella* sp.; *Ulva* sp.; *Laurencia* sp.	2-6
Aplysia dactylomela (A)	23-28	*Ulva* sp.; *Spyridia filamentosa*; *Laurencia* sp.	7
Aplysia juliana (A)	23-28	*Ulva* sp.; *Enteromorpha intestinalis*	7
Aplysia oculifera (A)	24	*Ulva* sp.; *Enteromorpha intestinalis*	8
Elysia chlorotica (S)	10	*Vaucheria litorea*	9-10
Elysia timida (S)	20	*Acetabularia acetabulum*	11-12
Elysia viridis (S)	10-18	*Codium fragile; C. tomentosum*	13-16

A - Anaspidea, N - Nudipleura, S –Sacoglossa; 1) Carroll and Kempf, 1990; 2) Smith and Carefoot, 1967; 3) Kriegstein et al., 1974; 4) Capo, et al., 2002; 5) Capo et al., 2009; 6) Smith, et al., 2011; 7) Switzer-Dunlap and Hadfield, 1977; 8) Plaut et al., 1995; 9) West et al., 1984; 10) Green et al., 2000; 11) Marin and Ros, 1989; 12) Wägele et al., 2011; 13) Trowbridge, 2000; 14) Trowbridge and Todd, 2001; 15) Unpublished data.

2.4.3 Feeding preferences and nutrition

Feeding preferences within the Heterobranchia are known to vary largely, with some species being able to feed on a range of prey, while others display a stenophagous feeding regime (preying on a single species). Nonetheless, all Heterobranchia species seem to be insatiable, displaying a voracious appetite and often requiring a daily supply of food. In order to assure the suitable feeding of stocked specimens, both quantitatively and qualitatively, prey organisms must be easy to collect from the wild or to culture in captivity. It is therefore of paramount importance to know before-hand the dietary preferences of the target sea slug species to be cultured, as well as how easy it will be to collect or culture its prey.

Feeding specificity is usually higher in shelled Sacoglossa (Jensen, 1980), although some species shift their dietary preferences throughout development (Thompson and Jarman, 1989; Trowbridge and Todd, 2001). Nudibranchia can also be stenophagous, preying only on a single genus or species (Carroll and Kempf, 1990). At present, molecular techniques can allow researchers to determine the feeding regime of sea slugs (in the case of species with unknown feeding regimes) by analysing DNA barcodes of their gut content. The dietary algal prey of sacoglossan sea slugs may also be investigated by analysing the chloroplast DNA from whole animals (Händeler et al., 2010). Not all sacoglossans feed on algae, as some species feed exclusively on embryos of other molluscs (Jensen, 1997). Table 2.2 summarizes the dietary items required for successfully stocking breeding pairs of some of the most important sea slug species for research.

Several protocols are already available to culture some of the macroalgae commonly employed to feed juvenile stages of *A. californica* (e.g. *Gracilaria ferox*, *Agardhiella subulata*, *Ulva* spp. and *Laurencia* spp.) (Capo et al., 1999, 2002; Smith et al., 2011). Similarly, sea anemones have also been

successfully propagated in captivity to culture the nudibranch *S. neapolitana* and *A. stephanieae* (Leal et al., 2012d; Schlesinger et al., 2009, respectively). For prey organisms with challenging life cycles and/or unsuitable for captive culture, such as certain sponges, ascidians, invertebrate embryos or even other sea slugs, the only option to sustain breeding pairs is to collect and stock their prey. While live preys are commonly employed, frozen sponges have already been successfully used as food items for certain nudibranchs (e.g., genus *Felimare*) (G. Calado, unpublished data).

A viable alternative to live feeds would be feeding sea slugs with inert microdiets. Significant efforts have been made in last years to design specific artificial diets suitable for commercial aquatic species such as fishes, crustaceans and bivalve molluscs (Luzardo-Alvarez et al., 2010). Successful advances such as diet binders, agglutination chemicals, the inclusion of different enzymes and pre-hydrolysed proteins among others, are good examples for future research in sea slug nutrition. It is known that lipids and fatty acids (FA) play an important role in developing embryos of molluscs (Joseph, 1982). However, few studies are currently available on this topic for sea slugs (Martínez-Pita et al., 2006). As most marine invertebrates, sea slugs cannot synthesize certain FA de novo, which must be derived from their dietary prey or provided through symbiotic relationships with microalgae or algal chloroplasts (Zhukova, 2007). FA profiles on early developing embryos are primarily regulated by broodstock nutrition and clearly reflect the pool of fatty acids available for parental diets (Leal et al., 2012d; Martinez-Pita et al., 2005).

2.4.4 Reproduction

Sea slugs display complex reproductive modes and strategies (Ghiselin, 1966). They are hermaphrodites with internal cross-fertilization (Beeman, 1970; Painter et al., 1985). Allosperm resorption has been shown to occur in several species (Rivest, 1984) and can be assumed to be widespread due to the presence of a gametolytic gland in most groups (Schmitt et al., 2007). Sea

slugs typically donate and receive sperm reciprocally in the head-to-tail cross-position (Carefoot, 1987). Besides this standard insemination mode, a variety of alternatives exist. Some species form mating chains (Angeloni, 2003; Switzer-Dunlap and Hadfield, 1984; Yusa, 1996), alternate sex roles (Anthes et al., 2006; Michiels et al., 2003), or transfer sperm via externally attached spermatophores (Karlsson and Haase, 2002). Hypodermic insemination, in which sperm is injected through the partners' body surface, is also widespread, particularly among Sacoglossa (Angeloni, 2003; Jensen, 1999; Rivest, 1984; Schmitt et al., 2007). The duration of different mating phases, such as courtship behaviour and the timing of the penial gland eversion are known to be species-specific (Reise, 2007). Species in genus *Aplysia* frequently mate with several partners (Angeloni et al., 2003; Yusa, 1996) and readily mate with a second partner immediately after an initial mating encounter of 30–40 min (Ludwig and Walsh, 2008). As a consequence of this behaviour and reproductive anatomy, these organisms display sperm competition, as well as post-copulatory female choice (Michiels, 1998; Yusa, 1994). Furthermore, size-differences between mating partners have been shown to influence mating behaviour in sea slugs (Angeloni and Bradbury, 1999; Angeloni et al., 2003; Gianguzza et al., 2004). In this way, a mixture of strategies or gender preferences can be employed by these organisms, depending on the unique set of circumstances associated with each mating encounter (Anthes et al., 2006).

In order to achieve good results on broodstock reproduction the following issues must be carefully addressed: 1) food must never be a limiting factor, nor negatively affect water quality parameters (Plaut et al., 1995; Capo et al., 2002); 2) pairing similar sized animals may increase the number of produced embryos (mated specimens will reproduce both as male and female), as size-differences can influence mating behaviour and small sized animals are more prone to mate in female role (Angeloni and Bradbury, 1999; Angeloni et al., 2003; Anthes et al., 2006); 3) stocking groups of breeding organisms is advisable, as long as animal density is maintained between the limits that induce matting strategies and do not suppress somatic growth (e.g., between

5 and 7 animals per breeding tank for *Aplysia*; see Capo et al., 2002). Animals stocked at high densities may display slower growth rates and contrasting final weights, but often display a synchronous onset of sexual maturity independently of stocking densities (Capo et al., 2002).

2.4.5 Embryos incubation

The number of embryos produced per spawning in sea slugs is species specific and depends on parental size (Switzer-Dunlap and Hadfield, 1979; Hadfield and Switzer-Dunlap, 1984). Switzer-Dunlap and Hadfield (1979) found that the lifetime egg production of certain anaspideans to be up to 272 × 106, while small sacoglossans have been reported to produce between 1000 and 2250 eggs (Chia, 1971). The shape of egg masses, as well as the attachment mode, may vary among families and genera, and may even be a useful feature to distinguish between sea slug species (Franz, 1975). Eyster (1986) reviewed the ultrastructure of nudibranch egg capsules, while the histology and ultrastructure of egg masses of several Heterobranchia was reviewed by several authors (Wägele, 1989, 1996; Klussmann-Kolb and Wägele, 2001).

While the majority of sea slug readily spawns on any submerged surfaces, some species may display more specific requirements. As an example, *S. neapolitana* will only lay its embryos if the substratum is clean (Schlesinger et al., 2009). For sacoglossan sea slugs, oviposition is usually facilitated if the host algae is present in the breeding tank (sea slugs will lay their embryos on the surfaces of the algae) (Franz, 1975). For some species, portable egg-laying substrates can be used as they provide shelter for breeding pairs, increase the available area for attaching egg masses and allow a better monitoring of developing embryos. These shelters can be made of PVC tubes, which are set into breeding tanks (Schlesinger et al., 2009). The inspection of these structures can be facilitated if PVC pipes are split in two longitudinally and hold together with rubber bands; by removing the rubber bands it is possible to perform a close inspection of the embryos attached to the inner

walls of the PVC pipe. Another alternative often employed to culture *A. stephanieae* is to set inside broodstock tanks clay pots placed upside down, which are drilled in their bottom surface; breeding pairs commonly lay their embryos inside the pot, which can later be easily removed and inspected (Banger, 2011). Some small sea slugs from the genus *Aeolidiella, Cuthona* and *Calma* can also spawn at the air-water interface but egg masses must be submerged artificially to develop and avoid contact with air (G. Calado, unpublished data). It is a common practice to carefully take/detach the egg masses from the substrate and transfer them to sterile beakers, or Petri dishes filled with filtered seawater, which are latter placed in an incubator (to control for temperature and photoperiod) until the hatching of veliger larvae. Recent advances were made by Banger (2011) on the development of a system that avoids the physical contact with egg masses during incubation. The “Banger breeding chamber” consists of an outer chamber that houses an inner chamber holding breeding sea slugs and a deep “flow through sand bed” (Fig. 2.4A). The deep “flow through sand bed” provides a natural barrier that avoids newly hatched larvae or juveniles to be drained through the outflow and damaged by filtration systems. It is well known that sea slug’s egg masses are commonly sensitive to external factors, namely water quality and circulation. In static incubation systems, water changes are usually performed daily or every other day to prevent water quality deterioration. Temperature, light, pH and salinity should be identical to that of parental stocking tanks and maintained constant during the incubation period (a feature that is more easily achieved when employing flow-through or recirculated systems than in static incubation tanks). The most suitable option to aerate sea slugs egg masses during incubation appears to be species-specific, as Carrol and Kempf, (1990) recommend a gentle air bubbling for *A. stephanieae* and Capo et al., (2009) advocate the use of a vigorous aeration to incubate the embryos of *A. californica*.

2.5 Larviculture

Larval culture is often a challenge for the production of most marine invertebrates, and most sea slugs are no exception to the rule. Researchers studying the early life history of Heterobranchia of interest for biomedical research provided detailed descriptions of their larviculture trials (Kriegstein et al., 1974; Strenth and Blankenship, 1978; Paige, 1988), particularly those targeting *Aplysia* (Kriegstein, 1977; Switzer-Dunlap and Hadfield, 1977). Larviculture of other ecologically important sea slugs (e.g. *Alderia modesta, Hermissenda crassicornis, Doridella obscura, Adalaria proxima, Dendronotus frondosus, Dollabella auricularia*) has also been described by several authors (Clark, 1975; Perron and Turner, 1977; Switzer- Dunlap and Hadfield, 1977; Harrigan and Alkon, 1978; Krug and Zimmer, 2000; Sisson, 2005). However, scientific information on larval feeding is still extremely limited (Switzer-Dunlap and Hadfield, 1977; Hubbard, 1988; Plaut et al., 1995; Avila et al., 1997). While a small number of sea slugs can already be mass cultured in captivity (Capo et al., 2002; Schlesinger et al., 2009, Banger, 2011), the small size displayed by the newly hatched larvae of most species, along with their unknown feeding requirements, are still a challenge for researchers. The need to develop suitable systems/techniques for rearing the larval forms of sea slugs is essential for a successful commercial scale production of these organisms. It is, therefore, urgent to gain further knowledge on sea slugs larviculture system design, larval feeding, and nutrition, as well as optimal culture conditions.

2.5.1 Larval development modes

As for several other marine invertebrates, the initial development of sea slugs occurs in protected egg masses, which is often followed by a free-swimming larva – the veliger (Oyarzun and Strathmann, 2011). Sea slugs may undergo metamorphosis inside the egg capsules and crawl out as adults (direct development), or metamorphose outside the capsules (indirect development) (Carroll and Kempf, 1990). If they come out from the egg capsule as larvae,

they may develop without feeding stages (lecithotrophic development, e.g., *Adalaria proxima*) (Thompson, 1976) or they may need to feed on phytoplankton for days, weeks, or even months (planktotrophic development, e.g., *Aplysia californica*) (Capo et al., 2009). Although the nutritional modes of marine invertebrate larvae are typically ranked in a dichotomy between planktotrophy or lecithotrophy (Strathmann, 1985), sea slugs are known to exhibit poecilogony - the existence of variable larval development modes within the same species (e.g., *Alderia willowi, Elysia pusilla, Elysia cause, E. chlorotica*) (Allen and Pernet, 2007; Vendetti et al., 2012) (see Fig. 2.5). In other words, the type of larvae produced from the egg masses of certain sea slugs can be modulated through culture conditions; as an example, *Alderia willowi* is known to produce a higher proportion of lecithotrophic larvae under high temperature and salinity, whereas the production of plankthrophic larvae is favoured under lower temperature and salinity (Krug et al., 2012). Several studies on the larval development of sea slug species displaying planktotrophic larvae are available, particularly for nudibranchs (Hadfield and Miller, 1987; Todd et al., 2001), sacoglossans (Seelemann, 1967; Clark, 1975; Clark and Goetzfried, 1978; Harrigan and Alkon, 1978; West et al., 1984; Trowbridge, 1998; 2000; Krug and Zimmer, 2000) and aplysiids (Hadfield and Switzer-Dunlap, 1984; Thompson and Jarman, 1989). From an aquaculture point of view, the most desirable sea slug species for culture, in increasing order of preference, will be those displaying a short planktotrophic larval development, a lecithotrophic larval development, or a direct development (the most desirable for culture).

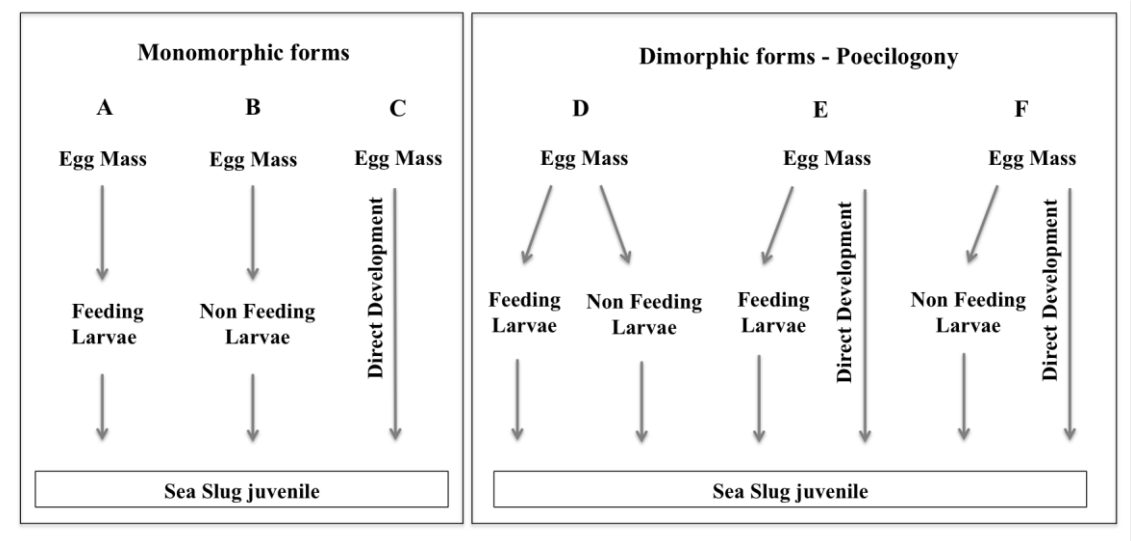

Fig. 2.5 Overview of monomorphic and dimorphic modes of development in sea slugs. Monomorphic forms: A) Feeding larvae (planktotrophic, require a suitable supply of phytoplankton after hatching to reach metamorphosis), B) Non-feeding larvae (lecitotrophic, no exogenous food is required after hatching to reach metamorphosis), and C) Direct development (metamorphosis occurs inside the egg capsule and an *imago* of the adult crawls from egg mass): Dimorphic forms (different modes of development can occur within the same egg mass - poecilogony): D) Feeding and non-feeding larvae can hatch from the same egg mass, E) The release of feeding larvae and *imagos* of the adult (direct development) can be recorded from the same egg mass, and F) The release of non-feeding larvae and *imagos* of the adult (direct development) can be recorded from the same egg mass.

2.5.2 Larviculture techniques

Earlier trials of sea slugs larviculture performed during the 1960s and 70s relied on static culture approaches (e.g., Franz, 1975; Kriegstein et al., 1974; Switzer-Dunlap and Hadfield, 1977 and references therein). While most research efforts have targeted the culture of sea slugs displaying planktotrophic larvae (Harris, 1970; Nadeau et al., 1989; Avila et al., 1997), it is not surprising to verify that the most successful larviculture experiments were achieved when addressing species displaying lecithotrophic larval development (Thompson, 1958; Swennen, 1961; Tardy, 1962, 1970; Harris, 1970). In general, researchers transfer newly hatched veligers to sterile dishes (Capo et al., 2009) or beakers (Trowbridge, 2000) for posterior culture. Filtered seawater is commonly used, with water changes being performed from 1 to 4 times a week with the help of mesh screens to retain cultured larvae (mesh screens of variable sizes can be easily constructed using PVC rings as frames) (e.g., (Franz, 1975; Carroll and Kempf, 1990; Avila et al., 1997; Trowbridge, 2000; Capo et al., 2009). Larvae retained on the mesh screen can

be washed to Petri dishes using a pipette and then poured into a new culture beaker with filtered seawater and algal food (Trowbridge, 2000). Young sea slugs larvae are positively phototactic and may be trapped at the air-water interface when swimming towards the top of culture vessels illuminated from above. The trapping of veligers in the air-water interface is due to surface tension and can be reduced by keeping culture vessels in the dark (Franz, 1975). Hurst (1967), Harris (1970) and Harrigan and Alkon, (1978) reported that cetyl alcohol can be successfully used to reduce larval mortality due to this phenomenon, namely by sprinkling flakes of this compound on the water surface of the culture vessel. Capo et al., (2009) successfully used an iodine-based surfactant to re-suspend any larvae entrapped at the air-water interface. Another bottleneck often faced during the larviculture of sea slugs is the susceptibility of cultured larvae to infections by bacteria, fungi, and protozoans. Cleanliness is the best solution to prevent this problem. Some authors use antibiotics, such as penicillin and streptomycin sulfate (Franz, 1975; Switzer-Dunlap and Hadfield, 1977) or anti septic solutions (e.g. poly-iodine complex and fish-grade Trizma (Capo et al., 2009), Chloramphenicol and EDTA (Harrigan and Alkon, 1978; Avila et al., 1997; Sisson, 2005) to suppress the growth of unwanted microorganisms. Several studies have already addressed the effect of initial larval density on growth, metamorphic competency and survival of sea slugs larvae (Bayne, 1965; Switzer-Dunlap and Hadfield, 1977; Avila et al., 1997; Capo et al., 2009; Schlesinger et al., 2009). The general trend of recording decreasing survival rates at increasing larval densities has been demonstrated for several species (Hubbard, 1988; Avila et al., 1997). This trend may be attributed to collisions among developing larvae, which may promote a range of deleterious effects, namely feeding inhibition and physical injuries. A higher rate of disease transmission under high larval densities was also suggested by Capo et al., (2009) in order to explain lower survival rates under such culture conditions.

2.5.3 Larval feeding

As previously referred, larvae can hatch either as feeding or non-feeding forms. Feeding veligers rely on the ingestion of phytoplankton for a variable period of time (weeks to months) until reaching metamorphosis. The duration of the larval phase is known to be species-specific and mainly regulated by culture conditions (e.g., water quality, temperature). The biggest constraint for the large-scale culture of sea slugs has long been diagnosed - the provision of an adequate larval diet, namely for species with larvae displaying undeveloped or rudimentary feeding structures (Franz, 1975). Feeding larvae will only grow at maximum rates if the preys provided fulfil all of their nutritional requirements (Pechenik and Heyman, 1987). “Non-feeding" veligers rely on yolk reserves provided by parental organisms to fuel their energetic demands until metamorphosis; while non-feeding larvae commonly develop in a relatively short period of time to metamorphosis, in some cases such larvae can feed secondarily and persist in the plankton for long periods (Kempf and Hadfield, 1985). Species that display direct development commonly produce larger sized juveniles than those resulting from the metamorphosis of planktotrophic or lecithotrophic veligers. Feeding protocols currently available for sea slugs comprise the supply of small-sized phytoplankton species (e.g., *Isochrysis, Tetraselmis, Rhodomonas*) to their developing larvae, either as pure or mixed diets (Harris, 1975; Harrigan and Alkon, 1978; Perron and Turner, 1977; Avila et al., 1997; Trowbridge, 2000; Schlesinger et al., 2009) (see Table 2.3). Previous studies have already demonstrated that species composition of microalgal diets (either monospecific or mixed), as well as their concentration, play a key role on the success of 618 sea slugs larviculture trials (Switzer-Dunlap and Hadfield, 1977; Hubbard, 1988; Capo et al., 2009; Schlesinger et al., 2009). Algal uptake in sea slugs veligers is regulated by the size and density of the microalgae provided (Chia and Koss, 1978). As shown for other organisms, larger microalgae may decrease total ingestion as a consequence of particle interference at the velar edge, longer handling time inhibiting the simultaneous ingestion of smaller particles or post-ingestive rejection (Strathmann, 1987). Most studies report algal concentrations ranging from 10

$\times 10^3$ (Chia and Koss, 1978; Plaut et al., 1995; Trowbridge, 2000) to 10×10^4 cell.ml^{-1} (Kriegstein et al., 1974; Switzer-Dunlap and Hadfield, 1977; Kempf, 1981; Paige, 1986; Avila et al., 1997) as suitable to culture sea slug veligers (Table 2.3). Nonetheless, under higher algal concentrations, it is common to record a faster growth, a higher survival and a shorter period required for larvae to reach metamorphic competence (Hubbard, 1988; Capo et al., 2009). Concerning the larviculture of *Aplysia*, static rearing conditions proved to be unsuccessful (Kriegstein et al., 1974; Capo et al., 1987; Nadeau et al., 1989), as water movement is needed to keep food in suspension so that it remains available for larvae under culture (accelerated particles are more likely to encounter the vela cirri and be ingested more easily). The use of roller bottles that maintain algal preys in constant suspension while providing a homogenous non-turbulent environment for rapid larval growth were a significant breakthrough for the successful larviculture of *Aplysia* (Capo et al., 2009).

Table 2.3 Larviculture conditions employed to raise sea slugs, with emphasis to larval diet, density (larvae mL^{-1}) and days required to reach metamorphis.

Species	Temp. (ºC)	Diet	Density	Metamorphosis	References
Aeolidiella stephanieae (N) [a]	21-26	No exogenous food required	-	0 or 13-15	1-2
Alderia modesta (S) [b]	25	Mixed: *Rhodomonas* sp., *Isochrysis galbana, Pavlova lutheri* (10^4 cell ml^{-1})	-	3-6	3
Aplysia californica (A) [c]	22	Mixed: *Isochrysis sp*. and *Chaetoceros muelleri* (1:1) (250x 10^3 cells ml^{-1})	0.5-1	35	4-5
Aplysia dactylomela (A) [d]	24-26	*Pavlova lutheri*, *I. galbana, Dunaliella tertiolecta, Nannochloris sp*.	0.8-1	30	6
Aplysia juliana (A) [e]	23-28	*Ulva* sp.	0.8-1	28	6
Aplysia oculifera (A) [e]	24	*I. galbana* (10^4 cell ml^{-1})	<1	28	7-8
Bursatella leachii plei (A) [f]	25	*I. galbana* (10^4 cell ml^{-1})	1-5	20	9
Elysia chlorotica (S) [g]	10	*I. galbana* (10^4 cell ml^{-1})	-	25	10-11
Elysia timida (S) [d]	20	No exogenous food required	-	0	12
Elysia viridis (S) [d]	15	*Rhodomonas baltica* (10^4 cell ml^{-1})	-	28-30	13
Hermissenda crassicornis (N) [h]	12	Mixed:*Isochrysis sp*. and *Rhodomonas salina* (1:1) (10-25 x10^3 cell ml^{-1})	1-4	42	14
Phestilla sibogae (N) [e]	24-27	*Pavlova lutheri* (10^4 cell ml^{-1})	-	24 -29	15
Spurilla neapolitana (N) [i]	24	Mixed:*Isochrysis galbana* (10^5 cell ml^{-1}) + *Tetraselmis tetrathele* (10^3 cell ml^{-1})	4	25	16

A - Anaspidea, N - Nudipleura, S –Sacoglossa; a) cultured in Petri dishes and in recirculated systems (described by Banger, 2011) using Millipore-filtered aged natural seawater and artificial seawater, respectively; b) cultured in beakers and requiring 20 mM K^+ in the form of KCl to induce metamorphosis (Yool, et al., 1986); c) cultured in roller bottles with aeration and filtered sea water containing chloramphenicol and Na_2EDTA; d) cultured in beakers using filtered sea water; e) cultured in beakers with filtered sea water containing antibiotics (e.g., Penicillin G and Streptomycin sulfate); f) cultured in beakers with artificial seawater; g) cultured in beakers with artificial seawater containing antibiotics; h) cultured in roller bottles with filtered sea water containing chloramphenicol and EDTA; i) cultured using the double beaker method (as described by Strathman, 1987) with filtered sea water containing antibiotics; 1) Carroll and Kempf, 1990; 2) Banger, 2011; 3) Krug, 1998; 4) Capo et al., 2002; 5) Capo et al., 2009; 6) Switzer-Dunlap and Hadfield, 1977; 7) Plaut et al., 1995; 8) Kempf, 1981; 9) Paige, 1988; 10) West et al., 1984; 11) Rumpho et al., 2011; 12) Marin and Ross 1989; 13) Trowbridge, 2000; 14) Avila, et al., 1997; 15) Kempf and Hadfield, 1985; 16) Schlesinger, et al., 2009.

2.5.4 Metamorphosis and settlement cues

Planktonic larvae preferentially metamorphose in response to specific cues (Pawlik, 1992; Avila, 1998; Krug and Zimmer, 2000; Trowbridge and Todd et al., 2001) (see Table 2.4). However, there are several sacoglossan sea slugs (e.g., *Tenellia fuscata*, *Toranatina canaliculata*) that do not require any specific stimulus to trigger metamorphosis (Franz, 1975).

In the laboratory, sea slugs larvae may settle and metamorphose in response to a variety of artificial or natural cues (e.g., aqueous extracts from dietary prey). An increase in seawater concentration of potassium can trigger metamorphosis in some sea slug species (Yool et al., 1986; Todd et al., 1991). Nevertheless, this stimulus is not a universal substitute for natural cues promoting metamorphosis (Pechenik et al., 1995; Pechenik and Rice, 2001). Sea slug larvae will also metamorphose when exposed to neuroactive agents, such as choline (Todd et al., 1991) or organic compounds (e.g., acetone, ethanol, methanol) (Pechenik et al., 1995; Avila, 1998).

Water agitation may also be very effective to increase metamorphosis when natural inducers are present (Pechenik et al., 1995). A step increase in water temperature can induce an increase in the percentage larvae metamorphosing, but may also negatively affects latter life stages (Avila, 1998). Most Heterobranchia are known to be feeding specialists, with adult preys commonly acting as the metamorphic trigger for developing larvae (Avila, 1998). Metamorphosis of several sea slugs is known to be stimulated by chemical cues released from their invertebrate (Carroll and Kempf, 1990; Hadfield and Koehl, 2004; Ritson-Williams et al., 2003 and references therein; Krug, 2009) or algal prey (Kriegstein et al., 1974; Nadeau et al., 1989), as these cues will probably indicate that newly metamorphosed juveniles will be provided a suitable environment for their grow-out. It is also important to highlight that, at least for some herbivorous sea slug species, both host and non-host algae may induce competent larvae to metamorphose (Trowbridge, 2000).

The introduction of invertebrate or algal species known to trigger metamorphosis must be carefully timed, as only competent larvae (larvae which already have enough energetic reserves to undergo metamorphosis) will be receptive to any potential cues and metamorphose (Franz, 1975; Kempf and Willows, 1977; Avila et al., 1997). Between and within-culture variations in the timing of larval competence are well known and inevitable, being largely inherent to larval variability (Kempf, 1981; Plaut et al., 1995). The source of such variability has been attributed, among other aspects, to genetic differences, dietary or water quality deficiencies, the use of antibiotics and bacterial loads (Avila, 1998).

Table 2.4 Sea slugs already cultured in captivity and respective settlement cue(s) employed to trigger metamorphosis (all species listed under settlement cues are algae, unless indicated otherwise).

Species	Settlement cue(s)	References
Aplysia californica (A)	*Gracilaria ferox*; *Agardhiella* sp.; *Ulva* sp.; *Laurencia* sp.	1-2
Aplysia juliana (A)	*Ulva* sp.	3
Bursatella leachii plei (A)	*Microcoleus lyngbyaceous, Schyzothrix calcicola, Porphyrosyphon notarisii*	4
Corambe obscura (N)	*Electra crustulenta* (bryozoan)	5
Dendronotus frondosus (N)	*Obelia geniculate* (hydroid)	6
Elysia chlorotica (S)	*Vaucheria litorea*	7
Elysia viridis (S)	*Codium fragile, C. tomentosum, Cladofora rupestris*, conspecifics	8
Elysia timida (S)	*Acetabularia acetabulum*	9
Hermissenda crassicornis (N)	*Tubularia crocea and Pennaria* sp. (hydroid); *Metridium senile* and *Haliplanella luciae* (anemone)	10
Phestilla sibogae (N)	*Porites* sp. (coral)	11-13
Phestilla melanobranchia (N)	*Tubastrea aurea* (coral)	14

A - Anaspidea, N - Nudipleura, S –Sacoglossa; 1) Pawlik, 1989; 2) Nadeau, et al., 1989; 3) Switzer-Dunlap and Hadfield, 1977; 4) Paige, 1988; 5) Perron and Turner, 1977; 6) Paige, 1988; 7) Rumpho et al., 2011; 8) Trowbridge and Todd, 2001; 9) Marín and Ros, 1993; 10) Avila, 1998; 11) Hadfield, 1977; 12) Ritson-Williams et al., 2003; 13) Ritson-Williams et al., 2009; 14) Ritson- Williams et al., 2007.

2.6 Juvenile grow-out

2.6.1 Feeding and nutrition

Sacoglossans are specialized suctorial feeders, with most species being stenophagous herbivores (Jensen, 1994) that may switch their food preference during grow-out (Thompson and Jarman, 1989; Trowbridge, 2004). The same level of specialization is valid for the majority of nudibranchs, with their diets being commonly limited to a single species (Thompson and Jarman, 1989). An electronic register of the worldwide food habits of nudibranchs was created by Gary R. McDonald and James W. Nybakken and can be consulted on www.theveliger.org webpage.

In general, aplysiids are more generalist feeders and can feed upon one or more species of algae (Carefoot, 1987). Increasing evidences suggest that feeding preference for certain sea slugs are related to the secondary metabolites they will acquire by feeding upon a certain prey (Barile et al., 2004). Artificial diets have already been successfully used to raise juvenile sea slugs. As an example, a diet made up of chemicals and set in agar with a small amount of water extract of the green alga *Ulva fasciata* promoted acceptable growth levels and even induced spawning in the sea hare *A. dactylomela* (Carefoot, 1980).

Studies specifically addressing the nutritional requirements of juvenile sea slugs during grow-out seem to be inexistent in the scientific literature. The information retrieved from ecological based works is also scarce, being limited to one study addressing lipid classes and fatty acids (FA) in two nudibranch genera (*Felimida* and *Phyllidia*) (Zhukova, 2007). In the previous study, phospholipids were shown to be the dominant lipid class, followed by sterols. A wide diversity of fatty acids was also recorded, with the typical marine *n*-3 polyunsaturated fatty acids (PUFA) comprising only a small proportion of the total pool of FAs (0.6 to 1.3%). On the others side, *n*-6 PUFA represented up to 25% of the total pool of FAs. It is, therefore, urgent to promote further research on the nutrition of juvenile sea slugs and determine if formulated

diets can be a reliable alternative to the use of live/frozen preys during grow-out.

2.6.2 Culture systems

The recent need for controlling animal quality and information on their age, parental background and reproductive state, was the main driver stimulating research on the grow-out of sea slugs. Successfully controlling water temperature, light, stocking density and food quantity and quality are pointed as the key-issues for culturing juvenile sea slugs at a commercial scale (Capo et al., 2009). Several factors have already been identified as potential growth inhibitors: 1) the release of animal pheromones that trigger mass spawning and/or suppress somatic growth (Audersirk, 1979; Levy et al., 1997); 2) high ammonia levels promoted by animal waste that are detrimental to the health of stocked organisms (Handy and Poxton, 1993); 3) toxic compounds secreted from uningested prey in response to grazing stimulus (Handy and Poxton, 1993; Toth and Pavia, 2001); and 4) high prey biomass that may deplete from seawater important nutrients required for the somatic growth of juvenile sea slugs (Capo et al., 2009). It has been suggested that crowding *per se* does not affect the timing for sexual maturity as long as food is available, but food limitation *per se* may negatively affect the onset of sexual maturity (Plaut et al., 1995; Capo et al., 2009). Grow out systems used for juvenile sea slugs are usually the same used for broodstock maintenance. The most successful systems employed so far for growing sea slugs are those by Banger (2011) for the mass grow-out of the ornamental nudibranch *A. stephanieae* (Fig. 2.4A) and by Capo et al., (2009) for the commercial scale culture of the sea hare employed in biomedical research *A. californica* (Fig. 2.4B).

2.6.3 Live shipping

As there are no specific live shipping protocols for sea slugs, we recommend the use of identical procedures to those described by Wabnitz et al. (2003),

which are commonly employed to ship marine ornamental invertebrates. At their origin, sea slugs should be quarantined and starved for at least 48 hours prior to shipment; this procedure will ensure that they do not excrete any undesirable compounds into the water of the shipping container. As most invertebrates, sea slugs should be packed in polyethylene bags with rounded bottom, filled with one-third seawater and two-thirds oxygen, sealed and placed in a polystyrene box (for insulation and shock resistance). Another method employed for shipping small sized sea slugs is the use of plastic bottles or vials. To avoid excessive risks, a maximum travel time of 48 hours is recommended for shipments of live sea slugs. At destination, newly arrived specimens should be slowly acclimated to the water chemistry of their new stocking system, with the dripping acclimation method commonly being a suitable solution. It is also important to highlight that that shipping water should always be discarded and never added to the new husbandry system that will house the sea slugs.

2.7 Concluding remarks

While new advances have been achieved in the lasts decades in the culture of sea slugs, large scale production is still restricted to a reduced number of species. Commercial scale production has only been implemented for *A. californica*, although recent data using small-scale recirculated culture systems (e.g. for culturing *A. stephanieae*) open good perspectives for the production of several other species. Future research efforts should target the standardization of larviculture systems and feeding protocols, as well as the clarification of the mechanisms involved in the trigger of metamorphosis for the most desirable species for captive culture. Another topic that should be investigated is the potential to replace live, freshly harvested or frozen prey by formulated diets that can be customized to meet species-specific nutritional requirements of adult and juvenile sea slugs. The successful establishment of reliable culture systems and protocols would certainly provide the necessary

know-how to prompt the large scale production of the most demanded sea slugs for academic research or commercial purposes.

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Chapter 3 Anesthetizing solar-powered sea slugs: a need for photobiological studies of kleptoplasts using PAM fluorometry

Abstract

Photosynthetic sea slugs have the ability to “steal” chloroplasts (kleptoplasts) from marine macroalgae and keep them structurally intact and physiologically functional. The photosynthetic activity of these symbioses has been assessed using Pulse Amplitude Modulated (PAM) fluorometry. However, the movement of these sacoglossan slugs can impair specific photobiological studies on kleptoplasts. Thus, immobilizing sacoglossan slugs, while not interfering with the photosynthetic activity, would be a methodological advance for research on this field. We evaluated the effect of two anaesthetics, eugenol and MS-222, on the photosynthetic activity of kleptoplasts and on the behaviour of kleptoplasts-bearing slug *Elysia viridis*. Anaesthetics promoted sea slug muscle relaxation with no touch reaction in approximately 6 min. Sea slugs immobilized for 120 min completely recovered after anaesthetic removal. No significant differences were found on photosynthetic parameters measured immediately (0-1 min) after immobilization. The effective quantum yield of photosystem II of *E. viridis* after 120 min of immobilization was significantly decreased by 12% in the MS-222 treatment, while eugenol promoted no significant effect. Photosynthetic activity assessed by rapid light-response curves (RLC) of relative electron transport rates (rETR) revealed a significant decrease in both initial response to light (-34%) and maximum rETR ($rETR_m$) (-60%), after 120min of immobilization using MS-222. After 120 min of immobilization with eugenol, the initial response to light significantly decreased 15% and $rETR_m$ decreased 27%. We conclude that, whenever photobiological studies employing PAM fluorometry require immobilization of photosynthetic sea slugs, eugenol can be used as a powerful anaesthetic with little impact on kleptoplasts photosynthetic activity.

Keywords
Anaesthetic; *Elysia*; kleptoplast; PAM fluorometry; photobiology.

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3.1 Introduction

Sacoglossan sea slugs, mainly from the family Plakobranchoidea, have developed the capacity of acquiring phototrophic-mediated carbon. Rather than hosting endosymbiotic microalgae as nudibranch (Wägele and Johnsen, 2001), sacoglossans graze on macroalgae and sequester plastids into tubule cells of their digestive diverticula (Kawaguti and Yamasu, 1965), a mechanism often named kleptoplasty or kleptoplastidy (for review see Johnson, 2011).

Chlorophyll (Chl) fluorescence is a rapid and non-intrusive method widely used to study photosynthesis (Baker and Oxborough, 2004) replacing to a certain extent the use of oxygen evolution or radiolabeled CO2 fixation in the study of this process. The commercial availability of reasonably cheap, easy to use and portable modulated fluorometers, extended the use of Chl fluorescence analysis to a wide range of photosynthetic organisms, including photosynthetic sea slugs (e.g. Evertsen et al., 2007; Vieira et al., 2009; Jesus et al., 2010; Schmitt and Wägele, 2011). However, PAM fluorometry was developed for higher plants and not envisioned for studying motile organisms. In order to accurately address the photophysiology of kleptoplasts in these “solar-powered” organisms, it is important to maintain the target animal immobilized during measurements. Some authors have immobilized sea slugs by carefully placing the animal in the well of a concavity microscope slide, filled with seawater and covered with a coverslip (Vieira et al., 2009; Serôdio et al., 2010). Nevertheless, E. viridis individuals are still able to slightly move within this limited space. Other authors have simply preferred to continuously adjust the animal to the fixed PAM fluorometer’s optical fiber (Jesus et al., 2010; Schmitt and Wägele, 2011) or place the animal in small vials (Evertsen et al., 2007).

While the methods described above can be satisfactory when a short saturating light pulse is applied to access, for instance, the maximum quantum yield of photosystem II (PSII) of a dark adapted sample (Fv/Fm, see Table 3.1 for notation), the measurement of more complex PAM fluorometry parameters can be compromised by the animal movement. A good example of a photobiological parameter that can be biased by sea slug movement is the characterization of the

kinetics of induction and relaxation of Chl a fluorescence. This type of measurement is commonly used to study the operation of photoprotective processes and the occurrence of photoinhibition in plants and algae (Niyogi, 1999; Müller et al., 2001). The lowering of fluorescence yield as a result of photoprotective or photoinhibitory processes is quantified by the non-photochemical quenching (NPQ) of Chl a fluorescence based on the variation of maximum fluorescence from dark-adapted to light-adapted state (Fm and Fm' respectively; see Table 3.1 for notation) (Müller et al., 2001). If sea slugs are not fully immobilized, even slight movements of the target animal will cause non-physiological changes in the steady-state fluorescence signal which, as a consequence, will compromise the relation between the maximal fluorescence of dark-adapted and light-adapted samples to be used, for instance, in NPQ calculations.

Steady-state light-response curves (LC) and/or rapid light-response curves (RLC) of relative electron transport rate (rETR, see Table 1 for notation) in photoacclimation studies can also be affected by sea slugs movement. Both type of light-response curves are constructed by exposing the sample to increasing light steps for a certain period of time in each irradiance level and calculating rETR at each of those levels. rETR is calculated by multiplying the effective quantum yield of photosystem II (PSII) at a certain irradiance by that same given irradiance. However, light-dependent behaviour in sea slugs has been reported (Giménez-Casalduero and Muniain, 2008; Jesus et al., 2010; Schmitt and Wägele 2011) and it is expected that at least some sea slugs will use their lateral body flaps (parapodia) to cover their dorsal surface as a protection to excessive light. Therefore, if the animal is able to move during fluorescence measurements it will be able to regulate incident light by closing/opening the parapodia introducing potential sources of error when calculating rETR (= E × ΔF/Fm'), especially at high light: 1) it is very likely that the effective quantum yield of PSII (ΔF/Fm') will be overestimated due to the animal photoprotective behaviour; and 2) the irradiance reaching the kleptoplasts will be animal behaviour-dependent with the consequence that the actual light reaching kleptoplasts is variable and different from that used in the rETR calculation (E).

Given that, specific parameters of PAM fluorometry can be significantly biased by animal movements, the use of anaesthetics to immobilize *Elysia viridis* individuals was investigated. Hypothermia has been a common method to diminish animal movement, sometimes with little or no regard to the animal's well-being. However, hypothermia would not be suitable in the study of sea slugs bearing kleptoplast due to low temperature-induced susceptibility of PSII to photoinhibition (Falk et al., 1996 and references therein). Magnesium chloride has been used before in sacoglossa (Clark et al., 1981), but it was found to interfere with photosynthetic functions of PSII (Liang et al., 2009) and thus not tested in the present study. MS-222 (tricaine methanesulphonate) has been for decades one of the most commonly used fish anaesthetics (Rombough 2007; Kiessling et al., 2009) and eugenol, the major constituent of clove oil, has been introduced as an eco-friendly alternative to anesthetize fish (Palić et al., 2006; Ghanawi et al., 2011). For these reasons, the effects of 0.1 ml L-1 eugenol and 0.8 g L-1 MS-222 on the photosynthetic activity of kleptoplasts in E. viridis were tested.

3.2 Materials and Methods

3.2.1 Biological material

Adults of the sea slug *Elysia viridis* (Montagu, 1804) and its dietary prey, the macroalgae *Codium tomentosum* Stackhouse, 1797, were collected on an intertidal flat in the northwest of Portugal (Aguda beach, 41º02'52.29''N and 8º39'14.43''W). *E. viridis* individuals and *C. tomentosum* were maintained in recirculating seawater under low light (water surface incident light: 20 µmol photons m^{-2} s^{-1}) at 18°C on a 14h light:10h dark photoperiod. *C. tomentosum* was replaced every two to four weeks.

3.2.2 Preliminary test: immobilization and survival of *E. viridis* exposed to different concentrations of eugenol and MS-222

Sea slugs full length was measured in order to select an experimental size range between 9 and 12 mm (typical size for adult *E. viridis* collected in our sampling area). To select the ideal concentration of each anaesthetic, a batch of

concentrations from 0.025 to 0.1 ml L^{-1} eugenol (Sigma-Aldrich) and 0.04 to 0.8 g L^{-1} MS-222 (Tris, pH 8) (Sigma-Aldrich) was used to test i) full immobilization versus time of exposure and ii) post-exposure locomotion recovery. The selected concentrations, 0.1 ml L^{-1} eugenol and 0.8 g L^{-1} MS-222, were chosen based on i) the rapid immobilization of *E. viridis* individuals in the range size of 9 to 12 mm observed at these concentrations ii) and full post-exposure motility recovery in a short period of time. Recovery from anaesthetic exposure was done by transferring the sea slugs to a new beaker containing clean sea water (0% anaesthetic) and monitoring the time to full recovery. We considered that sea slugs would have fully recovered when normal locomotion (e.g. climbing the submerged walls of the beaker without falling) was observed.

3.2.3 Experimental set-up

E. viridis adults measuring 9 to 12 mm full length were selected and placed under low light (20 µmol photons m^{-2} s^{-1}) and room temperature (20°C) for 30 min. These light and temperature conditions remained the same throughout the experiment. Six sea slugs per treatment were exposed to 0.1 ml L^{-1} eugenol, 0.8 g L^{-1} MS-222 or no anaesthetic in 50 ml of seawater. The effective quantum yield of PSII, $\Delta F/F_m'$ (see Table 3.1 for notations), was measured i) every ten minutes during low light and room temperature acclimation, ii) immediately after immobilization (IAI) or at a similar time for the control treatment and iii) during 120 min of the immobilization period. Rapid light-response curves (RLC, see description below and Table 1 for notation) were measured at the following time points: before exposure (BE), immediately (0-1 min) after immobilization (IAI) and 120 minutes after immobilization (120AI). For consistency between the control and anesthetized sea slugs, all measurements shown in Figs. 3.1 to 3.3 were done using a concave slide and a coverslip. More specifically, each sea slug was removed from the respective treatment, placed in the centre of the well filled with seawater and covered with a coverslip. The same part of the sea slug that was exposed to low light between measurements (sideways with closed parapodia or flat body with open parapodia, depending on the position taken by the sea slug when

immobilized) was the same as that facing the optical fiber during the measurements.

For the continuous record of PAM fluorometry measurements shown in Fig. 3.4, the sea slug was placed in a petri-dish containing 25 ml of seawater with 0.1 ml L^{-1} eugenol (just enough to cover the sea slug). The optical fiber was placed 1 mm above the water surface and covering most of the sea slug body. Measurements were done continuously with no movement of either the sea slug or the optical fiber. This procedure was necessary to assure the exact same spot was measured throughout the experiment as required for NPQ calculation (see Table 3.1 for notation). Since NPQ cannot be determined without a full immobilization of the sea slug, this parameter was not measured on sea slugs trapped between the lamina and the coverslip. Therefore, NPQ was only measured in anesthetized sea slugs placed in a Petri dish and 1 mm below the optical fiber.

3.2.4 Fluorescence measurements

Chl *a* fluorescence was measured using a Pulse Amplitude Modulation (PAM) fluorometer (Schreiber et al., 1986) comprising a computer operated PAM-Control Unit (Walz, Effeltrich, Germany) and a WATER-EDF-Universal emitter-detector unit (Gademann Instruments GmbH, Würzburg, Germany). Measuring, actinic and saturating light were provided by a blue LED-lamp (peaking at 450 nm, half-band width of 20 nm), and were emitted at a frequency of 18 Hz when measuring F_o (see Table 3.1 for notation) or 20 kHz when measuring other fluorescence parameters. The light delivered by the fluorometer and the fluorescence emitted by the sample were conducted by a 6 mm-diameter Fluid Light Guide fiber optics bundle in direct contact with the coverslip covering the sea slugs or 1mm distance from the water surface.

3.2.5 Rapid light-response curves (RLC) parameters

RLC were constructed by exposing the samples during ten seconds to 12 increasing irradiance levels (E, from 12 to 920 µmol photons m^{-2} s^{-1}). For every

irradiance level, the relative electron transport rate (rETR = $E \times \Delta F/F_m'$, see Table 3.1 for notations) was calculated and rETR versus E curves were constructed. The initial slop (α) and maximum rETR ($rETR_m$) of RLC were estimated by fitting the Eilers and Peeters (1988) model. The model was fitted iteratively using MS Solver and the curve fit was very good with r > 0.99 for a total of 54 light curves.

3.2.6 Statistical analysis

Differences in the times for immobilization and post-exposure recovery were tested using one-way analysis of variance (ANOVA). Effective quantum yield ($\Delta F/F_m'$), the initial slope (α) and the maximum rETR ($rETR_m$) of RLC were tested using two-way analysis of variance (ANOVA) for effects of anaesthetic and exposure time. Multiple comparisons among pairs of means were performed using Tukey's HSD. All statistical analyses were performed using the software Statistica 10 (StatSoft Inc., USA).

Table 3.1 Notation used in the text.

α	Initial slope parameter of the rETR vs. E curve
ΔF	Variable fluorescence ($=F_m' - F_s$) (dimensionless)
$\Delta F/F_m'$	Effective quantum yield of PSII (dimensionless)
E	Spectrally averaged ambient PAR (400–700 nm (μmol photons m^{-2} s^{-1})
rETR	Relative Electron Transport Rate ($= E \times \Delta F/F_m'$) (dimensionless)
$rETR_m$	Maximum relative Electron Transport Rate of the rETR vs. E curve (dimensionless)
F_o, F_m	Minimum and maximum fluorescence emitted by a dark-adapted sample (arbitrary units)
F_s, F_m'	Steady-state and maximum fluorescence emitted by a light-adapted sample (arbitrary units)
F_v/F_m	Maximum quantum yield of PSII of a dark-adapted sample (dimensionless)
LC	Light Curves: steady-state rETR vs. E curve
NPQ	Non-Photochemical Quenching of chlorophyll *a* fluorescence [$=(F_m-F_m')/F_m'$] (dimensionless)
RLC	Rapid light-response curves: rETR vs. E curve

3.3 Results

3.3.1 Immobilization and survival of *E. viridis* exposed to anaesthetics

There were no significant differences in time for immobilization (P=0.0626) and time for post-exposure recovery (P=0.5415) between sea slugs anesthetized with 0.1 ml L^{-1} eugenol or 0.8 g L^{-1} MS-222. Anaesthetics promoted sea slug muscle relaxation with no touch reaction (here considered the time of immobilization) in 3.8±1.7 and 7.7 ± 4.1 min (average ± s.d., n=6 individuals), in eugenol and MS-222 respectively. After 120m of exposure to the treatments, all sea slugs fully recovered locomotion after 20.0 ± 12.9 and 15.8 ± 9.7 min (average ± s.d., n=6 individuals) of post-exposure to eugenol and MS-222, respectively.

3.3.2 Effective quantum yield of PSII ($\Delta F/F_m'$)

After an acclimation to low light (20 µmol photons m^{-2} s^{-1}) and room temperature (20 °C) for 30 min before the experimental treatments, *E. viridis* adults exhibited an $\Delta F/F_m'$ before exposure (BE) of 0.77 ± 0.02, 0.78 ± 0.01 and 0.77 ± 0.02 (average ± s.d., n = 6 individuals) for control, eugenol and MS-222 respectively. Individuals exposed to seawater alone (control treatment) showed no significant difference (P=1.000) in the measured effective quantum yield of PSII ($\Delta F/F_m'$) throughout the duration of experiments (Fig. 3.1). Also, no significant differences (P=1.000 for control, eugenol and MS-222 treatments) were found between treatments BE and immediately after immobilization (IAI). At 120min of immobilization, $\Delta F/F_m'$ had decreased in anesthetized sea slugs to 0.73 ± 0.03 and 0.68 ± 0.03 (average ± s.d., n=6 individuals) for eugenol and MS-222, respectively. This decrease was gradual and, at each time of exposure, no significant differences in $\Delta F/F_m'$ were found between control and eugenol treatments (P=0.0750 at time 120 min of immobilization; P between 0.1747 and 1.0000 for any other times). The same result was found for sea slugs immobilized with MS-222 in the first 50 min after immobilization time (P=1.0000 at times BE, IAI, 10 and 20min after immobilization; P= 0.8939, 0.6285 and 0.2546 at times 30, 40 and 50 min after immobilization, respectively). However, at 60 min after immobilization

time, a significant difference (P=0.0232) in $\Delta F/F_m'$ was found between control and MS-222 treatments.

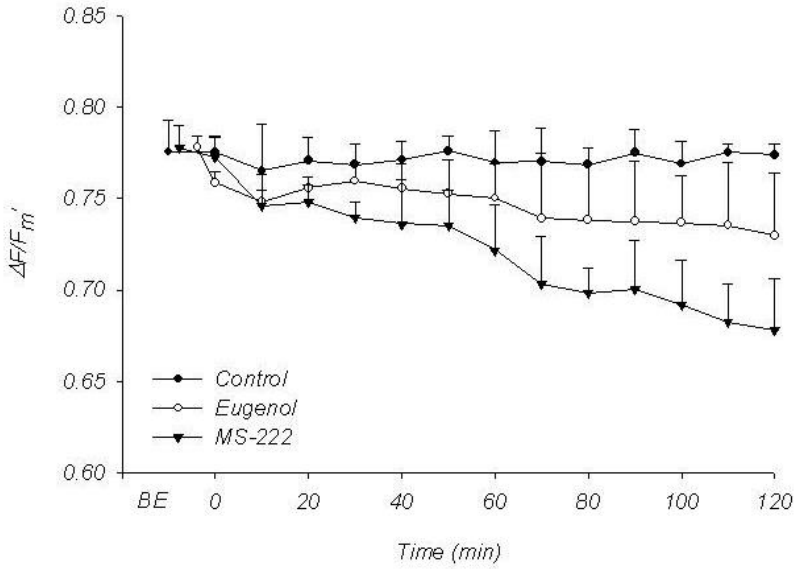

Fig. 3.1 Effects of 0.8 g L^{-1} MS-222, 0.1 ml L^{-1} eugenol in seawater and 0% anaesthetic (control) on the effective quantum yield of PSII ($\Delta F/F_m'$) of kleptoplasts in *E. viridis*. The time before exposure (BE) correspond to the last measurements taken before exposure to different treatments (10 min in control, 3.8min in eugenol and 7.7 min in MS-222 treatments), time 0 min correspond to time immediately (0-1min) after anaesthetic immobilization, remaining time correspond to exposure time after immobilization of *E. viridis*. Control: closed circles; Eugenol: open squares; MS-222: closed triangles. Bars represent the standard deviation (n = 6 individuals).

Between 60 and 120 min of immobilization all differences between control and MS-222 treatments were also significant (P=0.00004 for all comparisons). The decrease in $\Delta F/F_m'$ at 120 min of immobilization was 5.4 and 12.1% in eugenol and MS-222 treatments, respectively.

The minimal and maximal fluorescence of light-adapted individuals (F_s and F_m', respectively) used to calculate $\Delta F/F_m'$ (Fig. 3.1) were plotted against time and showed that F_m' remained reasonably stable (data not shown) while F_s was the main factor decreasing $\Delta F/F_m'$ during the exposure time. The latter correlation is represented in Fig. 3.2 where the same averaged $\Delta F/F_m'$ from Fig. 3.1 are plotted against the corresponding averaged F_s at each stage of the experiment: i) before immobilization (3 points: one time point every 10 min between 0 and 30 min of acclimation to low light and room temperature before exposure to anaesthetic

treatments) and ii) after immobilization (13 points: one time point every 10 min between 0 and 120 min of the immobilization period). Fig. 3.2 shows that $\Delta F/F_m'$ decrease was related to the increase in F_s in both eugenol and MS-222 immobilization. In both eugenol and MS-222, F_m' was reasonably stable during all period of animal immobilization. In the control treatment, both F_s and F_m' were stable throughout the experiment.

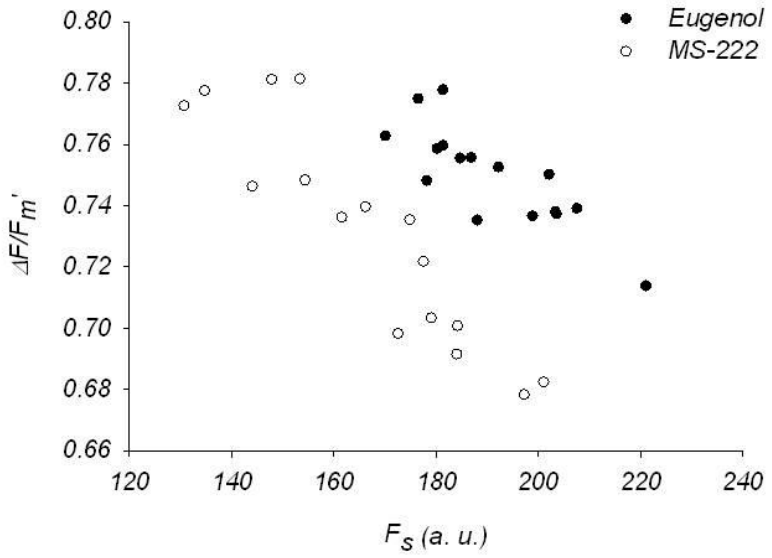

Fig. 3.2 Effective quantum yield of PSII ($\Delta F/F_m'$) and respective minimal fluorescence of light-adapted kleptoplasts (F_s) in *E. viridis* exposed to 0.8 g L^{-1} MS-222 and 0.1 ml L^{-1} eugenol at different time points before exposure to different treatments and during 120 min after immobilization. A number of replicas (*n*): 6 individuals. Maximal standard deviations measured were ± 62 and ± 55 for F_s in eugenol and MS-222, respectively, and ± 0.04 and ± 0.03 for $\Delta F/F_m'$ in eugenol and MS-222, respectively.

3.3.3 Rapid light-response curves (RLC) parameters

Individuals exposed to seawater alone (control treatment) showed no significant difference (P=1.0000) in the RLC parameters estimated ($rETR_m$ and α) throughout the duration of experiments (Fig. 3.3). When comparing different treatments at times BE and IAI, no significant differences were found in both photosynthetic parameters α (P=1.0000 for eugenol treatment; P=0.9999 and P=0.7465 for times BE and IAI in MS-222, respectively) and rETR (P=1.0000 for eugenol treatment; P=0.9697 and P=0.5941 for times BE and IAI in MS-222, respectively) (Fig. 3.3).

After 120 min of immobilization, both anaesthetics showed a significant effect on the photosynthetic parameter α (P=0.0001) with a decrease of 15% in eugenol and

34% in MS-222, relatively to control values (Fig. 3.3A). Regarding $rETR_m$, sea slugs anesthetized for 120 min with eugenol showed a decrease of 27% when compared to control sea slugs (Fig. 3.3B). This decrease in rETRm was not significant (*P*=0.0617). On the contrary, sea slugs anesthetized with MS-222 displayed a significant decrease (*P*=0.0001) on $rETR_m$ values after 120 min of immobilization (59.9%) when compared to control specimens (Fig. 3.3B).

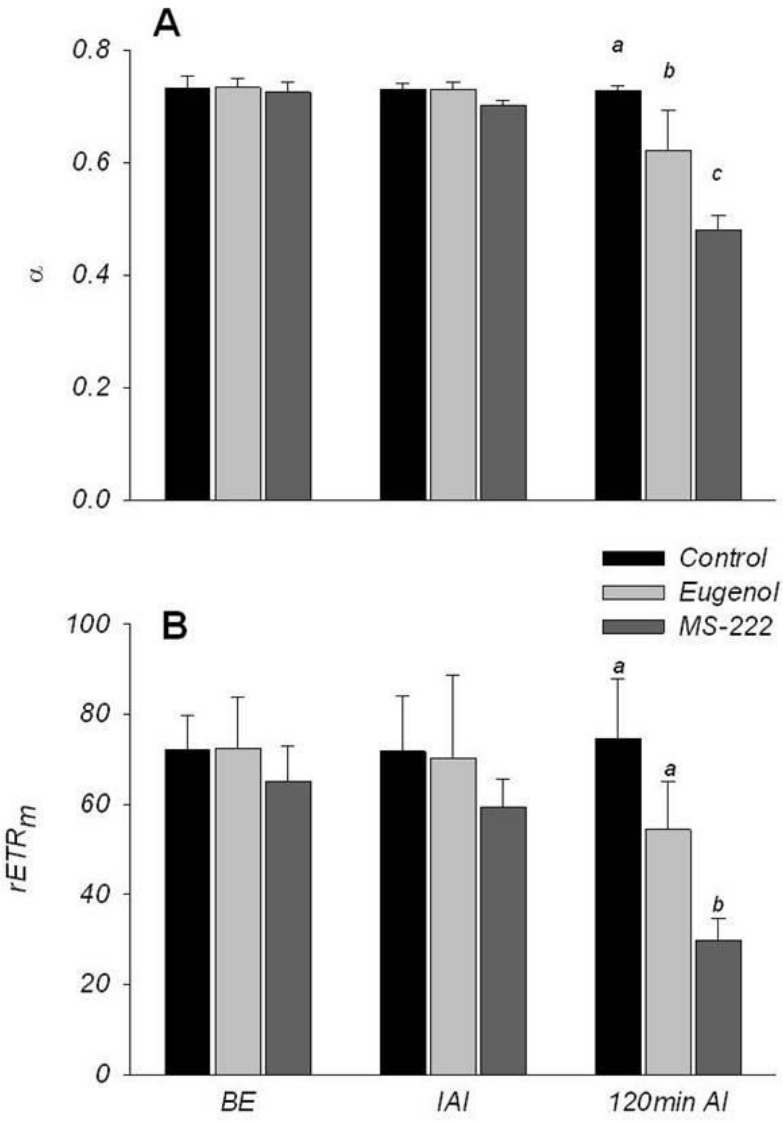

Fig. 3.3 Effects of 0.8 g L^{-1} MS-222, 0.1 ml L^{-1} eugenol in seawater and no anaesthetic (control) on rapid light-response curves (rETR vs. *E* curves) parameters measured in kleptoplasts of *E. viridis*. The initial slope (α) (Fig. 3A) and the maximum relative electron transport rate ($rETR_m$) (Fig. 3B) were measured before exposure (BE), immediately after immobilization (IAI) and 120 min after immobilization (120min AI). Bars represent the standard deviation (*n* = 6 individuals). Different letters (a, b and c) indicate significant differences between measurements in that group (see text for details).

3.3.4 Example of anaesthetic application

As an example of the need for sea slugs immobilization, we show a light stress experiment (Fig. 3.4) where we aimed to calculate the non-photochemical quenching [NPQ=F_m–F_m')/F_m'] and the maximal and effective quantum yield (F_v/F_m and ΔF/F_m' respectively, see table 3.1 for notation) at different stages of the experiment: i) dark adapted stage; ii) low light period for induction of light reaction; iii) high light period to induce a light stress; iv) and finally, the recovery when the light stress is over and the sample is transferred to dark condition. For calculation of F_v/F_m and ΔF/F_m' the permanence of the sea slug in the exact same position would not be crucial since those parameters derive from a ratio between absolute values. However, the light conditions that reach the sea slug would be different from those desired if the sea slug could hide or change parapodia position. For NPQ calculations, the exact position of the sea slug must be assured to guarantee that ground and maximal fluorescence are comparable at any time of the experiment. Fig. 3.4A shows that, when *E. viridis* individuals are immobilized (with eugenol), Chl *a* fluorescence can be recorded continuously in response to different light treatments and that any changes in fluorescence result from changes in the kleptoplasts physiological state rather than changes in the sea slug position. The photosynthetic parameters F_v/F_m, ΔF/F_m' and NPQ calculated from the given example of a continuous Chl *a* fluorescence record (Fig. 3.4A) is presented in Fig. 3.4B. NPQ was zero in dark and low light conditions and increased to 0.69 in 9 min of exposure to high light (619 µmol photons m^{-2} s^{-1}). After 24 min of relaxation in dark conditions, NPQ decreased by 7.6%, with no decrease observed for the first 14 min. F_v/F_m of the dark-adapted individual was 0.71 and decreased to nearly zero ΔF/F_m' in 2 min of exposure to high light (619 µmol photons m^{-2} s^{-1}). In the following 24 min of relaxation in dark conditions, F_v/F_m increased to 47.2% and 75.0% of the initial value after 2 and 24 min of post-light-stress, respectively.

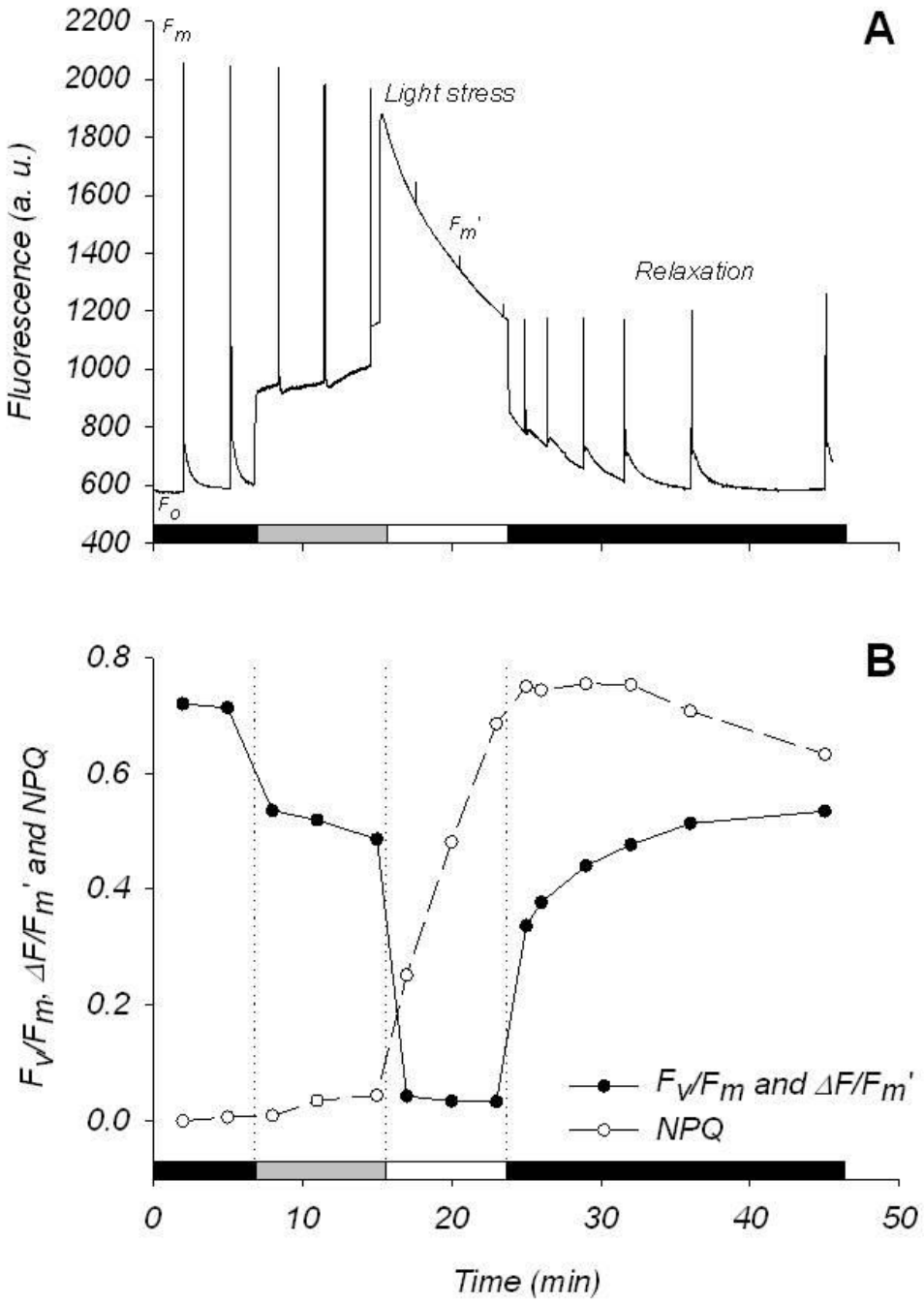

Fig. 3.4 Chlorophyll *a* fluorescence trace from an immobilized *E. viridis* individual (A) and respective F_v/F_m, $\Delta F/F_m'$ and NPQ (B) using 0.1 ml L^{-1} eugenol in seawater. In the presence of weak measuring light (1st dark bar, 2 data points) the minimal fluorescence of a dark adapted sample is seen (F_o). When a saturating light pulse is given, the photosynthetic light reactions are saturated and fluorescence reaches a maximum level (F_m). Upon continuous illumination with low light (grey bar: 16 µmol photons m^{-2} s^{-1}, used for activation of light reactions before the light stress, 3 data points) followed by moderately excessive light (white bar: 619 µmol photons m^{-2} s^{-1}, light stress, 3 data points) a combination of non-photochemical processes (e.g. NPQ) lowered the fluorescence yield. NPQ can be seen as the difference between F_m and the measured maximal fluorescence after a saturating light pulse during illumination (F_m'). After switching off the light (2nd dark bar: recovery, 6 data points), recovery of F_m' is expected to reflect relaxation of NPQ components.

3.4 Discussion

3.4.1 Choice of anaesthetic and respective concentration

Eugenol generally induced anesthesia faster at lower concentrations than MS-222, as shown before for fish (Munday and Wilson, 1997; Keene et al. 1998). In this study we measured similar anaesthesia times using 0.1 ml L^{-1} eugenol and 0.8 g L^{-1} MS-222, with a very high variation in sea slugs anesthetized with MS-222. Due to the wide use of eugenol in coral reef ecology, the effects of this anaesthetic have been tested on coral health and growth (Frisch et al., 2007, Boyer et al., 2009). Our results were in accordance with these authors, showing that low doses of eugenol had little or no effect on the photosynthetic efficiency of *E. viridis*. In the coral *Pocillopora damicornis*, low concentrations of eugenol (0.05 ml L^{-1}) had no effect on color or photosynthetic efficiency, irrespective of exposure time (1-60 min) (Frisch et al., 2007). Higher concentrations (0.5 ml L^{-1}) had variable effects with 10 min of exposure resulting in bleaching and reduced photosynthetic efficiency, longer exposure or higher concentrations caused total mortality (Frisch et al., 2007). Boyer and co-workers (2009) found similar results in three other species of corals (*Acropora striata*, *Pacillopora verrucosa* and *Porites australiensis*), with growth and occurrence of bleaching in 0.7 ml L^{-1} of eugenol in seawater not differing significantly from the control treatment (seawater). Higher concentrations (1.4 and 2.8 ml L^{-1}) of eugenol in seawater, as well as the optional dilution of eugenol in ethanol, showed deleterious effects on both coral growth and occurrence of bleaching (Boyer et al., 2009).

3.4.2 Experimental set-up and the problematic of a “real” control treatment

Ideally, the control treatment would allow the comparison of photosynthetic parameters between animals exposed to anaesthetics and animals in seawater alone, leaving aside the photobehaviour effect so that only the substances effect could be tested. The experimental design using a concave slide and a coverslip (Vieira et al., 2009) seemed the best option when trying to immobilize the animals without an anaesthetic. Nevertheless, most *E. viridis* individuals would still move

within that limited space during measurements of $\Delta F/F_m'$ and RLC construction. Moreover, they were allowed to move their parapodia while exposed to light (20 µmol photons m^{-2} s^{-1}) during the experiments. On the other hand, anesthetized sea slugs always kept the same position (sideways with closed parapodia or flat body with open parapodia, depending on the position taken by the sea slug when immobilized) were continuously exposed to the same low light in between measurements. Thus, the problem of comparing control (reduced motility) and immobilized animals may exist regarding some parameters, particularly in RLC measurements, and so a cautious approach is recommended.

For the same reasons mentioned above, it would not be possible to perform true steady-state LC in the control treatments due to closing/opening of parapodia or body turn of tested individuals during measurements. Since differences in the information given by RLC and LC (Cruz and Serôdio, 2008) were not relevant for the present study, RLC are the best option when comparing electron transport rates between motile and anesthetized *E. viridis* individuals. The use of an anaesthetic may allow the construction of LC but, considering the present results, LC must be constructed immediately after immobilization (IAI) and within the shortest period of time. The effects of prolonged immobilization in rETR are further discussed below.

Observations of animal movement during RLC construction indicate that *E. viridis* individuals tried to escape more actively the concave slide trap at higher irradiances, possibly to avoid excessive light, reinforcing the hypothesis that animal behaviour could be of photoprotection value (Doonan and Gooday, 1982; Giménez-Casalduero and Muniain, 2008; Jesus et al., 2010; Schmitt and Wägele, 2011).

3.4.3 Effect of eugenol and MS-222 on $\Delta F/F_m'$ and RLC parameters

It is important to note that the arrangement of pericardial veins along the parapodia in sacoglossa probably functions as a “negative gill” for uptake of CO_2 and release of O_2 (Clark et al., 1981). These apparent adaptations for gas exchange suggest that CO_2 transport could limit symbiotic photosynthesis, and

considering that the lack of movement could reduce carbon availability, it might account for the decrease in the photosynthetic parameters measured in prolonged immobilization of *E. viridis* individuals. However, more data would be necessary to confirm this speculative hypothesis. For instance, uptake of radiolabeled $^{14}CO_2$ in motile vs. immobilized sea slugs or enhancement of CO_2 concentration in the sea water solution could be used to address the hypothesis that photosynthesis is limited by CO_2 transport which in turn is influenced by the movement of the sea slug and/or carbon utilization.

The absence of photobehaviour in immobilized individuals could also account for the observed decrease in photosynthetic parameters. *E. viridis* individuals and respective macroalgae prey, *C. tomentosum*, were photoacclimated to low light conditions in the laboratory. Therefore, saturating light pulses and, more importantly, the exposure to excessive light during construction of RLC could be enough to induce a decrease in $\Delta F/F_m'$, α and $rETR_m$. Although the concave slide and coverslip trap reduces motility in control specimens, they can still move and close parapodia. The slight movement inside the trap could be enough to reduce kleptoplasts exposure or at least alternate which kleptoplasts are exposed to excessive light. It was expected that any effect of exposure to excessive light during RLC construction and saturating light pulses would be dissipated in the measurements interval. However, as the example of a light stress experiment indicates (Fig. 3.4), kleptoplasts do not rapidly recover from light stress photodamage. Therefore, the accumulation of saturating light pulses and RLC construction could account for some of the decrease in the photosynthetic parameters.

RLC can cause some photodamage to kleptoplasts, however, this was not the main factor counting for a decrease in photosynthetic efficiency. When F_s and F_m' used to calculate $\Delta F/F_m'$ (Fig. 3.1) was plotted against time (data not shown), it was evident that F_m' remained reasonably stable and that F_s was the main factor decreasing $\Delta F/F_m'$ during exposure time (Fig. 3.2). If photodamage would be the main cause for a reduction in photosynthesis efficiency, this should be seen as a decrease in F_m' relatively to F_m. Since F_s was the main factor responsible for lowering photosynthetic efficiency (Fig. 3.2), it may be speculated that the

reduction of the first electron acceptors in the electron transport chain, and therefore a lower capacity for photochemistry, is the main factor reducing $\Delta F/F_m'$ and not photodamage.

$\Delta F/F_m'$ provides an estimate of the effective quantum efficiency of PSII photochemistry in the light adapted state. The effective rate constant for photochemistry is proportional to the fraction of open PSII reaction centers (Baker and Oxborough, 2004). Therefore, under most non-stress conditions, the effective rate constant for photochemistry is defined by the effective rate constant for Q_A oxidation (Q_A being the first electron acceptor quinone on the photosynthetic electron transfer chain) which, in turn, is highly dependent on the rate at which carbon assimilation is able to utilize the NADPH and ATP that are produced as the result of photosynthetic electron transfer (Baker and Oxborough, 2004). Since experiments were carried on in low light-adapted individuals, the ability of processes downstream of PSII to utilize the products of electron transport should be active and, therefore, playing a minor role in defining the PSII operating efficiency. While this seems to hold true for control individuals and for measurements made immediately after immobilization, a gradual decrease in the photosynthetic efficiency (Fig. 3.1), which is mainly explained by an increase in F_s (Fig. 3.2), was seen in prolonged immobilization of *E. viridis* individuals. Since RLC depends on rETR values which in turn depend on $\Delta F/F_m'$ (rETR = $\Delta F/F_m' \times E$), a decrease in the latter will be amplified when multiplied by the irradiance at each light step of the RLC. Consequently, the decrease in RLC parameters at 120 min of immobilization can be explained using the same argument to explain the gradual decrease observed in $\Delta F/F_m'$: a decrease in carbon availability or other factors limiting the use of products of electron transport, induced an accumulation of reduced quinone species resulting in F_s decrease and lowering of $\Delta F/F_m'$.

3.5 Final remarks

In conclusion, eugenol and MS-222 promoted the same muscle relaxation needed for PAM fluorometry measurements and no mortality was observed for the concentrations and exposure times tested in the present work. Eugenol showed

less effects on the photosynthetic efficiency and appears to be the best eco-friendly option available in the anaesthetics market. Therefore, whenever photobiological studies employing PAM fluorometry require immobilization of “solar-powered” sea slugs, we recommend the use of low doses of eugenol. It is important to retain that long immobilization periods (e.g. >120 min) should be avoided, as they can bias experimental results by negatively affecting on photosynthetic parameters of kleptoplasts retained by sacoglossan slugs. Researchers employing this new methodology, particularly when studying different sea slug species, are advised to run preliminary trials to confirm the suitability of the anaesthetic product, as well as its dosage.

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Chapter 4 Ontogenetic development and chloroplast acquisition in solar-powered sea slugs under ocean climate change

Abstract

Solar-powered animals are amongst the most puzzling organisms in the marine environment. Although some groups have been widely studied (e.g. corals), little information is available on how other less charismatic photosynthetic animals will adapt to ocean changes. This study is the first to evaluate the impact of future ocean conditions on the fitness of tropical photosynthetic sacoglossan sea slugs through different life stages of development. Adults of Elysia clarki were exposed (30 days) to conditions simulating present-day and predicted scenarios of ocean acidification (ΔpH=0.4) and warming (+4 ºC). Egg masses were incubated under the same conditions as adult broodstock until 15 days after metamorphosis. Exposure to ocean acidification and warming scenarios led to a significant decrease in the number of spawned egg masses, as well as on their membrane thickness. Moreover, a significant decrease in the volume of embryo capsules was accompanied by an increase in embryo volume. These findings suggest that sea slugs shifted their allocation of energy towards the quality of embryos rather than to the structures that confer them protection from environmental challenges. Climate change-related variables significantly reduced the survival and length of veligers and caused an increase in the incidence of deformities. In contrast, chloroplast acquisition by juvenile slugs was not impacted under future ocean conditions. The lower reproductive output of adults, as well as the poorer condition of early life stages recorded, allows us to anticipate the occurrence of negative impacts on the recruitment of these sea slugs populations in the oceans of tomorrow.

Keywords

acidification, warming, paternal effects, offspring, chloroplast acquisition, sacoglossans.

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4.1 Introduction

Over the last decades, anthropogenic pressures on the planet have resulted in an unprecedented increase in atmospheric carbon dioxide (CO_2) concentration. Indeed, CO_2 pressure (P_{CO_2}) in the atmosphere has raised 40% since 1750 (from 280 to 390 ppm, Hartmann *et al.*, 2013), and a further rise up to 940 ppm is expected by the year 2100 (Pörtner *et al.*, 2014). As a consequence, approximately 25% of emitted CO_2 is being dissolved in the ocean. A decrease of 0.1 units in the pH of surface waters was observed over the last decades, with projections indicating a further decrease between 0.14 and 0.42 units by the end of the 21st century (Pörtner *et al.*, 2014). Another consequence of the escalation of atmospheric P_{CO_2} is the increase of global surface temperatures. The ocean is absorbing some of the heat from the atmosphere at a rate above 0.1 ºC per decade, with predictions pointing out to an increase of up to 2.7 ºC by the end of the present century (Pörtner *et al.*, 2014).

Only in the last years researchers began to understand how increasing ocean temperature and P_{CO_2} might affect marine life. A large body of literature has investigated the effects of ocean P_{CO_2} increase and temperature on the physiology (Anestis *et al.*, 2007, Pörtner & Farrell, 2008, Rosa & Seibel, 2008, Todgham & Stillman, 2013), development and growth (Byrne & Przeslawski, 2013, Kroeker *et al.*, 2013a, Kurihara *et al.*, 2008a, Przeslawski *et al.*, 2005, Rosa *et al.*, 2014a, Wolfe *et al.*, 2013), calcification (Gianguzza *et al.*, 2013, Lischka *et al.*, 2011, Pimentel *et al.*, 2014, Watson *et al.*, 2009), and reproduction (Byrne *et al.*, 2009, Havenhand & Buttler, 2008) of marine taxa. Overall, most studies have shown that organisms can be negatively impacted by ocean acidification and warming, either when using a single or multi-stressor approach.

Interestingly, photosymbiotic sea slugs and acoel worms, from temperate habitats, seem to be resistant to predicted future ocean acidification scenario for 2100, at least for short periods of time, with neither negative nor positive effects being recorded on their fitness and/or photosymbiotic associations (Dionísio *et al.*, 2015, Dupont *et al.*, 2012).

An important question that arises in ocean climate research is whether parental organisms exposed to ocean acidification and warming can affect the phenotypic

traits of their offspring. In fact, it has already been proved that environmental changes during reproductive conditioning of marine species can influence fecundity and offspring survival (Muranaka & Lannan, 1984, Przeslawski & Webb, 2009, Rossiter, 1996). Concerning marine invertebrates, previous research has shown that climate change-related variables have a negative impact on the number and quality of offspring, namely on egg production, incidence of larval malformations and survival and developmental rate (Davis *et al.*, 2013, Hettinger *et al.*, 2013, Hettinger *et al.*, 2012, Kurihara *et al.*, 2008b, Parker *et al.*, 2010, Rosa *et al.*, 2014c). Nevertheless, in some species, offspring has displayed the ability to acclimate to environmental stressors after parental exposure to climate change scenarios (Donelson *et al.*, 2012, Dorey, 2013, Parker *et al.*, 2012, Sunday *et al.*, 2014). Understanding the capacity of species to acclimate to expected pH and temperature increases is paramount for making predictions about the biological impacts of ocean climate change, particularly for tropical species, that are expected to have less capacity for acclimation since they have evolved in a more stable environment (Donelson *et al.*, 2012).

Solar-powered sacoglossan sea slugs from genus *Elysia* spp. are gastropod molluscs with high economic and ecological value (Dionísio *et al.*, 2013), but so far they have been neglected from ocean acidification and warming research. One of the most remarkable features of these organisms is their ability to retain within the cells of their digestive glands photosynthetically active chloroplasts (kleptoplasts) “stolen” from their algal food sources (Cruz *et al.*, 2015, Rumpho *et al.*, 2000). These organisms thus constitute an excellent model to study one of the most puzzling features observed in the animal kingdom: the mollusc-plastid association.

To our knowledge, there is no information on the potential effects of climate change-related variables on different life-history stages of solar-powered sea slugs. In the present study, we evaluated, for the first time, how ocean acidification (ΔpH=0.4) and warming (+ 4 ºC) may affect the offspring of the tropical photosynthetic sea slug *Elysia clarki,* namely its early ontogenetic development and chloroplast acquisition.

4.2 Material and Methods

4.2.1 Exposure of adults to different climate change scenarios

One hundred specimens of the tropical sea slug *Elysia clarki* (41.1 ± 3.8 mm of total length) were collected off the Florida Keys coastline and shipped to the Laboratório Marítimo da Guia by Tropical Marine Centre, a marine aquarium wholesaler recognized for its efforts on the sustainable collection and trade of reef organisms and promotion of animal welfare. Upon arrival, organisms were randomly placed in four recirculating systems, each one composed by 250 L aquaria. Each system was filled with 0.2 µm filtered natural seawater, and equipped with mechanical (100 µm filter, Tropical Marine Centre, UK), chemical (REEF-Skim Pro 400, Tropical Marine Centre, UK) and biological (bioballs, Fernando Ribeiro, Portugal) filtration, as well as with UV irradiation (Vecton 600, Tropical Marine Centre, UK). Ammonia and nitrite levels were monitored weekly using colorimetric test kits (Aquamerk, Merck Millipore, Germany), and kept within recommended levels for the stocking of tropical sea slugs. Overhead tank illumination was provided through dimmable LED artificial lightning apparatus (Aquabeam 1500 Ultima NP Ocean Blue, TMC Iberia, Lisbon, Portugal), consisting of 5 x white XP-G LEDs (9000 K) and 5 x XP-E blue LEDs (50000 K), suitable for marine set ups. Photosynthetically active radiation (PAR) was measured using FluorPen FP100 light meter (Photo System Instruments, Czech Republic) and maintained at 150 ± 15 µmol photons m^{-2} s^{-1} at water surface. Photoperiod was set to 14 h light: 10 h dark. The macroalgae *Bryopsis plumosa* (previously acclimated for 2 days to the same conditions of stocked sea slugs to which they were going to be supplied) was provided *ad libitum* as dietary prey. Food was never a limiting factor and was selected according to species dietary regime (Curtis *et al.*, 2007).

After an acclimation period of two weeks at control conditions (26 °C and pH 8.0, corresponding to the ambient temperature and pH conditions at the collection site), adults were randomly divided into five 5-L tanks per treatment (n=5 individuals per tank, n=25 individuals per treatment). Organisms were then exposed during five days to a gradual increase of P_{CO_2} and temperature. After that period, organisms were exposed for four weeks to four different treatments simulating present-day and predicted climate change scenarios of ocean acidification and warming: (i) Control treatment - normocapnia (~400 µatm, pH 8.0) and average sea

temperature; (ii) Acidification treatment - hypercapnia (~1100 µatm, pH 7.6) and control temperature; (iii) Warming treatment - the respective warming scenario (+4 °C, 30 °C) and normocapnia; and (iv) Acidification + Warming treatment - the warming scenario and hypercapnia.

Water temperature and pH were adjusted automatically by using a Profilux controlling system (GHL, Germany) connected to individual temperature and pH probes. Temperatures were automatically upregulated by heaters and downregulated using cooling systems (HC-1000A, Hailea, China). pH was monitored every 2 seconds and adjusted automatically via solenoid valves, being downregulated through the injection of a certified CO_2 gas mixture (Air Liquid, Portugal) via air stones and upregulated by aerating the tanks with filtered air. Salinity was kept at 35.0 ± 1.0 throughout the experiment. Seawater carbonate system speciation (see Table S4.1) was calculated weekly based on total alkalinity (see Sarazin *et al.*, 1999), pH, temperature and salinity measurements, by using the CO2SYS software developed by Lewis and Wallace (1998), with dissociation constants from Mehrbach (1973).

4.2.2 Exposure of egg masses to different climate change scenarios

A preliminary experiment was carried out in order to characterize the embryonic development of *E. clarki* (see Table 4.1). The different developmental stages were established based on the appearance or disappearance of specific morphological structures, such as cilia, velum, shell, eyespots and propodium, among others (according to Thompson, 1967, Trowbridge, 2000).

Sea slugs are simultaneous hermaphrodites with internal cross-fertilization. In order to achieve good results on broodstock reproduction the following issues were carefully addressed: 1) food was never a limiting factor; 2) similar sized animals were paired in each tank; 3) groups of animals were stocked and their density maintained between optimal limits for the species (for detailed information see Dionísio *et al.*, 2013, reproduction section).

Egg masses produced during adult exposure to different climate change scenarios were transferred to individual rearing boxes placed inside the tanks. Egg masses were reared under the same conditions as their parental organisms. Development

was monitored on a daily basis from the moment of egg mass deposition until 15 days post-hatching (after they had reached the juvenile stage). Juveniles were fed *ad libitum* with their preferred dietary prey, *Bryopsis plumosa*. The presence of food was assured before the juvenile stage was reached, in order to stimulate metamorphic competence as sea slugs may undergo metamorphosis inside the egg capsules (a process termed intracapsular metamorphosis, without the occurrence of a plancktotrophic veliger stage with newborns crawling out of the egg capsule already as an *imago* of adults; also termed direct development) (Dionísio *et al.*, 2013).

Table 4.1 Developmental stages of *Elysia clarki* from embryo to juvenile. Days denote the minimum number of days after egg mass deposition at 26 ºC.

Development stage	Description	Days
	Before hatching	
Pre-veliger	Division stage, blastula and gastrula	0
Veliger	Development of shell, cilia and foot	3
	Development of nephrocyst and otocyst	5
	Development of eyespots	6
	Development of black/purple pigmentation, operculum and propodium	8
	Intracapsular metamorphosis	9
	Capsule discard	12
	After hatching	
Juvenile	Metamorphic shell discard	13
	Acquisition of chloroplasts	14

4.2.3 Effects of different climate change scenarios on egg masses and embryos

The presence of egg masses was inspected daily during the four week exposure period of adult sea slugs. Egg masses were counted, collected (by carefully detaching the egg masses from the substrate using a plastic nail and photographed under a microscope (DM1000, Leica, Germany) equipped with a digital camera (DFC 450, Leica, Germany). The manipulation of egg masses was kept to a minimum in order to avoid causing any damage. Three egg masses were randomly selected per treatment. The mean egg mass membrane thickness of

each egg mass was determined based on 20 measurements taken along the membrane (Fig. S1). The length (L) and width (W) of at least 10 embryo capsules per egg mass were measured immediately after deposition (at the uncleaved stage). The radius (R) of at least 10 embryos per egg mass was also measured. All measurements were made using ImageJ software (1.8v, USA). Embryo capsule volume (ECV) was then determined assuming a prolate spheroid shape ($ECV = 4/3\ \pi\ W^2\ L$), while embryo volume was calculated assuming a spheroid shape ($EV = 4/3\ R^3$).

4.2.4 Effects of different climate change scenarios on veligers

The development time between the day of egg mass deposition and the day of hatching was determined for four egg masses per treatment. At day 8 after deposition, egg masses were observed under the microscope (DM1000, Leica, Germany). Survival was assessed by counting the number of live and dead veligers in each egg mass. The presence of deformities was inspected in each living veliger (before intracapsular metamorphosis), namely: undeveloped or abnormal shell, abnormal or absent velum, undeveloped propodium, elongated body and complete body deformity. The percentage of larvae with deformities was then determined for each egg mass.Three to five egg masses were randomly selected per treatment. Veligers (at least 15 per egg mass) were photographed at day 8 for morphometric analysis. Shell length was measured across the aperture, along with the propodium diameter. According to Trowbridge (2000) and Bickell and Kempf (1983), propodium size can be used as an indicator of metamorphic competence for veligers, since the full development of this structure, along with the growth of a dense cover of cilia in the central surface of the foot, enables the crawling behaviour of juvenile slugs. All measurements were performed using the ImageJ software (1.48v, USA).

4.2.5 Effects of different climate change scenarios on juveniles

As other opisthobranchs, *E. clarki* metamorphosis involves the loss of several morphological structures, such as the velum, shell, and operculum (Bickell & Kempf, 1983). In this sense, the timing of the loss of these structures was

inspected on a daily basis. Moreover, whenever crawling was initiated (immediately after hatching), animals from four different egg masses per treatment were photographed under the microscope. The acquisition of chloroplasts was also checked daily from hatching until 15 days post-hatching (Fig. 4.6). Juvenile sea slugs were photographed when feeding on macroalgae, acquiring green pigmentation as a result of incorporation of intact chloroplasts into specific cells lining the digestive gland. Images were then processed for total length measurements using ImageJ software (ImageJ 1.48v, USA).

4.2.6 Statistics

Two-way ANOVA were conducted to evaluate the impact of ocean acidification, warming and the potential synergy between these two scenarios on the number of egg masses produced, their membrane thickness, capsule and embryo volumes, development time, survival, shell length, propodium diameter, incidence of deformities, and juvenile length. Only the mean measures of each egg mass were used as replicates. Before any analysis, percentage data (i.e., survival and deformities) were square-root transformed. The assumptions of normality and homogeneity of variances were verified using the Kolmogorov-Smirnov and the Levene's tests, respectively. Subsequently, post-hoc tests (Tukey HSD, Fisher LSD or Unequal N HSD) were performed. All statistical analyses were done considering a significance level of 0.05, using STATISTICA 12.6 software (StatSoft Inc., USA).

4.3 Results

4.3.1 Effects of different climate change scenarios on egg masses and embryos

The number of egg masses laid by adults (Fig. 4.1a) was not significantly affected by warming ($F=3.7$, $p=0.0698$). In contrast, the number of egg masses was significantly affected by pH ($F=25.5$, $p=0.0001$), causing a decrease of 68% when compared with control condition. The membrane thickness of egg masses ($n=60$ per treatment) (Fig. 4.1b) was significantly affected by acidification ($F=8.79$, $p=0.0180$), with pH being the only factor significantly affecting this trait. Embryo capsule volume ($n=60$ per egg mass) (Fig. 4.1c) was not significantly affected by

synergistic conditions of acidification and warming (F=2.2, p=0.1759), neither by warming (F=0.7, p=0.4257). However, embryo capsule volume was significantly affected by acidification (F=6.5, p=0.0347). Embryo volume (n=60 per egg mass) was significantly affected by both decreasing pH and increasing temperature with significant effects of synergistic conditions (F=0.7, p=0.4429). When compared to control conditions, embryo volume increased 33% under the future synergistic scenario (Fig. 4.1d). Embryos were bright white in colour at oviposition but, with subsequent development, they assumed a yellowish colour. This pattern did not change between experimental treatments.

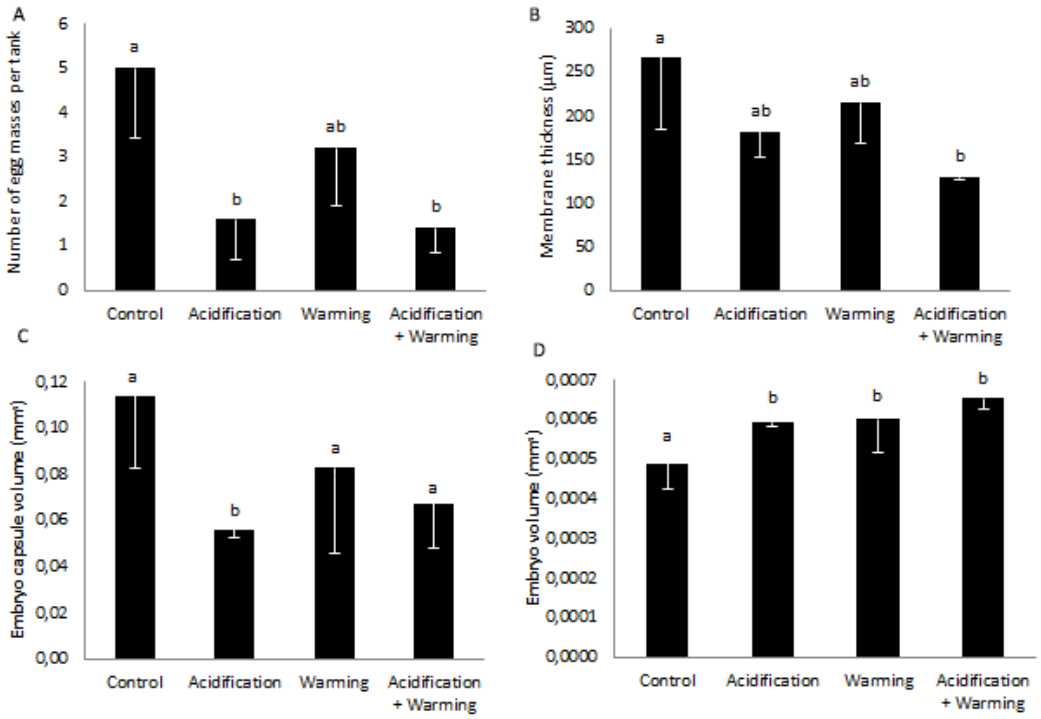

Fig. 0.1 Effects of ocean acidification and warming on *Elysia clarki* egg masses and embryos. (a) Number of egg masses; (b) membrane thickness of egg masses; (c) embryo capsule volume; and (d) embryo volume, under different climate change scenarios: Control (26 ºC, pH 8.0); Acidification (26 ºC, pH 7.6); Warming (30 ºC, pH 8.0); and Acidification + Warming (30 ºC, pH 7.6) treatments. Values are given as means ± SD. Different letters represent significant differences between treatments (p<0.05).

4.3.2 Effects of different climate change scenarios on veligers

E. clarki embryogenesis (n=4 egg masses per treatment) (Fig. 4.2a) lasted 13 ± 0 days under control conditions. The duration of intracapsular development decreased with increasing temperature to 11-12 days, although this trend was not statistically significant (F=0.08, p=0.3700).

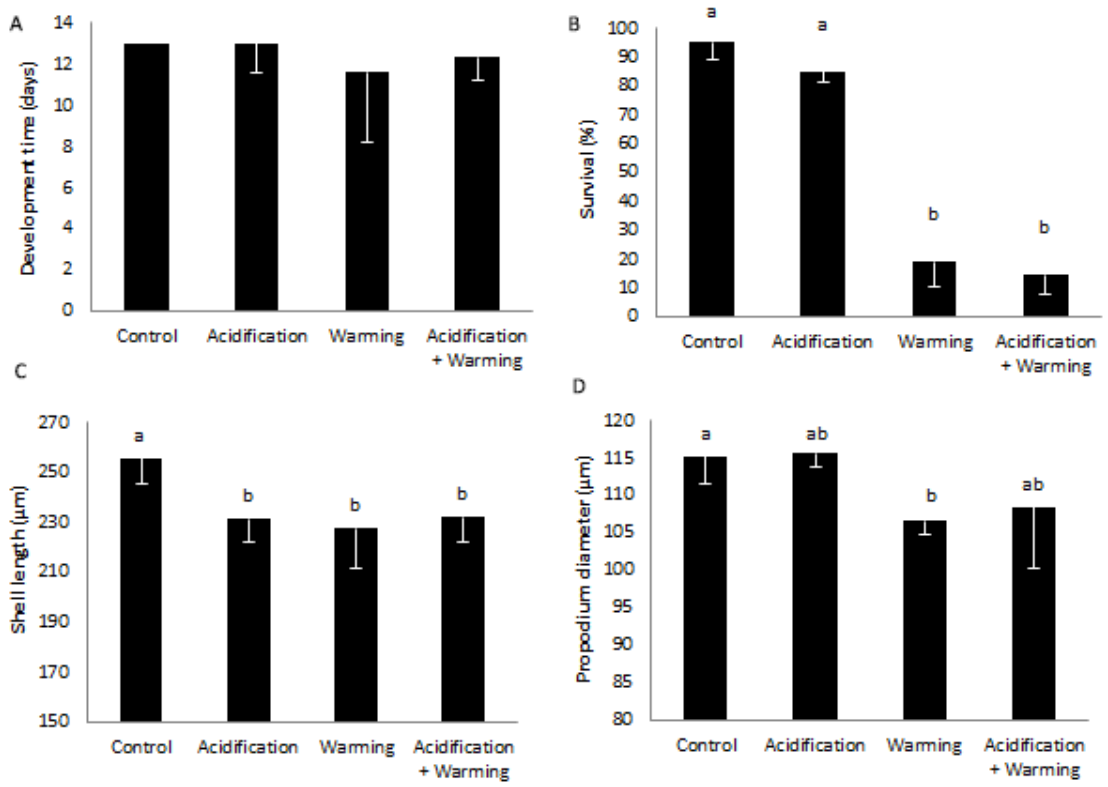

Fig. 0.2 Effects of ocean acidification and warming on *Elysia clarki* veligers. (a) Development time; (b) survival; (c) shell length; and (d) propodium diameter, under different climate change scenarios: Control (26 ºC, pH 8.0); Acidification (26 ºC, pH 7.6); Warming (30 ºC, pH 8.0); and Acidification + Warming (30 ºC, pH 7.6) treatments. Values are given as means ± SD. Different letters represent significant differences between treatments (p<0.05).

The survival of veligers (Fig. 4.2b) was 95.2 ± 1.5% when organisms were exposed to control conditions. Both pH and temperature significantly decreased survival (F=6.5, p=0.0189 and F=632.4, p=0.00001, respectively), with temperature being the major driver affecting it. In fact, while acidification slightly decreased the survival to 85%, warming led to a reduction in survival to less than 20% in both control and low pH conditions (19 and 14.5%, respectively).

Shell size (at least n=15 per treatment) was also affected by future ocean conditions (Fig. 4.2c). Both pH (F=6.0, p=0.0401) and temperature (F=11.3, p=0.0099) had a significantly negative effect on shell length, with a significant interaction between these factors (F=13.0, p=0.0069), although they did not act synergistically. On the other hand, propodium diameter (n=30 per egg mass) (Fig. 4.2d) was not affected by synergistic conditions of acidification and warming (F=0.08, p=0.7791), neither by pH (F=0.17, p=0.6883) but significantly and negatively affected by temperature (F=8.78, p=0.0181).

The most common deformities observed during *E. clarki* development are presented in Figure 4.3 and included elongated malformed body, abnormal velum (enlarged), and malformed propodium and shell (irregular shape).

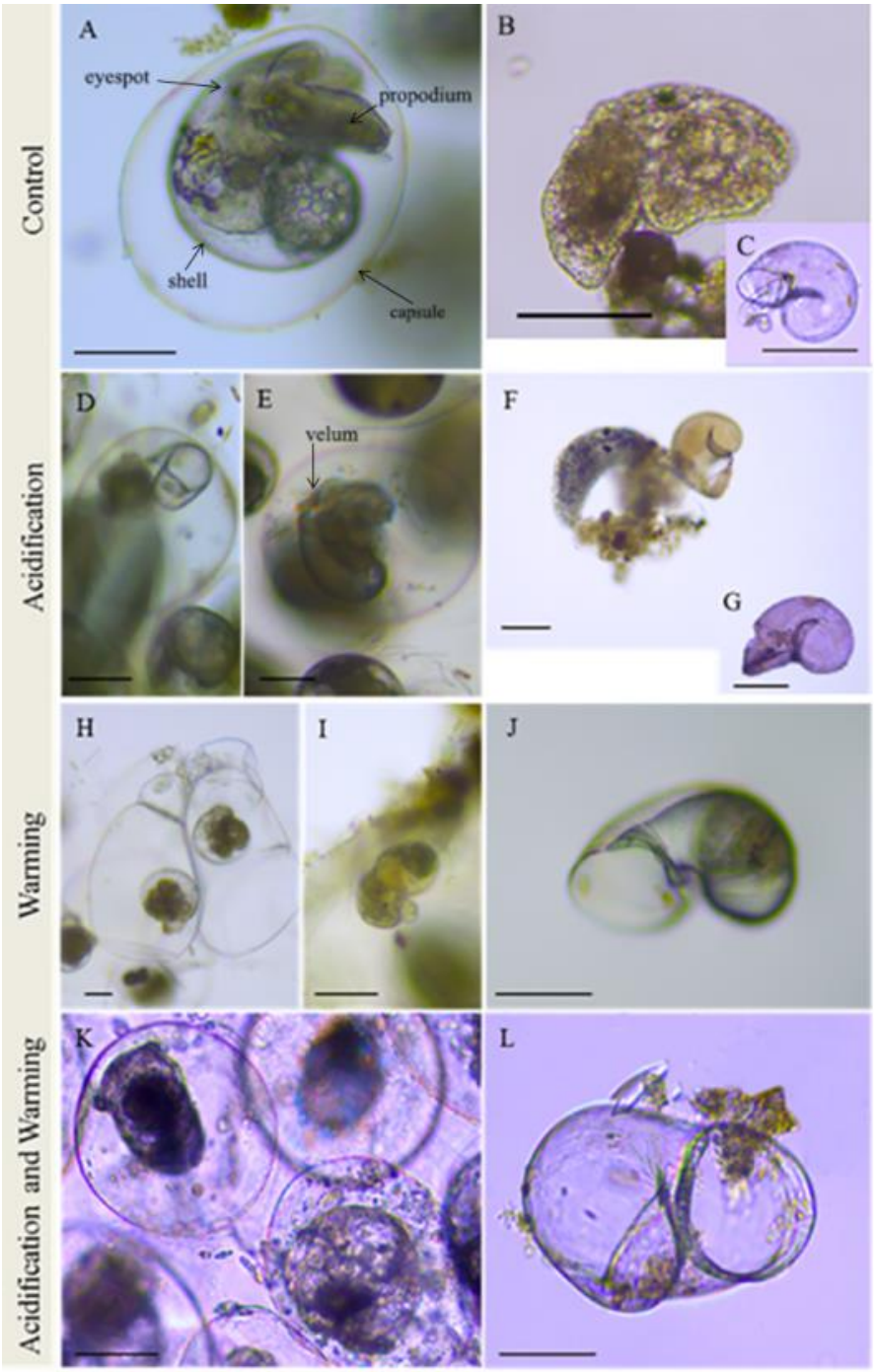

Fig. 0.3 Most common deformities observed during *Elysia clarki* development, under different climate change scenarios: Control (26 ºC, pH 8.0); Acidification (26 ºC, pH 7.6); Warming (30 ºC, pH 8.0); and Acidification + Warming (30 ºC, pH 7.6) treatments. (a) Normal intracapsular metamorphosis; (b) newly-metamorphosed juvenile actively exploring algal surfaces; (c) shell after hatching; (d) abnormal development (body and shell deformities); (e) abnormal shell, velum and propodium; (f) juvenile after hatching and respective damaged shell; (g) shell loss; (h) abnormal capsules; (i) abnormal veliger; (j) abnormal shell; (k) abnormal development (body and shell deformities); (l) abnormal shell. Scale bar: (a), (d-l) 100 µm, (b) and (c) 200 µm.

The incidence of deformities (at least n=145 veliger's surveyed per treatment) (Fig. 4.4) was not affected by the interaction between acidification and warming (F=0.20, p=0.5963), but was significantly affected by warming (F=26.1, p=0.0002) and acidification (F=27.4, p=0.0001), with a clear synergistic effect of both factors. The percentage of deformities increased from 1.5 ± 2.1% under control conditions to 27.8 ± 14.0% under acidification and to 28.5 ± 21.0% under warming, reaching 61.0 ± 7.8% under the future synergistic scenario.

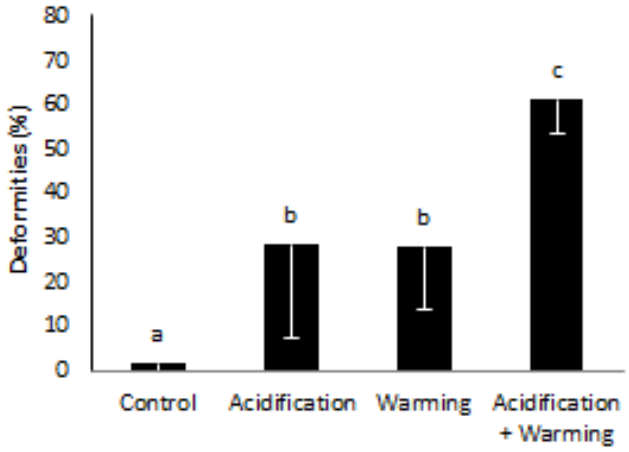

Fig. 0.4 Effects of ocean acidification and warming on the incidence of deformities on *Elysia clarki* veligers, under different climate change scenarios: Control (26 ºC, pH 8.0); Acidification (26 ºC, pH 7.6); Warming (30 ºC, pH 8.0); and Acidification + Warming (30 ºC, pH 7.6) treatments. Values are given as means ± SD. Different letters represent significant differences between treatments (p<0.05).

4.3.3 Effects of different climate change scenarios on juveniles

Juvenile length (at least n=15 per treatment) (Fig. 4.5) was not affected by the interaction between acidification and warming (F=0.8, p=0.3971), neither by temperature (F=3.7, p=0.0904). In contrast, juvenile length was significantly affected by pH (F=38.4, p=0.0003), decreasing 21% when compared to control conditions. The loss of the operculum usually occurred simultaneously or immediately after the loss of the shell, regardless of the treatment. Newly-metamorphosed juveniles were all aposymbiotic.

Within a few hours after metamorphosis, juveniles actively explored algal surfaces and began to feed (Fig. 4.6a, b and c). In all experimental treatments, chloroplast acquisition was observed and maintained during at least 15 days (Fig. 4.6d-e).

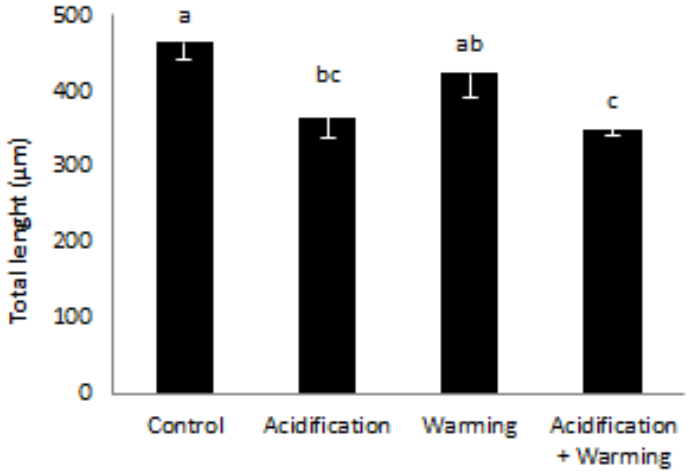

Fig. 0.5 Effects of ocean acidification and warming on the length of recently-metamorphosed juveniles of *Elysia clarki*, under different climate change scenarios: Control (26 ºC, pH 8.0); Acidification (26 ºC, pH 7.6); Warming (30 ºC, pH 8.0); and Acidification + Warming (30 ºC, pH 7.6) treatments. Values are given as means ± SD. Different letters represent significant differences between treatments ($p<0.05$).

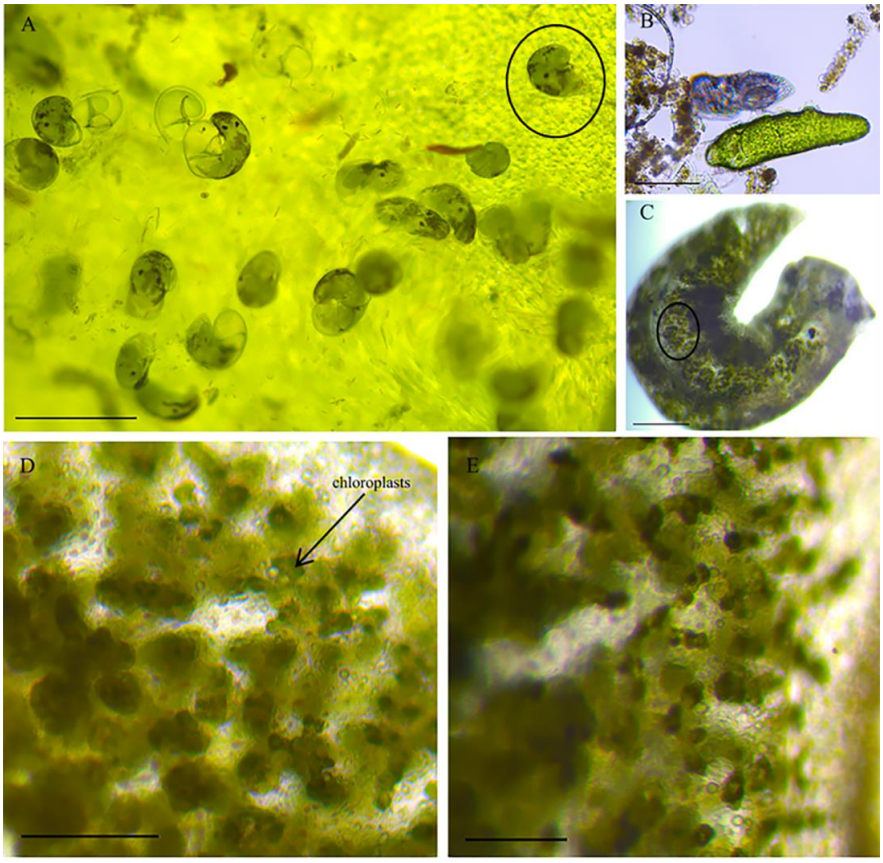

Fig. 0.6 Chloroplast retention ability of *Elysia clarki* juveniles, under different climate change scenarios: (a) juveniles crawling out of the shell, actively exploring algal surfaces and feeding (black circle); (b) newly-metamorphosed juveniles actively exploring algal surfaces; (c) juvenile with chloroplasts spread along their body; (d) juvenile with chloroplasts spread along their body at the control (26 ºC, pH 8.0) and (e) Acidification (26 ºC, pH 7.6). Scale bar: figure (a), (c) 500 µm and (b), (d), (e) 200 µm.

4.4 Discussion

The present study found that the environmental conditions to which breeding adults of the photosynthetic sacoglossan sea slug *E. clarki* were exposed during reproduction shaped the condition of their progeny. The major biological outcomes are not positive and will likely reduce the recruitment of tropical photosynthetic sea slugs. More specifically, acidification and warming had a negative impact on egg mass release, as well as on survival, growth and incidence of deformities during the early stages of ontogeny.

Increased pH was the factor that most affected the number of egg masses released, the membrane thickness of egg masses and also embryo capsule volume. Since both spawning and capsule production are energetically costly (Pechenik, 1979), results suggest that parental organisms exposed to ocean climate change have decreased the energy allocated on both the number and morphological structure of egg masses (such as egg mass membrane thickness). This is possibly explained by the fact that, under stressful environmental conditions, organisms have to deviate part of their available energy from non-essential processes (e.g., growth and reproduction) and prioritize the fuelling of primary processes (e.g. respiration), including those involved in the minimization of environmental stress (Wieser, 1994). Moreover, adults under acidified and warming conditions seem to have allocated extra energy to improve the quality of their embryos. In fact, embryos were 21 to 33% larger in volume under increased P_{CO_2} and temperature conditions. Increased egg size has been previously described as a common response of adults exposed to environmental stresses, including temperature and acidification (Allen *et al.*, 2008, Moran & McAlister, 2009, Parker *et al.*, 2012). This may represent an adaptive strategy since larger egg size is broadly correlated with higher energy content, reduced dependence on exogenous food, and increased growth and survival (Moran & McAlister, 2009), thus conferring a competitive advantage that may persist throughout development. However, this strategy generally carries high costs to fecundity (maternal organisms adaptively adjust offspring size and phenotype across life-history stages, see detailed information in Allen *et al.*, 2008). As already observed in other marine molluscs (Rawlings, 1994), photosynthetic sea slugs under future climate change scenarios seem to prioritize energy allocation towards embryos quality,

rather than to the structures that may confer them protection from environmental stresses and predation.

Increased temperature and P_{CO_2} also affected embryonic development. Higher temperatures are known to accelerate development and consequently decrease development time in some species (Przeslawski *et al.*, 2005). Also, acidification proved to lower survivorship and developmental rate at moderate temperatures in encapsulated mollusc embryos, but these effects showed to be alleviated by warmer temperatures (Davis *et al.*, 2013). In the present study, the duration of intracapsular development decreased with temperature from 13 to 11-12 days (different from the duration periods observed by Curtis *et al.*, 2007), but this effect was not statistically significant. The duration of *E. clarki* development was also not significantly affected by high P_{CO_2}, which goes against the findings of previous studies with gastropods that have reported slower development under hypercapnic conditions (Byrne & Przeslawski, 2013, Li *et al.*, 2013).

Larval survival was significantly reduced under the future synergistic scenario, decreasing in 81 percentage points when compared to the present-day scenario. However, warming was the main driver for such effect, indicating that this species may be on the edge of its upper thermal tolerance limit. Intracapsular oxygen availability is one of the main factors affecting embryo development of marine gastropod species, mostly those which aggregate its offspring in the way of encapsulation, due to the low oxygen diffusion rate that the capsule wall allows (Cancino *et al.*, 2011). At higher temperatures, embryo metabolism is higher and intracapsular oxygen levels become lower, which may affect survival, normal embryonic development and rotational behaviour (Cancino *et al.*, 2011, Deschaseaux *et al.*, 2010, Goldberg *et al.*, 2008b, Strathmann & Strathmann, 1995). Increased temperature and lower oxygen levels may also explain the atypical rotational behaviour observed on encapsulated veligers from all climate change scenarios (Goldberg *et al.*, 2008a, Shartau *et al.*, 2010). The significantly higher larval mortality observed under future ocean conditions is of great concern, since under natural conditions the mortality of juvenile gastropods is already estimated to exceed 90% (Gosselin & Qian, 1997). An increase in larval mortality will unquestionably affect recruitment and negatively affect the shaping of adult populations (Parker *et al.*, 2012).

Besides the impact on survival, future ocean conditions also affected the size of *E. clarki* veligers and juveniles. Both acidification and warming decreased significantly the shell length of veligers. The negative impact of high P_{CO_2} on shell size has already been documented for other molluscs (Lischka *et al.*, 2011, Noisette *et al.*, 2014, Watson *et al.*, 2009). Moreover, shell length did not increase with temperature as would be expected due to faster growth. Instead, shell length decreased, indicating a potential reallocation of energy from shell formation to the support of temperature-related increases in maintenance costs under warming. This shift in the allocation of the energy of developing larvae exposed to environmental stress has already been observed in other studies (Mackenzie *et al.*, 2014). The propodium diameter of veligers also decreased under warmer conditions. The development of the propodium is a sign of larval competence and acquisition of crawling behaviour (Kriegstein *et al.*, 1974). Reduced propodium size at elevated temperature may affect crawling and impair settlement (Kriegstein *et al.*, 1974), and ultimately lead to a lower juvenile survival. Juvenile size was also affected by climate change conditions. Juveniles were more than 20% smaller under hypercapnia. Seawater pH has already been suggested to be a growth-limiting factor for other gastropods (Glass & Darby, 2009, Naylor *et al.*, 2014). Moreover, the lack of a significant thermal effect on juvenile size was not expected, since growth is expected to increase with temperature.

Future ocean conditions also affected the normal development of *E. clarki*, by significantly increasing the incidence of deformities. Under higher temperature and P_{CO_2}, the incidence of deformities was 40 times higher than under control conditions, affecting more than 60% of veligers. The occurrence of abnormal shells is a common response in mollusc larvae exposed to high P_{CO_2}, due to a reduced availability of carbonate ions (Noisette *et al.*, 2014). Larval abnormalities observed in this study may have affected survival in the laboratory, even though *E. clarki* larvae are not free swimming. It is likely that in the wild, larval survival may even be lower for other sacoglossan species displaying free-swimming larval stages, as abnormal larvae are expected to be more vulnerable to predation.

Despite all the negative effects that ocean acidification and warming had on *E. clarki* development, there was one important feature that seems not be impacted (at least on a short experiment) – the capacity to sequester functional chloroplasts

“stolen” from their algal prey. The acquisition and maintenance of photosymbionts under high temperature and P_{CO_2} was described for temperate species such as the sea slug *Elysia viridis* (Dionísio *et al.*, 2015) and the photosynthetic acoel worm *Symsagittifera roscoffensis* (Dupont *et al.*, 2012) under short-term (hours to a few days) ocean acidification experiments.

Altogether our results suggest that future ocean conditions may impact the fitness of the sea slug *E. clarki* across different life-history stages, from adults to embryos, veligers and juveniles. The lower reproductive output of adults and the poorer condition of early life-stages will have a negative impact on recruitment and further affect the species persistence in a changing ocean. Embryos were mostly affected by acidification, while larval and juvenile stages were mainly affected by both increased temperature and P_{CO_2}. Yet, chloroplast acquisition by young juvenile specimens seems not be affected by future ocean conditions. To our knowledge, this is the first time that early life stages of photosynthetic sea slug have been investigated in relation to environmental stressors associated with climate change. Long term experiments are now required to understand if and how (using PAM fluorometry) climate change scenarios may impact the mechanisms that allow *E. clarki* and other sacoglossan sea slugs to maintain “stolen” plastids fully functional inside metazoan cells.

4.5 Acknowledgements

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Chapter 4 - Supplementary material

Fig. S1. ***Elysia clarki*** **embryo capsules and egg mass membrane thickness. Scale bar 100 µm.**

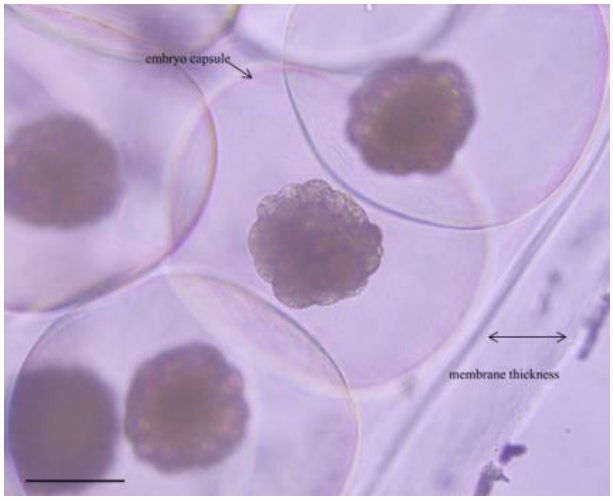

Table S4.1 Seawater carbonate chemistry during the exposure of *Elysia clarki* adults and early life stages to different temperature and pH conditions. Values for P_{CO_2}, $\Omega_{aragonite}$ and $\Omega_{calcite}$ were calculated from salinity, temperature, pH and total alkalinity (TA), using CO2SYS software. Values are given as mean ± SD.

Treatment	Temperature (°C)	pH (total scale)	TA (µmol kg^{-1})	P_{CO_2} (µatm)	$\Omega_{aragonite}$	$\Omega_{calcite}$
Adults						
Control	26.0 ± 0.1	8.0 ± 0.1	2075.9 ± 48.3	393.4 ± 9.6	2.97 ± 0.08	4.51 ± 0.10
Acidification	26.0 ± 0.1	7.6 ± 0.1	2028.6 ± 37.1	1144.7 ± 21.3	1.30 ± 0.02	1.96 ± 0.03
Warming	30.0 ± 0.1	8.0 ± 0.1	2063.9 ± 29.1	398.4 ± 5.9	3.30 ± 0.08	4.93 ± 0.10
Acidification + Warming	30.0 ± 0.1	7.6 ± 0.1	2059.0 ± 27.9	1181.6 ± 16.3	1.51 ± 0.02	2.23 ± 0.03
Early stages						
Control	26.0 ± 0.1	8.0 ± 0.1	1952.5 ± 53.3	381.6 ± 9.2	4.13 ± 0.10	2.73 ± 0.06
Acidification	26.0 ± 0.1	7.6 ± 0.1	1837.9 ± 36.3	1032.2 ± 20.8	1.78 ± 0.03	1.17 ± 0.02
Warming	30.0 ± 0.1	8.0 ± 0.1	2004.5 ± 21.3	386.3 ± 4.3	4.75 ± 0.05	3.18 ± 0.03
Acidification + Warming	30.0 ± 0.1	7.6 ± 0.1	1856.9 ± 27.3	1053.9 ± 15.9	2.05 ± 0.03	1.38 ± 0.02

Table S4.2 Results of two-way ANOVAs evaluating the effects of temperature and pH during the early development of *Elysia clarki*. Significant values (p < 0.05) are marked in bold.

	df	MS	F	p
Number of egg masses				
Temperature (T)	1	5.0	3.7	0.0698
pH	1	33.8	25.5	**0.0001**
T x pH	1	3.2	2.4	0.1397
Error	16	1.3		
Membrane thickness				
Temperature (T)	1	8324.8	3.36	0.1041
pH	1	21788.3	8.79	**0.0180**
T x pH	1	0.01	0.01	0.9983
Error	8	2476.1		
Embryo capsule volume				
Temperature (T)	1	0.0000	0.70	0.4257
pH	1	0.0041	6.45	**0.0347**
T x pH	1	0.0014	2.20	0.1759
Error	8	0.0006		
Embryo volume				
Temperature (T)	1	0.00004	7.53	**0.0253**
pH	1	0.00002	6.13	**0.0383**
T x pH	1	0.00001	0.65	0.4429
Error	8	0.00001		
Development time				
Temperature (T)	1	6.5	3.4	0.0884
pH	1	1.6	0.8	0.3700
T x pH	1	1.6	0.8	0.3700
Error	11	1.8		
Survival				
Temperature (T)	1	2.650	632.4	**0.0000**
pH	1	0.027	6.5	**0.0189**
T x pH	1	0.004	1.0	0.3290
Error	19	0.004		

Shell length				
Temperature (T)	1	561.0	11.32	**0.0099**
pH	1	296.7	5.99	**0.0401**
T x pH	1	644.0	13.00	**0.0069**
Error	8	49.5		
Propodium diameter				
Temperature (T)	1	189.4	8.78	**0.0181**
pH	1	3.7	0.17	0.6883
T x pH	1	1.8	0.08	0.7791
Error	8	21.6		
Incidence of deformities				
Temperature (T)	1	0.32	26.1	**0.0002**
pH	1	0.34	27.3	**0.0001**
T x pH	1	0.00	0.20	0.5963
Error	13	0.01		
Juvenile length				
Temperature (T)	1	2157	3.70	0.0904
pH	1	22329	38.38	**0.0003**
T x pH	1	466	0.80	0.3971
Error	8	582		

Chapter 5.1 Effect of ocean acidification on the temperate solar-powered sea slug, *Elysia viridis*

5.1.1 Extended Abstract

Ocean acidification is known to trigger deleterious effects on several marine photosynthetic invertebrates (Dupont et al., 2012). Photosymbiosis, whereby photosynthetic microorganisms or organelles live inside an animal (host) is widespread in the marine biota, underlying a wide range of ecologically and biogeochemically significant processes that remain largely unclear (Dupont et al., 2012). One of the most remarkable symbiosis is between sacoglossan molluscs and algal chloroplasts (Rumpho et al., 2011). These organisms are able to “steal” functional chloroplasts (termed kleptoplasts) from their algal prey and keep them functional inside digestive diverticula (Rumpho et al., 2011). The aim of this study was to investigate the impact of environmental hypercapnia on cellular structures of kleptoplastic animals. *Elysia viridis* (Sacoglossa) were exposed to different pH conditions (pH 8, 7.5, 6.8 and 6.1) for 24 h. Six animals were anaesthetized per treatment (Cruz et al., 2012) and the integrity of kleptoplasts was determined (*in vivo*) under optical and stereomicroscope (Figs. 5.1 and 5.2). Morphological modifications to the normal condition of digestive diverticula, chloroplasts and mortality, were checked every 8 h. Under normal conditions (pH 8.0) the symbiotic chloroplasts are packed tightly in the tubule cells, particularly close to cell walls (Fig. 5.2A, arrow). Tubule cells ramify throughout the body, giving its green appearance. The same condition was verified for pH 7.5 (Fig. 5.2B). Sea slugs subjected to pH 6.8 presented fragmented clusters of chloroplasts and overall color variation (Figs. 5.1, 5.2C). Mortality was only verified in animals exposed to pH 6.1 (100% mortality), presenting severe impacts at the cellular level after 24 h of exposure, including cellular burst (Fig. 5.1). Chloroplasts and their plastoglobuli spread across host cytoplasm (Fig. 5.2D, arrow) and cellular layers surrounding the plastid were absent. These preliminary results (short experiment) suggest that *E. viridis* kleptoplasts may resist to environmental hypercapnia down to pH 6.8, where the first sings of cellular modifications where detected. At pH 7.5 (future scenario, 2100) kleptoplastic sea slugs retain their endosymbionts in opposition to other well-established photosymbiotic groups.

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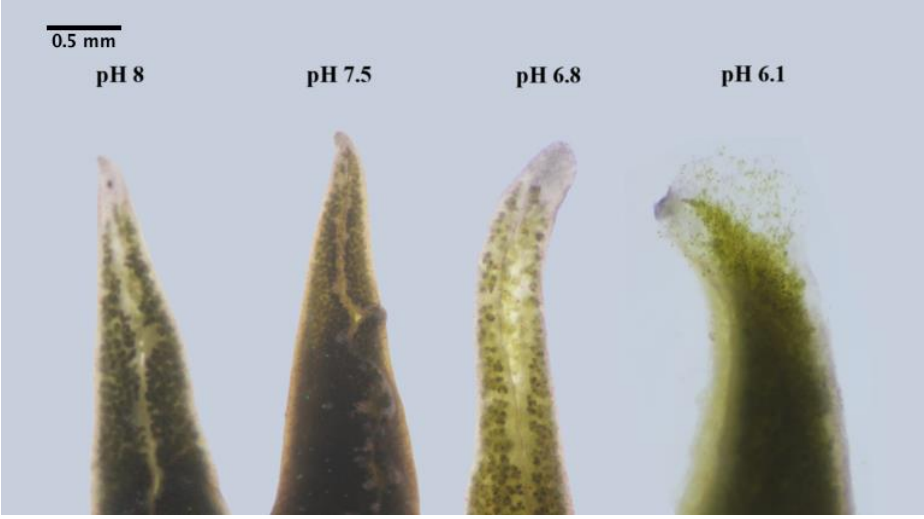

Fig. 5.1.1 Posterior sections of *E. viridis* exposed to different pH conditions for 24h (8, 7.5, 6.8 and 6.1) observed under optical stereomicroscope; green coloration is given by chloroplasts and evident burst is shown under pH 6.1. Scale bar: 0.5 mm.

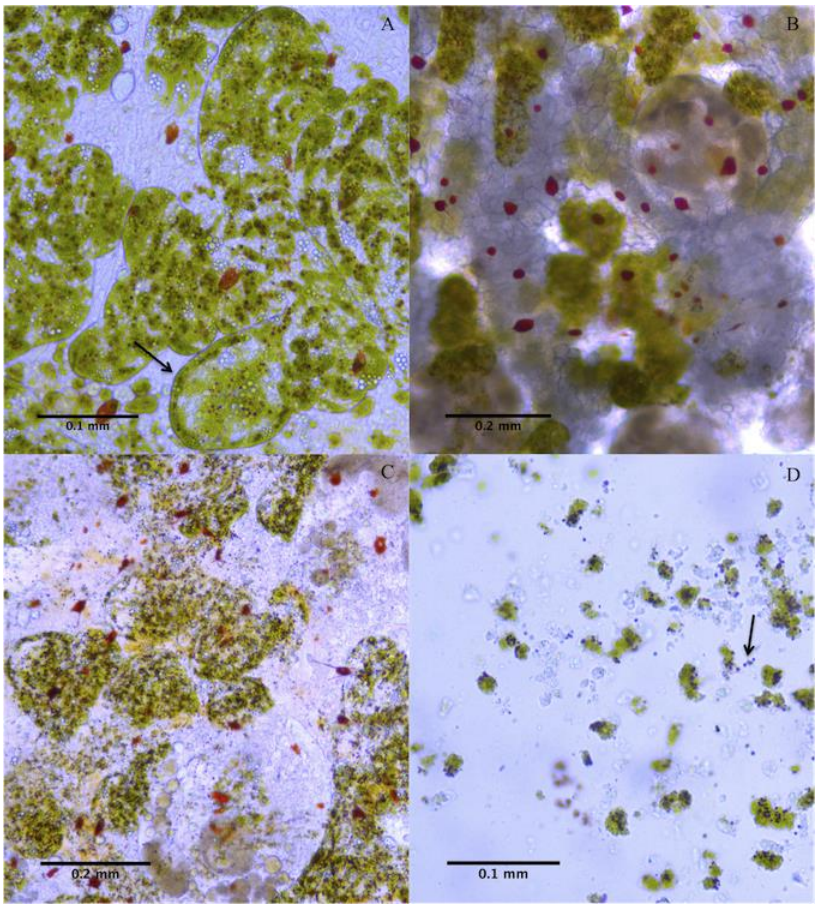

Fig. 5.1.2 Digestive diverticula from posterior sections of *E. viridis* exposed to different pH conditions for 24 h (A – pH 8; B – pH 7.5; C – pH 6.8; D – pH 6.1), observed *in vivo* under optical microscopy. Normal pH conditions (A) show numerous algal plastids within a cell (black arrow, A), contrasting with the absence of cellular layers surrounding the plastids, and plastoglobuli (black arrow) spread across host cytoplasm at the lowest pH (D). Scale bars: 0.1 mm (A, C) and 0.2 mm (B, D).

Chapter 5.2 Effect of ocean acidification and warming on tropical and temperate solar-powered sea slugs

Abstract

The impact of climate change on symbiotic relationships have been highly studied in corals but overlooked in less charismatic groups such as solar-powered sacoglossan sea slugs. These organisms display one of the most puzzling features observed in the animal kingdom - the mollusc-plastid association, which is based on their ability to retain photosynthetically active chloroplasts (kleptoplasts) “stolen” from their algal food sources. Here we analyse the impact of future scenarios of ocean acidification (∆pH=0.4) and warming (+4 ºC) on the survival, photophysiology (occurrence of bleaching, photosynthetic efficiency and metabolism) and stress defence mechanisms (heat shock and antioxidant responses) of tropical (*Elysia clarki*) and temperate (*Elysia viridis*) solar-powered sacoglossan sea slugs. Both species presented a reduced survival under acidified and warming conditions, but this reduction was more pronounced in *E. clarki*. The photophysiology of *E. viridis* remained stable under future ocean conditions, while *E. clarki* showed photoinhibition under high temperature and P_{CO_2}. Bleaching was observed in all tropical specimens exposed to warming, but not in *E. viridis*. Our findings also reveal that the stress defence mechanisms cope with environmental stress also varied among tropical and temperate species. The kleptoplasty resistance of temperate sea slugs may be in part related to the enhanced expression of heat shock and antioxidant enzyme responses. This study is the first reporting that tropical animals, other than corals, hosting photosynthetic symbionts bleach under future climate change scenarios. However, some temperate mollusc-kleptoplast associations appear able to cope and thrive in an acidified and warmer ocean.

Keywords

Climate change, kleptoplast, bleaching, photosymbiosis, oxidative stress, metabolism, mollusc-plastid association.

In preparation: Tropical, but not temperate, photosynthetic plastid-bearing molluscs bleach under ocean warming and elevated CO_2. To be submitted to Global Change Biology.

5.2.1 Introduction

Kleptoplasty is an exciting research topic as it represents a unique naturally occurring biological condition where chloroplasts are found living intra-cellularly in organisms phylogenetically distant from the algal host in which they evolved (Serôdio *et al*., 2014). This photosynthetic association results from the maintenance of photosynthetically competent chloroplasts – often termed "kleptoplasts" - sequestered from algae that remain structurally intact and temporarily functional (Pierce & Curtis, 2012, Wise *et al.*, 2007). Symbiont photosynthesis plays a major role in the nutrient acquisition of these associations (Tremblay *et al*., 2013). Kleptoplasty may be especially valuable in environments where other sources of nutrients are in short supply (Venn *et al*., 2008) or to overcome periods when food algae are either absent (e.g. during winter months, see Giménez Casalduero & Muniain, 2008) or calcifying (e.g. in the case of *E. timida*, see Giménez Casalduero & Muniain, 2008).

Over the last decades, anthropogenic pressures on the planet have resulted in an unprecedented increase in atmospheric carbon dioxide (CO_2) concentration. As a consequence, atmospheric CO_2 is being dissolved in the ocean, causing an increase in its acidity. A decrease of 0.1 units in the pH of surface waters was observed over the last decades, with projections indicating a further decrease between 0.14 and 0.42 units by the end of the 21st century. Another consequence of the escalation of atmospheric Pco_2 is the increase of global surface temperatures. The ocean is absorbing some of the heat from the atmosphere, with projections estimating an increase of up to 2.7 ºC by the end of the century (Pörtner *et al.,* 2014).

Future changes in ocean's physical and chemical properties are expected to influence marine taxa (Kroeker *et al.*, 2013b), with tropical organisms being shown to be more vulnerable to warming and acidification than temperate organisms (Nilsson *et al.*, 2009, Rosa *et al.*, 2014b). Impacts of climate change have been shown in a variety of organisms living in association with a photosynthetic organism (Anthony *et al.*, 2008, Rodolfo-Metalpa *et al.*, 2011, Watson, 2015). However, most studies have focused corals and their symbiotic relationship with

zooxanthellae. Under increasing Pco_2 and temperature, the association between metazoans and microalgae was shown to break down, a phenomenon known as bleaching, in both corals (Anthony *et al*., 2008) and foraminiferans (e.g. Hallock, 2000, Yellowlees *et al.*, 2008 for Foraminifera and corals, respectively). The photosynthetic efficiency of endosymbionts was also shown to decreased under acidified and warming conditions, both in corals (Anthony *et al.*, 2008, Reynaud *et al.*, 2003) or foraminiferans (Sinutok *et al.*, 2014). Moreover, future ocean conditions may further impact the survival and growth of photosynthetic endosymbionts, as observed in giant clams (Watson, 2015, Watson *et al*., 2012). Nevertheless, some photosymbiotic organisms have shown high resistance to future climate change, including corals (Palumbi *et al.,* 2014), sea slugs (Dionísio *et al.,* 2015) and acoel worms (Dupont *et al*., 2012).

Efficient antioxidant networks and increased levels of stress proteins have been described in autotrophs as protective mechanisms against environmental stress (e.g. Baird *et al.*, 2009, Foyer & Shigeoka, 2011, Gattuso *et al.*, 1999). As photosynthesis is a well-established source of reactive oxygen species (ROS), autotrophs must have an efficient antioxidant network in order to maintain high rates of photosynthesis (Foyer & Shigeoka, 2011). Despite their harmful potential, photosynthetic ROS are also powerful signalling molecules that are involved in several processes, such as growth, development and acclimatory responses to stress in plants (Foyer & Shigeoka, 2011). Increased ROS production not only decreases the activity of photosystem II (PSII), it also stimulates gene expression, particularly with regard to acclimation and defence genes (Foyer & Shigeoka, 2011). On the other hand, heat shock proteins (HSPs) of chloroplasts have also shown to be important to protect photosynthesis during heat, oxidative and photoinhibitory stress, by defending PSII reaction centres (Barua *et al.*, 2003, Heckathorn *et al.,* 2002, Nakamoto *et al.*, 2000). In photosynthetic symbionts such as corals, HSPs have also shown to be important to avoid bleaching (Baird *et al*., 2009).

The aim of the present study is to understand the potential effects of ocean acidification and warming on one of the most puzzling features observed in the animal kingdom: the mollusc-kleptoplast association. The impact of future climate

change scenarios on tropical (*Elysia clarki*) and temperate (*Elysia viridis*) sacoglossan sea slugs bearing kleptoplasts was evaluated based on their survival and growth, photosynthetic efficiency (Fv/Fm - PSII maximum quantum yield, and relETR - relative electron transport rate), metabolism (R – respiration, and NPP - net primary production), and oxidative stress response (HSP - heat shock protein, and GST - glutathione S-transferase).

5.2.2 Materials and Methods

5.2.2.1 Exposure of adults to ocean acidification and warming

One hundred specimens of the tropical sacoglossan sea slug *E. clarki* (41.1 ± 3.8 mm of total length) were collected off the Florida Keys coastline and shipped to Laboratório Marítimo da Guia (Cascais, Portugal) by Tropical Marine Centre, a marine aquarium wholesaler recognized for its efforts on the sustainable collection and trade of reef organisms and promotion of animal welfare. One hundred and forty-four specimens of the temperate sacoglossan sea slug *E. viridis* (13.3 ± 0.9 mm of total length) were hand collected in Cabo Raso (Cascais, Portugal).

Upon arrival, organisms were randomly distributed through recirculating life support systems, each one composed by 250-L aquaria. Each system was filled with 0.2-µm filtered natural seawater, and equipped with mechanical (100-µm filter, Tropical Marine Centre, Portugal), physicochemical (REEF-Skim Pro 400, Tropical Marine Centre, Portugal) and biological (bioballs, Fernando Ribeiro, Portugal) filtration, as well as with UV irradiation (Vecton 600, Tropical Marine Centre, Portugal). Ammonia and nitrite levels were monitored weekly using colorimetric test kits (Aquamerk, Merck Millipore, Germany), and kept within recommended levels. Overhead tank illumination was provided through dimmable LED artificial lightning apparatus (Aquabeam 1500 Ultima NP Ocean Blue, Tropical Marine Centre, Portugal), consisting of five white XP-G LEDs (9000 K) and five XP-E blue LEDs (50000 K). Photosynthetically active radiation (PAR) was measured using FluorPen FP100 light meter (Photo System Instruments, Czech Republic) and maintained at 150 ± 15 µmol photons m^{-2} s^{-1} at water surface, representing a mid-light intensity for both species. Photoperiod was set to 14 h

light: 10 h dark. The siphonaceous macroalgae *Codium tomentosum* and *Bryopsis plumosa* (previously acclimated for 2 days to the same conditions of stocked sea slugs) were provided *ad libitum* as food.

During the first two weeks, sea slugs were kept at control conditions, corresponding to the ambient temperature and pH at collection sites (26 °C and pH 8.0 for *E. clarki*, and 18 °C and pH 8.0 for *E. viridis*). After this acclimation period, *E. clarki* individuals were randomly divided into five 5-L tanks per treatment (n=5 individuals per tank, n=25 individuals per treatment), and *E. viridis* into three 5-L tanks per treatment (n=12 individuals per tank, n=36 individuals per treatment). Organisms were then exposed during five days to a gradual increase of P_{CO_2} and temperature. After that period, organisms were exposed for eight weeks to four different treatments simulating present-day and predicted climate change scenarios of ocean acidification and warming: (i) Control treatment - normocapnia (pH 8.0) and control temperature (26 ºC and 18 ºC for *E. clarki* and *E. viridis*, respectively); (ii) Acidification treatment - hypercapnia (pH 7.6) and control temperature; (iii) Warming treatment - the respective warming scenario (+4 °C, 30 °C and 22 °C for *E. clarki* and *E. viridis*, respectively) and normocapnia; and (iv) Acidification + Warming treatment - the warming scenario and hypercapnia.

Water temperature and pH were adjusted automatically by using a Profilux control system (GHL, Germany) connected to individual temperature and pH probes. Temperature was automatically upregulated by heaters and downregulated using cooling systems (HC-1000A, Hailea, China); pH was monitored every 2 seconds and adjusted automatically via solenoid valves, being downregulated through the injection of a certified CO_2 gas mixture (Air Liquid, Portugal) via air stones and upregulated by aerating the tanks with filtered air. Salinity was kept at 35.0 ± 1.0 throughout the experiment. Seawater carbonate system speciation (Table S5.1) was calculated weekly based on total alkalinity (see Sarazin *et al.*, 1999), pH, temperature and salinity measurements using the CO2SYS software developed by Lewis and Wallace (1998), with dissociation constants being according to Mehrbach (1973).

5.2.2.2 Survival

Survival at each treatment was checked daily during throughout the experimental trial (60 days).

5.2.2.3 Photo-physiological responses of kleptoplasts

The integrity of the symbiosis was evaluated at 0, 30 and 60 days of exposure based on the presence of green kleptoplasts inside the digestive tubules of the sacoglossan sea slugs. Kleptoplasts were qualitatively evaluated using morphological features, namely color and symmetry, as well as their distribution in the tubules. Images were taken using a binocular microscope (DM1000, Leica, Germany) equipped with a digital camera (DFC 450, Leica, Germany).

Variable chlorophyll *a* fluorescence was measured at day 60 using a PAM (Pulse Amplitude Modulated) fluorometer, comprising a computer-operated PAM-control unit (JUNIOR-PAM, Walz Heinz GmbH, Germany) and a WATER-EDF emitter-detector unit (Gademann Instruments GmbH, Germany). Actinic and saturating light was provided by a blue LED-lamp (450 nm peak, 20 nm half-band width) and supplied through a plastic fibre optic bundle (1.5 mm diameter) that was perpendicularly positioned to the surface of the sea slug parapodia. A saturation pulse of 2500 µmol photons m^{-2} s^{-1} with a duration of 0.8 s was applied to at least 8 slugs per treatment (previously anaesthetised as described in (Cruz *et al.*, 2012) to determine the fluorescence at both dark and light conditions. Sea slugs were dark-adapted for 30 minutes, and the minimum (F_o) and maximum fluorescence (F_m) in the dark-adapted state were used to determine the variable fluorescence ($F_v = F_m – F_o$) and the maximum quantum yield of PSII (F_v/F_m) (Schreiber *et al.*, 1986). Sea slugs were then light-adapted at 150 ± 15 µmol photons m^{-2} s^{-1} for 30 minutes. The minimum (F) and maximum fluorescence (F_m') in the light-adapted state were used to determine the variable fluorescence ($\mathbf{\Delta}F=F_m'–F$) and the PSII maximum quantum yield ($\mathbf{\Delta}F/F_m'$) in the light-adapted state. The relative electron transport rate (relETR) was then calculated as: relETR = $\mathbf{\Delta}F/F_m' \times PAR \times 0.5$, where PAR is the photosynthetically active radiation and 0.5 compensates for irradiance being split between two photosystems.

5.2.2.4 Sea slug metabolism

Oxygen consumption was determined 60 days after exposure to ocean future scenarios according to previously established methods (Rosa *et al.*, 2012, Rosa *et al.*, 2013, Rosa *et al.*, 2009). Sea slugs (n=6 per treatment) were individually incubated in sealed water-jacketed respirometry chambers (Strathkelvin, UK) containing 1-µm filtered and UV-irradiated seawater from the respective experimental treatment. Water volumes were adjusted in relation to animal mass (up to 3 mL) in order to minimize locomotion and stress but still allow for spontaneous and routine activity rates. Respiration chambers were immersed in Lauda water baths (Lauda-Brinkmann, Germany) to control temperature. Oxygen concentrations were recorded with Clark-type O_2 electrodes connected to a multi-channel oxygen interface (Model 928, Strathkelvin). Controls (blanks) were used to correct for possible bacterial respiratory activity. Two runs of 3 h were made per individual, one exposed to light and the other in complete darkness to inhibit photosynthesis. Incubations in the light or dark were made within the respective photoperiod of the animals. Oxygen concentration measurements (µmol O_2 L^{-1}) were transformed into µmol O_2 g^{-1} L^{-1} h^{-1} by taking into consideration the volume of the chamber and the wet weight of the slug. Respiration (R) was determined as the oxygen consumption rate in complete darkness, while net primary photosynthesis (NPP) was determined as the oxygen production rate in the light exposed conditions, according to Baker *et al.* (2015).

5.2.2.5 Oxidative stress response of sea slugs

The oxidative stress response was analysed based on both the HSP production (HSP70/HSC70) and the activity of the antioxidant enzyme (GST). A total of 3 samples (each one containing 3 slugs) were analysed per treatment.

The HSP70/HSC70 content was assessed by Enzyme-Linked Immunosorbent Assay (ELISA), by adapting the protocol from Njemini *et al.* (2005). Briefly, 10 µL of the homogenate supernatant was diluted in 250 µl of phosphate-buffered saline (PBS). Then, 50 µL of the diluted sample was added to 96-well microplates (Nunc-Roskilde, Denmark) and allowed to incubate overnight at 4 °C. On the next day, the microplates were washed in PBS containing 0.05% Tween-20. A total of 100

µL of blocking solution (1% bovine serum albumin, Sigma-Aldrich, USA) was added to each well and left to incubate at room temperature for 2 h. After washing the microplates, 50 µL of a solution of 5 µg mL^{-1} of primary antibody (anti-HSP70/HSC70, Acris, USA) was added to each well and then incubated at 37 ºC for 90 min. According to the manufacturer details, the primary antibody anti-HSP70/HSC70 has abroad range reactivity, including diverse fish species. The primary antibody reactivity for the species *E. clarki* and *E. viridis* was validated by Western blot. The non-linked antibody was removed by washing the microplates again. The alkaline phosphatase-conjugated anti-mouse IgG (Fab specific, Sigma-Aldrich, USA) was then used as a secondary antibody, by adding 50 µl of a solution at 1 µg mL^{-1} to each well and incubating the microplates for 90 min at 37 °C. After three additional washes, 100 µL of substrate (SIGMA*FAST*™ p-nitrophenyl phosphate tablets, Sigma-Aldrich, USA) was added to each well and incubated for 10-30 min at room temperature. Subsequently, 50 µL of stop solution (3 M NaOH) was added to each well, and the absorbance was read at 405 nm in a 96-well microplate reader. The concentration of HSP70/HSC70 in the samples was calculated from a curve of absorbance based on serial dilutions (between 0 and 2 µg mL^{-1}) of purified HSP70 active protein (Acris, USA). Results were expressed in relation to the protein content of the samples, which was determined according to Bradford (1976).

The activity of the antioxidant enzyme GST was determined according to Rosa *et al.* (2012) and Lopes *et al.* (2013), optimized for a 96-well microplate. This assay uses 1-chloro-2,4-dinitrobenzene (CDNB) as substrate, which conjugates with the thiol group of the glutathione (GSH) causing an increase in absorbance. A total of 180 µL of substrate solution (composed by 200 mM L-glutathione reduced in Dulbecco's PBS and 100 mM CDNB) was added to each well of a 96-well Nunclon microplate (Thermo Scientific Nunc, USA), along with 20 µL of GST standard or sample. Equine liver GST was used as a positive control to validate the assay. The enzyme activity was determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of GSH and CDNB. The absorbance was recorded every minute for 6 min, using a plate reader (BioRad, USA). The

increase in absorbance per minute was estimated and the reaction rate at 340 nm was determined using the CDNB extinction coefficient of 0.0053 ℇμM as follows:

$$\text{GST activity} = \frac{\Delta A340/\text{min}}{0.0053} \text{x} \frac{\text{Total volume}}{\text{Sample volume}} \; x \; \text{dilution factor}.$$

Results were expressed in relation to the protein content of the samples, which was determined according to the Bradford method (see Bradford, 1976).

5.2.2.6 Statistical analysis

All data were analyzed using generalized linear mixed models (see, e.g., Zuur et al. 2009). The distributional family used was Binomial (logit link function) for proportions (i.e., survival), Gaussian (identity link function) for quantities (i.e., F_v/F_m and relETR), and Gamma (log link function) for positive quantities with a severe positively skewed distribution (i.e., R and NPP). The sample size of oxidative stress variables was not enough to model HSP and GST as response variables, which were therefore analysed only through descriptive statistics. The initial mixed models included the species, temperature and pH as fixed effects, the corresponding second and third order interactions, and the tank as a random effect to account for possible dependency within tanks. Following the recommendation from Barr *et al.* (2013), the random effects were kept in the models irrespectively of the amount of variation they explained.

The most parsimonious models were selected based on the Akaike Information Criterion. Model residuals were checked for departures from the assumed distributions and no significant deviations were found. For Binomial models, odds ratios and confidence limits were determined to allow a more informative discussion of the results. Considering that odds define the ratio of the probability of success and the probability of failure, odds ratios were built by the ratio of odds between the two species (*E. clarki* vs *E. viridis*), temperatures (control temperature vs warming) or pH (normocapnia vs hypercapnia).

All statistical analyses were implemented in R (R Core Team, 2015), using the lme4 (Bates et al., 2015) and nlme (Pinheiro *et al.,* 2015) packages. Results were considered statistically significant at a significance level of 0.05.

5.2.3 Results

5.2.3.1 Survival

Sea slug survival (Fig. 5.2.1) was significantly affected by temperature (p=0.005) but not by pH (p=0.624). The odds of survival under control temperature were more than 7 times higher the odds of survival under warming. No mortality was observed under control conditions for both species. *E. clarki* survival decreased with warming to 40.0 ± 34.6 and 53.3 ± 30.6% under normocapnia and hypercapnia, respectively. *E. viridis* survival decreased with high temperature to 72.2 ± 9.6 and 41.7 ± 8.3% under normocapnia and hypercapnia, respectively. Moreover, no significant differences were found between species (p=0.152), but the interaction between species and pH was significant (p=0.004). While we cannot detect a pH effect on *E. clarki* survival, the survival of *E. viridis* decreased with acidification 69.4 and 30.6 percentage points under control temperature and warming, respectively.

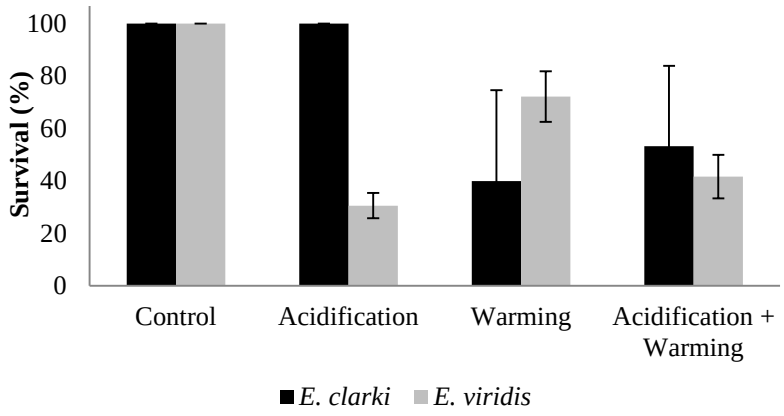

Fig. 5.2.1 Effects of ocean acidification and warming on survival (%) of tropical *Elysia clarki* and temperate *Elysia viridis*, under different climate change scenarios: Control (18 ºC and 26 ºC, pH 8.0); Acidification (18 ºC and 26 ºC, pH 7.6); Warming (22 ºC and 30 ºC, pH 8.0); and Acidification + Warming (22 ºC and 30 ºC, pH 7.6) treatments. Values are given as means ± SD.

5.2.3.2 Photo-physiological responses

Under control conditions, *E. viridis* kleptoplasts were packed tightly in the tubule cells surrounding the terminus of the tubule (Fig. 5.2.2a). Neither warming nor

acidification affected the colour and morphology of kleptoplasts (Fig. 5.2.2b). In contrast, *E. clarki* kleptoplasts under control conditions were mainly distributed in the tip of the tubule cells (Fig. 5.2.2c). Bleaching was observed in all the slugs exposed to high temperature, with the majority of the host tubule cells being recorded unfilled or displaying degraded kleptoplasts (Fig. 5.2.2d).

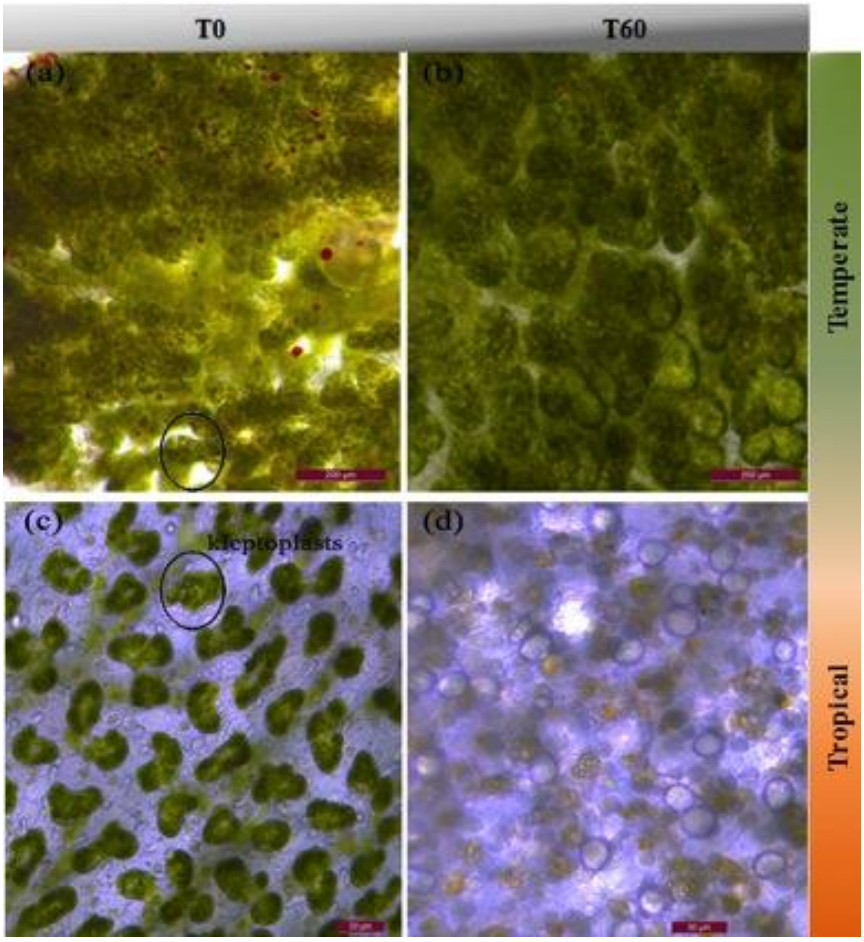

Fig. 5.2.2 Light micrographs of the termini of the digestive diverticula tubules of *Elysia viridis* (a-b) and *Elysia clarki*. (a) Kleptoplasts of *Elysia viridis* at T0 – Control (18 ºC, pH 8.0). Kleptoplasts are packed tightly in the tubule cells and ramify throughout the body. (b) Kleptoplasts of *Elysia viridis* at T60 – Acidification + Warming (22 ºC, pH 7.6); (c) kleptoplasts of *Elysia clarki* at T0 – Control (26 ºC, pH 8.0). The main area is traversed by small digestive diverticula; kleptoplasts are located along the length of the tubules as well in the tip of the tubule (black circle) (according to Curtis, 2006). (d) Bleaching of *Elysia clarki* kleptoplasts at T60 – Acidification + Warming (30 ºC, pH 7.6). Scale bar: (a-b) 200 µm, (c-d) 50 µm.

The photosynthetic efficiency of kleptoplasts (Fig. 5.2.3) was significantly affected by temperature ($p<0.001$ for F_v/F_m and relETR) and pH ($p=0.026$ for F_v/F_m and $p=0.001$ for relETR), but these effects varied between species ($p<0.001$ for F_v/F_m

and p=0.003 for relETR). Moreover, the interaction between species and temperature was also significant (p=0.022 for F_v/F_m and p=0.030 for relETR). While the F_v/F_m and relETR of *E. viridis* varied little among treatments, the photosynthetic efficiency of *E. clarki* decreased under both acidification and warming. More specifically, F_v/F_m decreased 35.9 and 55.3%, while relETR decreased 48.9 and 53.0% under acidification and warming, respectively. However, under the combined effect of acidification and warming, the negative impact of these variables was not cumulative, resulting in a significant interaction between temperature and pH (p=0.012 for F_v/F_m and p=0.005 for relETR).

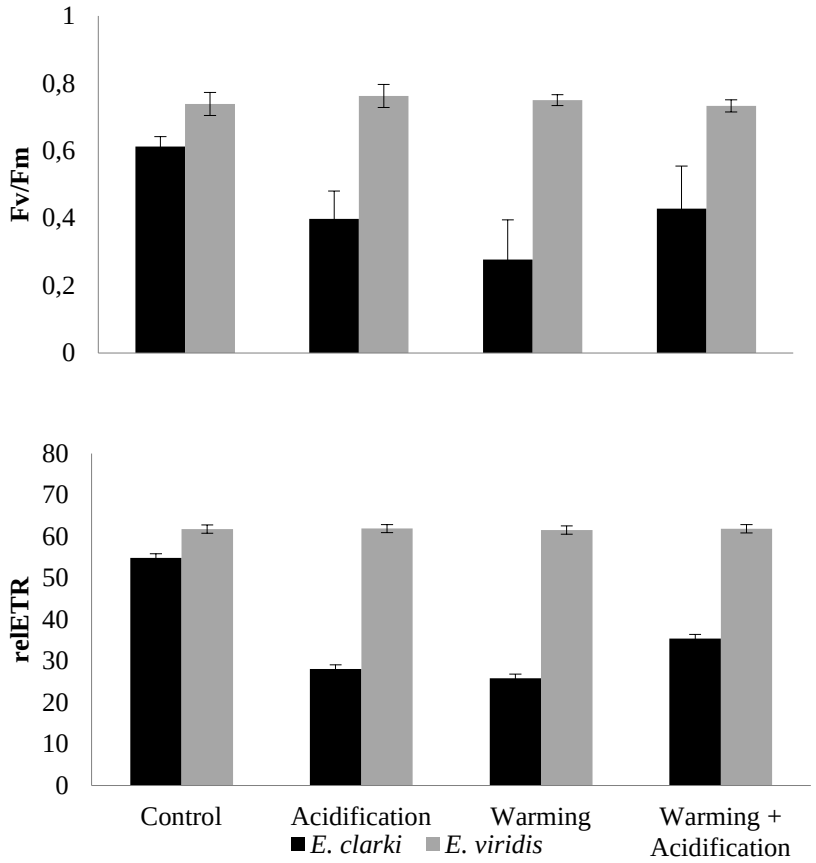

Fig. 5.2.3 Effects of ocean acidification and warming on the tropical *Elysia clarki* and the temperate *Elysia viridis*. (a) F_v/F_m, and (b) relETR under under different climate change scenarios: Control (18 ºC and 26 ºC, pH 8.0); Acidification (18 ºC and 26 ºC, pH 7.6); Warming (22 ºC and 30 ºC, pH 8.0); and Acidification + Warming (22 ºC and 30 ºC, pH 7.6) treatments. Values are given as means ± SD.

5.2.3.3 Metabolism

Sea slug metabolism (Fig. 5.2.4) was affected by future ocean conditions. R was significantly affected by pH ($p<0.001$), but not by temperature ($p=0.673$). Moreover, the interaction between species and pH was also significant ($p<0.001$ for R and NPP). On the other hand, NPP was significantly affected by both pH ($p<0.001$) and temperature ($p=0.043$). Moreover, the interactions between species and pH ($p<0.001$) and between species and temperature ($p=0.045$) were also significant. While *E. viridis* metabolism varied little or even increased, the metabolism of *E. clarki* decreased to values near zero under acidification and/or warming.

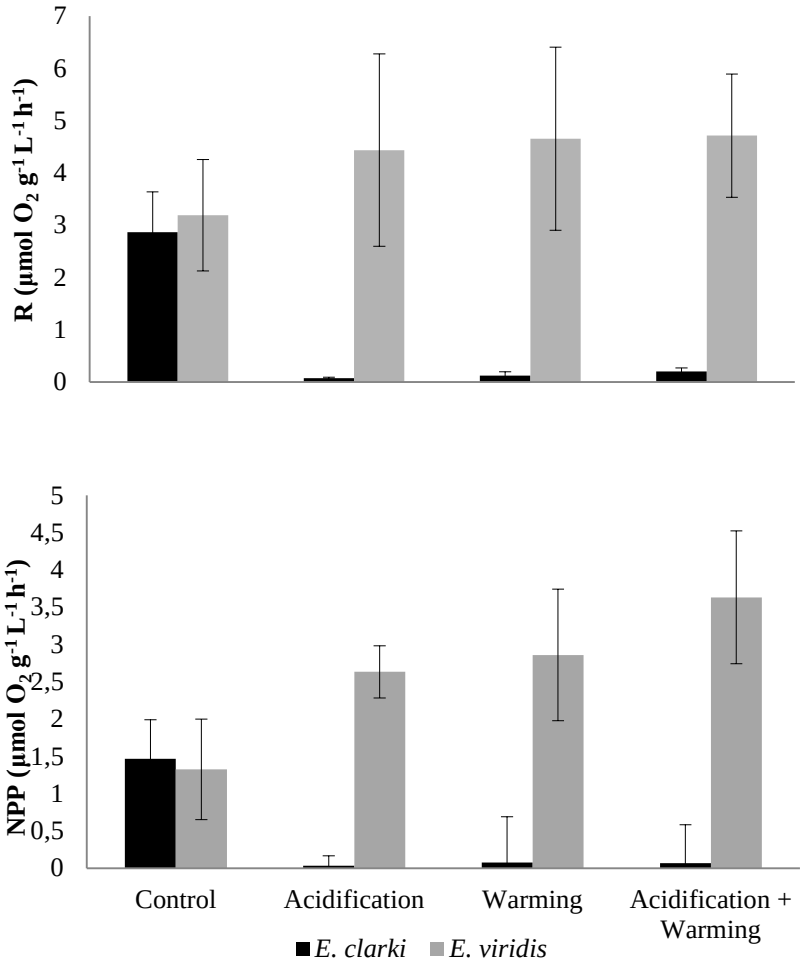

Fig. 5.2.4 Effects of ocean acidification and warming on the tropical *Elysia clarki* and the temperate *Elysia viridis*. (a) R, respiration and (b) NPP, Net primary production under different climate change scenarios: Control (18 ºC and 26 ºC, pH 8.0); Acidification (18 ºC and 26 ºC, pH 7.6); Warming (22 ºC and 30 ºC, pH 8.0); and Acidification + Warming (22 ºC and 30 ºC, pH 7.6) treatments. Values are given as means ± SD.

5.2.3.4 Oxidative stress response

The oxidative stress response of sea slugs (Fig. 5.2.5) was analysed based on descriptive statistics since it was not possible to apply mixed models due to the reduce number of replicates. Under control conditions, the mean HSP and GST levels of *E. viridis* were more than 5 times higher than those of *E. clarki*. Compared to control conditions, mean HSP values were 1.7 and 11.3 times higher under acidification and warming in *E. viridis* and *E. clarki*, respectively. On the other hand, mean GST values increased 58.8% in *E. viridis* but decreased 20.0% in *E. clarki*.

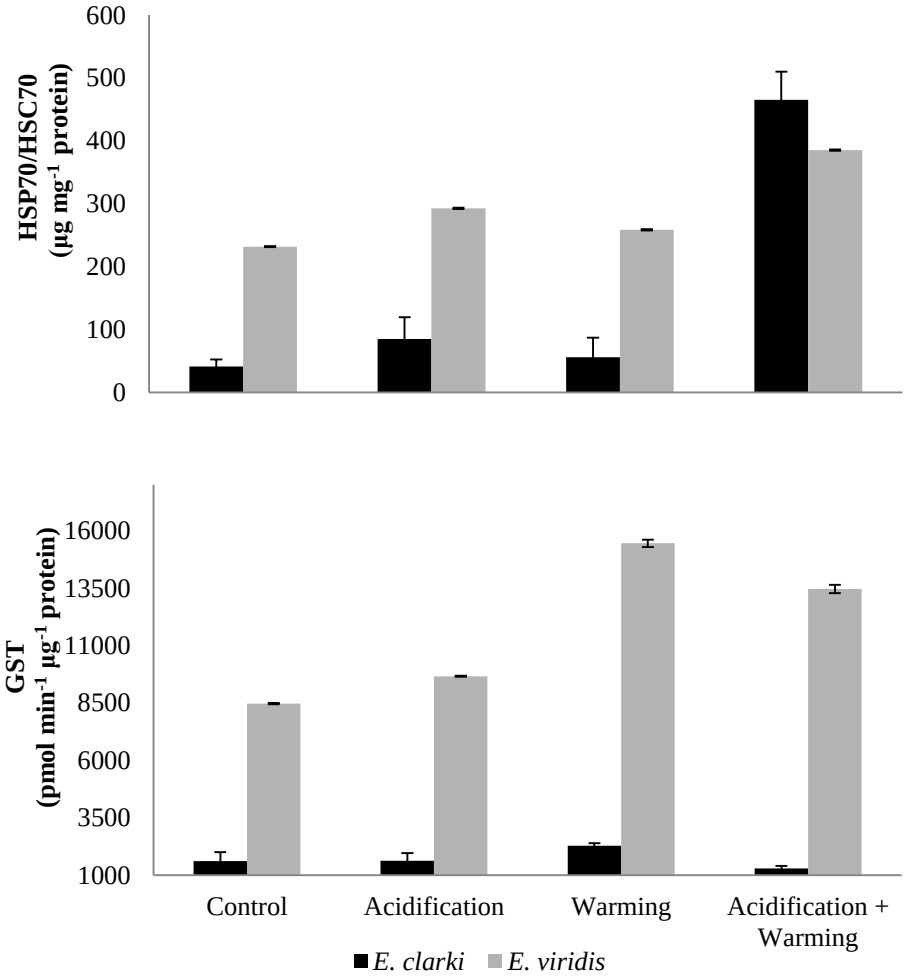

Fig. 5.2.5 Effects of ocean acidification and warming on the tropical *Elysia clarki* and the temperate *Elysia viridis* regarding their heat shock response (HSP) and antioxidant defense (GST). (a) HSP; (b) GST, under under different climate change scenarios: Control (18 ºC and 26 ºC, pH 8.0); Acidification (18 ºC and 26 ºC, pH 7.6); Warming (22 ºC and 30 ºC, pH 8.0); and Acidification + Warming (22 ºC and 30 ºC, pH 7.6) treatments. Values are given as means ± SD.

5.2.4 Discussion

In the present study, temperate and tropical sacoglossan sea slugs showed to be differently vulnerable to future ocean conditions. While in the temperate *E. viridis* the mollusc-plastid association was resilient to warming and acidification, in the tropical *E. clarki* this association was extremely sensitive to ocean conditions predicted to 2100. However, it is worth mention that, even though kleptoplasty in the temperate species was not impaired, sacoglossan sea slugs survival was. When compared to present-day conditions, *E. viridis* survival decreased 58.3% under the combined effect of acidification and warming.

Survival of both tropical and temperate sea slugs was negatively affected by acidification and warming. Warming was the main factor affecting the survival of these species, while pH only affected the survival of the temperate one. Elevated P_{CO_2} *per se* did not influence the survival of *E. clarki*. Similar results have been reported for tropical foraminiferans bearing photosymbionts (e.g. Schmidt *et al.*, 2014), but also for metazoans hosting temperate kleptoplasts (Dionísio *et al.*, 2015) and microalgae (Dupont *et al.*, 2012). In contrast, the survival of *E. viridis* decreased up to 70% under water acidification conditions. Intertidal species such as *E. viridis* are exposed to a higher range of daily pH fluctuations, tolerating pH values as low as 7.4 or more during the night, when photosynthesis does not occur and CO_2 from respiration accumulates in tidal pools (Cornwall *et al.,* 2013). Indeed, *E. viridis* exposed for 24 hours to a pH of 6.8 presented 100% survival but were not able to thrive at a pH of 6.1 (Dionísio *et al.*, 2015). The present study shows that despite being able to tolerate a short-term exposure to water acidification, *E. viridis* is unable to survive under long-term exposure to hypercapnia.

The integrity of the symbiosis displayed between sacoglossan sea slugs and their kleptoplasts was not identical for temperate and tropical specimens. While in *E. viridis* the mollusc-plastid association remained stable under future ocean conditions, warming led to chloroplast degradation and bleaching in *E. clarki*. The

disruption of the symbiotic association has already been recorded in cnidarian tropical species (Fitt *et al.*, 2001, Gates *et al.*, 1992), a scenario which is aggravated under the combined effect of warming and ocean acidification (Kaniewska *et al.*, 2015, Kroeker *et al.*, 2013b, Rodolfo-Metalpa *et al.*, 2011). In accordance, the photosynthetic efficiency of the tropical symbionts being hosted has also shown to decrease under warming. While the F_v/F_m and relETR remained stable in *E. viridis* under the different climate change scenarios that were tested in the present study, the kleptoplasts hosted by *E. clarki* showed a marked decrease in both photosynthetic parameters under acidification and/or warming conditions.

The metabolism of tropical and temperate sacoglossan sea slugs also responded differently to future ocean conditions. The metabolism displayed by *E. viridis* varied little or even increased when exposed to future climate change conditions, indicating that this species may be capable of performing high photosynthetic rates even under such environmental pressures. Enhanced rates of photosynthesis and respiration have been observed in temperate sea anemones and corals exposed to elevated P_{CO_2} (Gibbin *et al.*, 2014, Suggett *et al.*, 2012, Crawley *et al.*, 2010, Towanda & Thuesen, 2012). In contrast, in the tropical *E. clarki* both R and NPP decreased significantly to values near zero under acidification and/or warming. Metabolic depression is a widespread strategy to withstand environmental stress that is characterized by the shutting down of expensive processes to save energy and ensure long-term survival (Storey & Storey, 2004).

The higher or lower vulnerability of sacoglossan sea slugs to climate change conditions may be associated to their capacity to physiologically adapt to environmental shifts. Marine organisms possess a diverse set of physiological regulatory mechanisms that allows them to avoid deleterious effects caused by environmental disturbances, which include the heat shock and antioxidant responses (Lesser, 2006). Under control conditions, *E. clarki* presented lower HSP and GST levels than *E. viridis*. This is not surprising since intertidal organisms that experience highly variable thermal conditions (which is the case of *E. viridis*) activate the heat shock response more frequently to withstand thermal fluctuations. In contrast, marine organisms occupying stable thermal environments (such as *E. clarki*) do not need to cope with thermal fluctuations and may even

lack a heat shock response (Tomaneck, 2008). Similarly, basal GST levels of *E. clarki* were also much lower than those of *E. viridis*. Heat shock and antioxidant responses were enhanced under future ocean conditions. HSP levels increased in both species (1.7 and 11.3 times in *E. viridis* and *E. clarki*, respectively) under warming and acidification. Although *E. clarki* presented lower basal HSP levels than *E. viridis*, its response to environmental stress was much more pronounced. Increased expression of HSPs protects the cells against protein unfolding and damage due to environmental stress (Tomaneck, 2008) and has been observed in other photosymbionts exposed to warming and acidification (Heckathorn *et al.*, 2004, Moya *et al.*, 2015). On the other hand, mean GST values increased in *E. viridis* exposed to these environmental disturbances, especially under warming. In contrast, *E. clarki* showed a poor antioxidant defense capacity. These results agree with those found in a previous study with different species of photosynthetic sea slugs from the same genus. According to de Vries *et al.* (2015), the tropical *E. cornigera* accumulated ROS to a much higher degree than the temperate *E. timida,* suggesting contrasting antioxidant capacities between tropical and temperate species. Our results, along with the reduced photosynthetic efficiency of tropical *E. clarki* under acidification and warming, suggest that heat shock and antioxidant response may play an important role as mechanisms for stabilizing photosynthesis in stress situations as observed for corals (Bhagooli & Hidaka, 2004, Moya *et al.*, 2015).

Overall, our results revealed that the mollusc-plastid association in tropical habitats is much more vulnerable to warming and acidification than in temperate regions. Under future ocean conditions, the temperate *E. viridis* showed photo-physiological tolerance to acidification and warming, but its survival was still negatively affected. In contrast, the tropical sacoglossan sea slug *E. clarki* showed a reduced photosynthetic efficiency and metabolic depression, which indicates kleptoplast photoinhibition and resulted in bleaching and reduced survival. We suggest that its greater vulnerability to future ocean conditions may be in part related to restrictions in heat shock and antioxidant defence mechanisms.

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Chapter 5 - Supplementary material

Table S5.1 Seawater carbonate chemistry during the exposure of *Elysia clarki* and *Elysia viridis* to different temperature and pH conditions. Values for P_{CO_2}, $\Omega_{aragonite}$ and $\Omega_{calcite}$ were calculated from salinity, temperature, pH and total alkalinity (TA), using CO2SYS software. Values are given as mean ± SD.

Treatment	Temperature (ºC)	pH (total scale)	TA (µmol kg^{-1})	P_{CO_2} (µatm)	$\Omega_{aragonite}$
Elysia clarki					
Control	26.0 ± 0.1	8.0 ± 0.1	2075.9 ± 48.3	393.4 ± 9.6	2.97 ± 0.08
Acidification	26.0 ± 0.1	7.6 ± 0.1	2028.6 ± 37.1	1144.7 ± 21.3	1.30 ± 0.02
Warming	30.0 ± 0.1	8.0 ± 0.1	2063.9 ± 29.1	398.4 ± 5.9	3.30 ± 0.08
Acidification + Warming	30.0 ± 0.1	7.6 ± 0.1	2059.0 ± 27.9	1181.6 ± 16.3	1.51 ± 0.02
Elysia viridis					
Control	18.19 ± 0.1	8.0 ± 0.1	2059.1 ± 180.3	466.1 ± 32.4	2.11 ± 0.14
Acidification	18.10 ± 0.1	7.58 ± 0.1	2215.0 ± 88.8	1370.0 ± 55.7	0.97 ± 0.03
Warming	21.95 ± 0.1	8.0 ± 0.1	2058.4 ± 155.2	329.9 ± 26.0	2.86 ± 0.22
Acidification + Warming	21.78 ± 0.2	7.59 ± 0.1	2268.0 ± 125.7	1381.2 ± 77.7	1.16 ± 0.0

Chapter 6 Final remarks and future directions

6.1 Final remarks

The main goal of this thesis was to increase the knowledge on the role of climate change in the physiology and photobiology of tropical and temperate mollusc-kleptoplast associations and their ecological implications. Overall, the work presented here provides: i) a comprehensive state of the art on the optimal culture conditions to maximize the production of sea slugs; ii) new methodological approaches to study the puzzling mollusc-kleptoplast association and; iii) an insight in how predicted ocean acidification and warming may impact the development and performance of tropical and temperate solar-powered sacoglossan sea slugs and their kleptoplasts.

Chapter 2 summarizes the major issues impairing the culture of sea slugs and presents relevant biological and ecological data that can assist in the development of protocols for culturing sea slugs. The methods here reviewed and optimized were successfully used in the subsequent chapters (3-5). **Chapter 3** presents a method that allowed improving the non-invasive and non-destructive survey of the photophysiological performance of kleptoplasts in motile organisms, such as sacoglossan sea slugs. The anaesthetic eugenol showed less effects on the photosynthetic efficiency and appears to be the best eco-friendly option available in the market for anaesthetising animals.

The effects of acidification and warming on early life stages of the tropical sacoglossan sea slug *Elysia clarki* are provided in **Chapter 4**. This study allowed to unravel key findings associated with the ontogenetic development of sacoglossan sea slugs, assessed for the first time in a climate change context. Different life-history stages of a tropical sacoglosan sea slug species were confirmed to be impacted by future ocean conditions, which is in accordance with previous works for marine invertebrates (Byrne & Przeslawski, 2013, Parker *et al.*, 2012). Furthermore, this study also showed that chloroplast acquisition by juvenile specimens does not seem to be impacted by future ocean conditions.

Cellular structures were investigated in a laboratory experiment addressing the effects of future hypercapnia on the morphology of temperate mollusc-kleptoplast

association (**Chapter 5.1**). Results suggest that the temperate *E. viridis* shows physiological mechanisms for environmental accommodation, as observed for other photosynthetic symbiosis (Palumbi *et al*., 2014). Lastly, this work culminated with an integrated approach that investigated the effects of acidification and warming on tropical and temperate sacoglossan sea slugs bearing functional kleptoplasts (**Chapter 5.2**). This study revealed that the mechanisms displayed to deal with stress varied between tropical and temperate sea slugs; as it has been shown for many other photosynthetic organisms (Dupont *et al.*, 2012, Hall-Spencer *et al.*, 2008, Palumbi *et al.*, 2014, Pandolfi, 2015, Rodolfo-Metalpa *et al.*, 2011, Schmidt, 2015, Suggett *et al.*, 2012). Under future ocean conditions, the temperate *E. viridis* showed photo-physiological tolerance to acidification and warming, but its survival was still negatively affected. In contrast, the tropical sacoglossan sea slug *E. clarki* showed a reduced photosynthetic efficiency and used metabolic depression as a strategy to cope with environmental stress (Storey & Storey 2004). Heat shock and antioxidant responses were also shown to be enhanced under future ocean conditions. These results agree with those found in a previous study with different species of photosynthetic sea slugs from the same genus (de Vries *et al*., 2015), suggesting different antioxidant capacities for tropical and temperate species. Temperate and tropical sacoglossan sea slugs also showed different vulnerabilities with regard to their symbiosis with algal chloroplasts. While in *E. viridis* the mollusc-plastid association remained stable under future ocean conditions, in *E. clarki*, warming led to chloroplast degradation and bleaching. We suggest that its greater vulnerability to future ocean conditions may be in part related to restrictions in heat shock and antioxidant defence mechanisms.

Four research questions were hypothesized in the introductory chapter (**Chapter 1**). Here, the answer to each specific hypothesis is revised in light of the results reported in this thesis. The research questions were:

1. How can we maximize the production of the tool organism *Elysia* spp. to study sea slug-kleptoplast association?

Research on sea slugs has steadily increased in the last decades as a result of their distinctive attributes. Remarkable photosymbiotic associations and unique chemical defences prompted the use of these organisms as biological tools for scientific research (ecological importance). These organisms have also experienced a growing demand for bio-prospection for new marine drugs, biomedical studies and also for the marine aquarium trade (economic relevance). To successfully culture and maximize the production of the tool organism, *Elysia* spp., a new culture system was adapted from the recirculating "breeding chamber" described by Banger (2011). Under laboratory conditions, the provision of adequate specific stimulus to trigger metamorphosis (i.e. specific diet such as *Bryopsis* and *Codium* for *Elysia clarki* and *Elysia viridis,* respectively) was essential to maintain the reproductive output of breeding pairs, offspring quality and minimize between within variations in the timing of larval competence.

2. How can we improve the non-invasive and non-destructive survey of the photophysiological performance of kleptoplasts in motile organisms such as sacoglossan sea slugs?

The movements of sacoglossan sea slugs impair the use of current methods to study photobiological responses in chloroplasts. Through the use of anaesthetics it was possible to assess the photosynthetic activity of kleptoplasts sequestered in sacoglossan sea slug. This method allowed to conclude that whenever photobiological studies employing PAM fluorometry are performed the complete immobilization of photosynthetic sacoglossan sea slugs is required. Eugenol showed less effects on the photosynthetic efficiency and appears to be the best eco-friendly option available to achieve such goal. These findings minimized the steady-state fluorescence signal bias, associated with the motion of sea slugs, thereby minimizing the consequences for the relation between maximal fluorescence of dark-adapted and light-adapted samples. Nevertheless, this

method still needs validation during the post-recovery period to gain a more in depth knowledge on the effects of the anaesthetics on sacoglossan sea slugs kleptoplasts.

3. Will future ocean conditions impact the fitness of the tropical photosynthetic sacoglossan sea slugs across different life-history stages? Will chloroplast acquisition, which ultimately leads to kleptoplasty, be at risk under a future ocean climate change scenario?

Exposure to future ocean conditions led to a significant decrease on the fitness of *E. clarki* across different life-stages, from adults to embryos, veligers and juveniles. Exposure to future climatic scenarios for the oceans of tomorrow led to a significant decrease in the number of spawned egg masses, as well as in their membrane thickness. Moreover, a significant decrease in the volume of embryo capsules was accompanied by an increase in embryo volume. These findings suggest that sacoglossan sea slugs shifted their allocation of energy towards the quality of embryos, rather than to the structures that protect them from environmental challenges. Future ocean acidification and warming significantly reduced the survival and length of veligers and caused an increase in the incidence of deformities. These findings have implications for recruitment and further affect the species persistence in a changing ocean. Yet, chloroplast acquisition by juvenile specimens seems to not be affected by future acidification and warming conditions.

4. How does acidification and warming affect the physiology and photobiology of tropical and temperate photosynthetic sea slugs?

Tropical and temperate solar-powered sacoglossan sea slugs respond differently to increased Pco_2 and temperature in the long term. Along with the reduced survival and photosynthetic efficiency of tropical *E. clarki* under acidification and warming, our results suggest that heat shock and antioxidant response may play

an important role as mechanisms for stabilizing photosynthesis in stress situations as observed for plants and corals (Bhagooli & Hidaka, 2004, Heckathorn *et al.*, 2002, Moya *et al.*, 2015). Specimens of *E. clarki* were observed to bleach as a consequence of kleptoplasts photoinhibition and poorer antioxidant defences to deal with environmental stress. *E. viridis* was revealed to be a more resilient host with the capacity to neutralize ROS, and equipped with the metabolic machinery for preventing further damage to proteins via HSPs, but its survival was still negatively affected. These features provide photo-physiological tolerance to future climate change scenarios, thereby revealing an adaptable mollusc-kleptoplast complex. The work presented here is the first reporting that other photosynthetic models, rather than cnidarian-dinoflagellate symbiosis, bleach under climate change future scenarios.

The results obtained have broad implications and provide us with critical information to anticipate negative impacts on the recruitment of photosynthetic sea slugs in the oceans of tomorrow. Nevertheless, geographic and taxonomic responses to climate change are highly variable and several key aspects of photosynthetic sea slug research remain to be addressed.

6.2 Future directions

Research in kleptoplastic symbiosis is still in a relatively early stage of development when compared with other animal–algae symbioses, namely corals. To guide the scope of future studies addressing the eco-physiological responses of solar-powered animals to changing ocean conditions, acidification and warming should be examined together with expected combinations of other environmental changes. Recent research suggests that combined anthropogenic eutrophication and climate change poses a new risk to coastal communities (Rabalais *et al*., 2009). The cumulative effects of global change, including climate warming and increased human population, as a result of intense industrialisation and agriculture, will probably continue and intensify the course of eutrophication in marine ecosystems. Global climate change will likely result in higher water

temperatures, stronger stratification and increased inflows of freshwater and nutrients to coastal waters in many areas (Suikkanen *et al*., 2013).

Based on the present findings, it is also crucial to evaluate the effects of future ocean conditions on the onset and post-bleaching recovery of marine photosynthetic symbionts. How can tropical and temperate animals re-establish their photosynthetic symbioses with plastids after damaging or bleaching events? Is this recovery favored in temperate waters rather than in the tropics?

Evolutionary responses to an increasing CO_2 concentration and temperature in photosymbiotic organisms should also be examined across generations. Acclimatization and its experimental equivalent, acclimation, can influence the ability of organisms to cope with the scenarios currently predicted for the oceans of tomorrow (Donelson *et al*., 2012; Munday *et al.,* 2013; Guillaume *et al*., 2015). Furthermore, understanding whether the remaining variation within species and life stages represent real biological differences among species, locally adapted populations, or acclimatory capacities, rather than experimental error, remains a critical area for future research. Is local adaptation driving to new biological diversity? If so, what are the evolutionary consequences?

Ocean acidification and warming is already underway and it is now predictable that it will, in combination with other stressors, have significant effects on marine ecosystems. Ultimately, only the reduction of atmospheric CO_2 levels provides the “solution” to ocean acidification and warming; nevertheless, there may be ways in which ocean acidification and warming research can become more “solution-oriented”, rather than simply “documenting the disaster”. Research in this field may need to be prioritized and by improving our understanding on the impacts of ocean climate change, it will be possible to identify the most vulnerable organisms and ecosystems needing the most urgent attention.

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