Study of environmental and biological factors that affect larval survival in sessile coastal marine organisms

Estudi dels factors ambientals i biològics que determinen la viabilitat larvària en els organismes sèssils litorals marins

Thesis doctoral

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Memòria presentada per Núria Viladrich Canudas per optar al títol de
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Per Tu,
que m’has ensenyat a somià

Resum

La reproducció sexual és un procés biològic fonamental per a la majoria de les espècies vives, sent essencial per a la perpetuació de les espècies i assegurar-ne la diversitat genètica. En invertebrats sèssils marins, com corals i gorgònies, aquest tipus de reproducció, a més, permet la dispersió dels individus, el que facilita la colonització de noves àrees i assegurar el flux de gens entre poblacions. En general, la reproducció sexual es caracteritza per un alt cost energètic, la qual cosa implica que els organismes han de trobar un equilibri entre el creixement, la supervivència i la reproducció.

La diferent assignació d'energia en la reproducció, la supervivència i el creixement es reflecteix en les diferents estratègies reproductives. Els invertebrats sèssils mostren dues estratègies reproductives principals: 1) la producció d'un gran nombre de petites larves, o 2) la producció de poques però grans larves. En aquest context, el nombre de larves pot influir en la quantitat d'energia invertida pels pares i per tant en la seva supervivència, mentre que la talla pot afectar a l'energia disponible/emmagatzemada en les larves i per tant a la supervivència de la descendència. La supervivència de les larves i la de les colònies parentals però, també pot dependre de la estratègia tròfica. En corals i gorgònies no simbiòtiques, l'adquisició d'energia deriva de la captura de matèria orgànica particulada en suspensió a la columna d'aigua, però la abundància d'aquest aliment pot presentar fluctuacions aleatòries. En les espècies simbiòtiques, el carboni autotròfic produït pels simbionts s'utilitza per cobrir la major part de les necessitats metabòliques de la colònia, no obstant, la seva obtenció està fortament relacionada amb la intensitat de la llum. Per tant, la combinació de autotròfia i heterotròfia (mixotrófica) és una forma de maximitzar l'adquisició de nutrients i l'èxit ecològic en ambients on la llum i la concentració de plàncton poden ser molt variables i sovint limitant. És important destacar que l'assignació energètica de la colònia a les larves també pot ser un punt clau per entendre el potencial de les noves generacions per colonitzar nous espais. Per tal d'entendre com el cost energètic de la reproducció afecta les diferents etapes del cicle de vida, la present tesi analitza la inversió energètica en la reproducció, els efectes d'aquesta inversió en la supervivència de les colònies parentals i la energia transferida a les larves, en tres espècies de gorgònies del Mar Mediterrani (*Corallium rubrum*, *Eunicella singularis* i *Paramuricea clavata*) d'acord amb la seva estratègia reproductiva i tròfica. Per a aquest objectiu, els mètodes clàssics (és a dir, la producció de gàmetes) es van combinar amb nous enfocaments bioquímics (és a dir, contingut de lípids i contingut i composició d'àcids grassos). És important destacar que a causa de la selecció de diferents estratègies tròfiques i reproductives, la present tesi representa un cas d'estudi que pot ser aplicat fàcilment en altres zones del món.

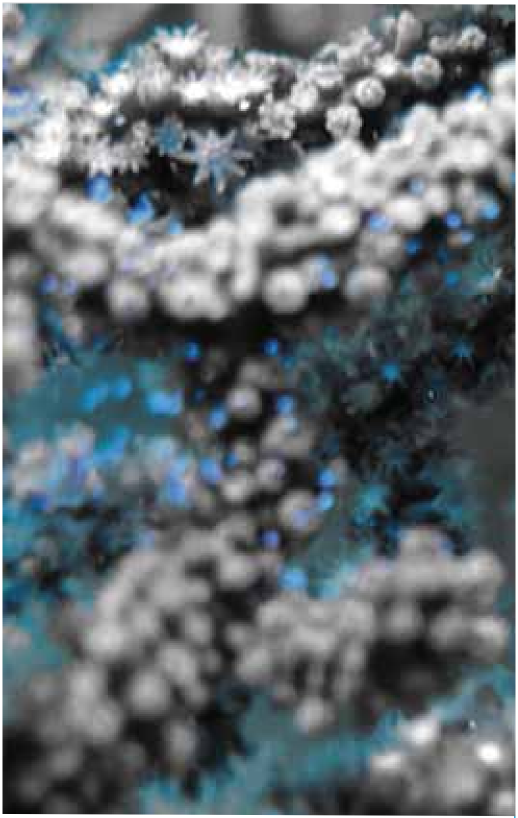
Abstract

Sexual reproduction is one of the fundamental biological processes for most living species and it is essential for the perpetuation of the species and to ensure genetic diversity. In marine sessile invertebrates, such as corals and gorgonians, sexual reproduction is also important because it allows the dispersal of individuals, facilitating the colonization of new areas and ensuring the flux of genes among populations. In general, sexual reproduction is characterized by a high energetic cost, which implies that organisms reproducing sexually have to find a trade-off between growth, survival and reproduction.

Different balances in the energetic investment in reproduction, survival and growth are reflected in the different reproductive strategies. Sessile invertebrates can display two main reproductive strategies: 1) the production of high numbers of small larvae, or 2) the production of few but large larvae. In this context, the number of larvae may influence parental energy reserves and thus, their survival, while the size may affect energy storage/availability in the larvae, and therefore, the offspring survival. However, the survival of both the larvae and the parental colony also depends on the nutritional condition. In non-symbiotic corals and gorgonians, the energy supply proceeds from the capture of particulate organic matter suspended in the water column, although its abundance can follow random fluctuations. In symbiotic species, autotrophic carbon produced by the symbionts is largely transferred to the colonies and used to cover most of their metabolic needs, but its availability is strongly related to light intensity. Therefore, combining autotrophy with heterotrophy (mixotrophy) is a way to maximize nutrient acquisition and ecological success in environments where light and plankton concentration can be very variable and often limiting. Significantly, the energy allocation from the maternal colony to the larvae may also be a key point to understanding the potential colonization by new generations. In order to understand how the energetic cost of reproduction affects the different stages of the life cycle, the present thesis analyses the energetic investment in reproduction, the effects of this investment on parental colonies and energy transfer to larvae in three gorgonian species of the Mediterranean Sea (*Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata*) according to their reproductive and trophic strategy. To this aim, classical methods (i.e. gamete production) were combined with new biochemical approaches (i.e. lipid content and FA content and composition). It is important to emphasize that due to the selection of different trophic and reproductive strategies, the present thesis represents a case study that is easily applicable to other areas of the world.

Contents

Introducction



VILADRICH N (2015) Doctoral Thesis pp 13-20

INTRODUCTION

Reproduction is the process by which new individual living organisms are generated. It is one of the fundamental biological processes of all living species and it is essential for perpetuating the different phylum. In general, reproductive mechanisms entail an energetic cost (Calow 1979). However, the amount of energy that an organism can devote to reproduction is limited, since the energy resources available are usually finite and need to be partitioned into different processes such as respiration, movement, growth, defence and reproduction (Giesel 1976; Lawrence 1985; Stearns 1992). To minimize the amount of energy allocation in reproduction, without reducing the colonization capacity, some organisms can reproduce asexually. However, in the long term (over several generations), asexual reproduction compromises the capability for adaptation to the environment due to the loss of genetic diversity introduced by sexual reproduction.

Sexual reproduction

Sexual reproduction is a mode of reproduction involving the fusion of female and male gametes. It is characterized by a high energetic cost, which implies that organisms reproducing sexually have to find a trade-off between growth, survival and reproduction. Different balances in the energetic investment in reproduction, survival and growth result in different reproductive strategies. Some traits of different reproductive strategies include the age and size at which an organism reaches reproductive maturity, how often it can reproduce during its lifetime or within a year, the duration its sexual fertility, the number and size of offspring produced, and the expected age-specific mortality and longevity (Kunz and Orrell 2004). A high number of different reproductive strategies exist, however, to simplify, it has been considered that a species can be either semelparous or iteroparous (Stearns 1976). In semelparous species, an individual allocates all its available energy to a single burst of reproduction and then dies (e.g. salmon; termed "big bang" reproduction). Conversely, iteroparous species are those that reproduce more than once, and their life history traits need to balance the energy allocated to reproduction and the one devoted to the maintenance of parental individuals. In iteroparous species, the principal objective of the reproductive strategy is to maximize the energy invested in reproduction in relation to available energy and parental life expectancy (Wootton 1984; Roff 1992; Pianka 2000). Energetic investment in reproduction can be focussed to increase the number and/or the survival of the offspring. This different allocation of energy is strongly related to the life history strategy of a species in the continuum between the two extremes of r-oriented and k-oriented strategies. R-oriented species are characterized by high fecundity (i.e. number of sexual products), low survival of offspring, and commonly show high resilience to disturbances. Conversely, k-oriented species are characterized by low fecundity with high survival of offspring, and low resilience but high resistance to disturbances. Moreover, different populations of the same species may experiment very different environmental conditions depending on the place where they live, which can strongly affect the fitness of individuals and consequently, the energy that they can allocate to maintenance, growth and reproduction. This results in differences among populations or time periods in fecundity as well as in the survival of parental organisms and offspring.

Sexual reproduction in corals and gorgonians

Marine sessile invertebrates, such as corals and gorgonians, are characterized by a complex life cycle with a sedentary adult phase and a mobile larval phase. For such species then, sexual reproduction is not only important for the maintenance of their populations, or so as to ensure adequate genetic variability, but also to allow the dispersal of species and, consequently, their capability to maintain gene fluxes among populations and to colonize new areas (Palumbi 1994; Heller and Zavaleta 2009; Hart and Marko 2010). Corals and gorgonians present lecithotrophic larvae, which are capable of developing based solely on the maternal provisions transferred during the oogenesis (Thorson 1950; Pechenik et al. 1990; Morgan 1995). Therefore, offspring survival capacity and larval dispersal depend, in part, on larval energy reserves transferred by the mother colony (Mousseau and Fox 1998; Roff 2002; Maestripieri and Mateo 2009). This maternal energy transfer can be related to the reproductive strategy of the species, and can affect both offspring and parental colony survival.

Corals and gorgonians can present three different reproductive strategies: 1) broadcast spawning, where sperm and oocytes are released and fertilized in the water column, 2) surface brooding, where the oocytes are retained by mucous material and fertilized on the surface of female colonies, and 3) internal brooding, where oocytes are fertilized inside polyps of female colonies, where the zygotes and early embryos are retained (Fautin 2002). While gorgonians exhibit similar proportions of broadcast spawners, brooders, and surface brooders (Ribes et al. 2007), scleractinians are mainly broadcast spawners (Harrison and Wallace 1990; Richmond 1997; Fautin 2002). Each of these reproductive strategies may affect fecundity, fertilization success and offspring survival. Broadcast spawner corals and gorgonians are characterized by high fecundity, and participate in synchronized mass spawning events, a strategy that may increase the fertilization success and reduce predation pressure on gametes (Alino and Coll 1989). However, in these species, parental colonies allocate low energy reserves in larvae and thus, offspring survival may be low (Harrison and Wallace 1990). Conversely, in brooder species, spawning synchronicity may not be as crucial as for broadcast spawners, since eggs are retained either inside the polyps or on the colony surface until fertilization occurs (Coma et al. 1995; Dahan and Benayahu 1997). Indeed, despite asynchronous spawning, the surface brooding gorgonians *Pseudopterogorgiaelizabethae* and *Paramuriceaclavata* are known to achieve relatively high levels of fertilization success (Lasker unpubl. Data; Gutierrez-Rodriguez and Lasker 2004; Linares et al. 2008). Moreover, internal brooding may be a strategy that compensates for low fecundity by enhancing offspring survival, by means of high-energy reserves transferred to the eggs as well as by providing protection from predation during larval development (Babcock 1990).

Besides the reproductive strategy, the offspring survival and dispersal may also be influenced by the nutritional condition of maternal colonies which depends on their trophic strategy (Grottoli et al. 2006; Gori et al. 2012) and food availability (Rossi et al. 2006; Gori et al. 2013). In non-symbiotic corals and gorgonians, the energy supply proceeds from the capture of zooplankton and particulate organic matter suspended in the water column (Giliand Coma 1998; and references therein), and in some cases, also from the uptake of dissolved organic matter (e.g. Al-Moghrabi et al. 1993). In symbiotic species, autotrophic carbon produced by the symbionts is largely transferred to the corals and used to cover most of their metabolic needs (Muscatine et al.1981,1984). However, heterotrophic nutrition has been highlighted as also very important for the energy supply in several symbiotic corals and gorgonians (e.g. Goreau et al. 1971; Sebens et al. 1996; Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004;Palardy et al. 2008). Combining autotrophy with heterotrophy (mixotrophy) is a way to maximize nutrient acquisition and ecological success in environments where light and plankton concentration can be very variable and often limiting (Muller-Parker and Davy 2001; Grottoli et al. 2006). The different trophic strategies (mixotrophic or heterotrophic) together with the reproductive mode (broadcast spawning, surface brooding or internal brooding) will be decisive in understanding the presence and survival of species in different areas and ecosystems. Importantly, the energy allocation from the mother colony to the offspring will also be a key point to understanding the potential colonization by the new generations, this process being poorly understood in marine sessile invertebrates in general, and in anthozoans in particular.

Understanding the energy allocation from the mother colonies to the offspring

Until now, reproductive investment in corals and gorgonians has mainly been estimated from reproductive effort, i.e. by quantifying fecundity and oocyte volume (e.g. Hall and Hughes 1996). However, this approach does not account for the energetic cost caused by gametes production or by tissue repair associated with spawning, and thus, the reproductive investment may be potentially understated (Calow 1979). Moreover, it is very difficult to understand how much of the available energy is invested in reproduction from the reproductive effort, and thus we cannot estimate how the energy investment in reproduction can affect parental colonies and larval survival.

To sustain metabolic demands, all living organisms require energy, which can be obtained from proteins, carbohydrates or lipid reserves according to the metabolic processes. Proteins are mainly used in structural, enzymatic, transport and regulatory functions in the cell (Bujnicki 2009). Carbohydrates represent the principal source of basic energy, as they are quickly catabolized to obtain immediate energy (Lehninger 1982). Instead, lipids constitute the main energetic reserves in animals, and can strongly affect the general fitness of individuals and their survival, reproduction and growth (Szmant and Gassmann 1990; Anthony and Connolly 2004; Rossi et al. 2006; Grimsditch and Salm 2006). Therefore, the quantification of lipid content in parental colonies before and after reproduction may be a useful method of studying the amount of energy allocated in reproduction as well as the energetic implications of the reproductive effort on parental organisms.

The total lipid content is the sum of several lipid compounds such as wax esters, phospholipids, triacylglycerols and free fatty acids (FA; Imbs 2013). Phospholipids are major constituents of cell membranes and are not involved in metabolism, whereas wax esters and triacylglycerols are considered stable energy reserves that can be oxidized to provide energy in form of free FA (Gurr et al. 2002). Free FA are characterized by high power efficiency (high ATP per FA molecule) (Sargent et al. 1988), and thus, their content can be used as a measure of metabolic demands. Indeed, FA content can increase under stress situations, such as starvation and thermal stress, in order to compensate for the increment of metabolic needs (Sargent et al. 1999). On the other hand, FA composition may reflect the nature of metabolic demands (i.e. energetic requirements) (Díaz-Almeyda et al. 2011; Imbs 2013; Viladrich et al. 2015), and the amount of the different kinds of FA (Saturated Fatty Acids (SFA), Mono Unsaturated Fatty Acids (MUFA), and Poly Unsaturated Fatty Acids (PUFA)) may determine the fitness of individuals. In fact, PUFA are highly energetic FA, essential for overcoming stress conditions, since they can be converted in many other FA (selective accumulation) (Müller-Navarra et al. 2000; Wacker and Von Elert 2001), while MUFA, but especially SFA, are mainly used for basic metabolic energy consumption (Sargent et al. 1999; Dalsgaard et al. 2003). Therefore, the quantification of free FA content and composition in parental colonies before and after reproduction may be a useful method of assessment of the metabolic effects of reproduction on parental organisms. Similarly, their content and composition in larvae may highlight the amount of used energy, and how this energy is mainly used by larvae.

Aims of the thesis

The present thesis analyses the energetic investment in reproduction, the energy transfer to larvae, and its effects on the parental colonies, in three of the main gorgonian species in the coastal areas of the Mediterranean Sea (*Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata*) according to their reproductive and trophic strategy. In order to take into account the temporal variability of environmental conditions, and its effects on the reproductive process in the same population, all the analyses, together with an environmental characterization of the study area, were carried out in two different years (2010-2011). As the thesis is based on samples collected in the same area, environmental features are the same for all the species and are presented in a pre-chapter describing the environmental and biological conditions during the sampling period (temperature, oxygen, settling pParticulate oOrganic mMatter, epibenthic zooplankton, etc.). The present thesis consists of one chapter describing the environmental features and three chapters containing new data and results based on several background hypotheses, followed by an overall discussion.

Pre-chapter: **Environmental features in the studied coastal area of Cap de Creus (May 2010 – November 2011)**

Chapter 1: **Energetic resource allocation for reproduction in temperate octocorals *Corallium rubrum* and *Paramuricea clavata*: Contrasting reproductive strategies of surface brooders versus internal brooders**

Chapter 2: **Energetic resource allocation for reproduction in temperate octocorals *Corallium rubrum*** and *Eunicella singularis*: Contrasting mixotrophic and heterotrophic strategies

Chapter 3: Differential energy allocation between maternal colonies and larvae in the octocorals *Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata*

Discussion

Box 1
Ecological importance of Mediterranean gorgonians

Gorgonians are among the main engineering species (*sensu* Jones et al. 1994) in several benthic communities in the Mediterranean Sea (Gili and Ros 1985; Harmelin and Garrabou 2005;Ballesteros 2006), where they play a crucial structural and functional role (Wildish and Kristmanson1997; Gili and Coma 1998). From a structural point of view, the three-dimensional complexity originated by their populations may change hydrodynamism at a local scale and, consequently, increase the residence time of suspended particles (Gili and Coma 1998). The main environmental features, such as current flow, food availability, and sediment re-suspension, vary widely within these complex structures, and this heterogeneity may increase the abundance and functional diversity of the associated fauna (Laborel et al. 1961; Harmelin 1995). From a functional point of view, gorgonians play a paramount role in sublittoral food webs, since they cause a significant flow of matter and energy from the pelagic to the benthic system (Gili and Coma 1998), by capturing plankton and particulate organic matter suspended in the water (e.g. Coma et al. 1994; Ribes et al. 1999, 2003; Rossi et al. 2004; Tsounis et al. 2006). The three Mediterranean gorgonians chosen in the present work represent case studies that can be extrapolated easily to other areas of the world due to its differential trophic and reproductive strategies.

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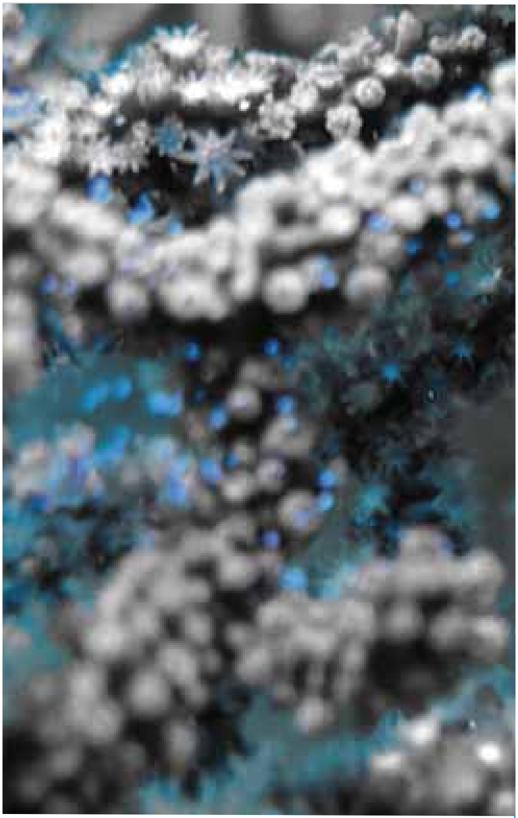
Prechapter

Environmental features in the studied coastal area of Cap de Creus (May 2010 – November 2011)



VILADRICH N (2015) Doctoral Thesis pp 21-32

Environmental features in the studied coastal area of Cap de Creus (May 2010 – November 2011)

Environmental features may be a key point to understanding the presence, distribution and population health status of species. As passive suspension feeders, corals and gorgonians are affected by environmental and biological factors like water temperature (Glynn et al. 1985; Oku et al. 2003), turbidity (Anthony and Fabricius 2000; Larsson et al. 2013), water currents (Chindapol et al. 2013; Madin and Connolly 2006), food availability (Meyers 1979; Dalsgaard et al. 2003; Rossi et al. 2006; Imbs et al. 2010) and, in the case of symbiotic species, by light intensity (Harland et al. 1992). However, the main studies on the consequences of biotic and abiotic factors have been performed in the case of stochastic events or in experimental assessment (Coma et al. 2006; Garrabou et al. 2009; Previati et al. 2010; Ezzat et al. 2013), omitting the effects caused by seasonal and inter-annual variations. The fluctuations of environmental features, even at small scale, exert an overriding role in species distribution, especially in passive suspension feeders (Cushing and Dickson 1976; Glémarec 1979; Southward and Boalch 1994; Wilkinson and Buddemeier 1994; Southward et al. 1995; Garrabou et al. 1998) and thus, the study of environmental conditions may be important to better understand the ecology of species.

In general, seasonal fluctuations are minimal in tropical seas and extreme in polar ones (Thomas 2004). Conversely, in temperate seas, such as the Mediterranean, seasonal variations of environmental conditions may be more gradual (Estrada 1996). In actual fact, the highest turbidity and flow currents ensue in winter, the highest temperature and light intensity occur in summer, whereas autumn and spring are considered seasons of transition (Estrada 1996; Coma et al. 2000). As a consequence of this strong seasonality, heterotrophic species suffer a stressful period in summer due to starvation, while autotrophic species undergo a reduction of autotrophic carbon input in winter (Coma et al. 2000; Rossi et al. 2006). Therefore, the degree of affectation depends on the trophic strategy.

On the other hand, environmental conditions can also show inter-annual variations determining yearly variability in growth rates, reproduction process and survival (Qian and Chia 1991; Gardner 2000; Bramanti et al. 2005; Gori et al. 2007). In this case, the degree of affectation depends on the ecology of species, and thus, knowledge of environmental conditions is important to better understand the life-history of organisms. Therefore, in order to take into account the temporal variability of environmental conditions, and its effects on the reproductive process, all the analyses were carried out in two different years (2010-2011) in the same populations. The following pages contain a description of the main biotic and abiotic factors in the study area, from May 2010 to November 2011.

Study Area

The present thesis was carried out at Punta s'Oliguera in Cap de Creus (42° 17'03'' N; 003° 17'95'' E, Northwestern Mediterranean Sea; Fig. 1), where *C. rubrum* population was at 25–30 m depth on rocky overhangs, *E. singularis* population at 12–16 m depth on sloping substrates and *P. clavata* population at 16–23 m depth on vertical rocky walls (Fig 1).

In this area, the environmental features (temperature, currents, dissolved oxygen, turbidity, suspended sediment fluxes (SSF) and zooplankton) were monitored from May 2010 to November 2011, concurrently with the monitoring of the populations of the three species. The final target was to identify the factors that may affect the annual dynamics of the gorgonian populations depending on the reproductive and trophic strategy.

Temperature

The seawater temperature could explain variations in physiological and metabolic processes of many species. In fact, it may regulate feeding rate (Ribes et al. 1999), polyp activity (Previati et al. 2010), gonadal development (Gori et al. 2007) and spawning timing (Gori et al. 2012) in gorgonian populations. Hence, in order to describe temporal and spatial variability, the seawater temperature at the sampling location was recorded hourly from June 2010 to March 2012 with Hobo Pro V2 temperature data loggers lo-

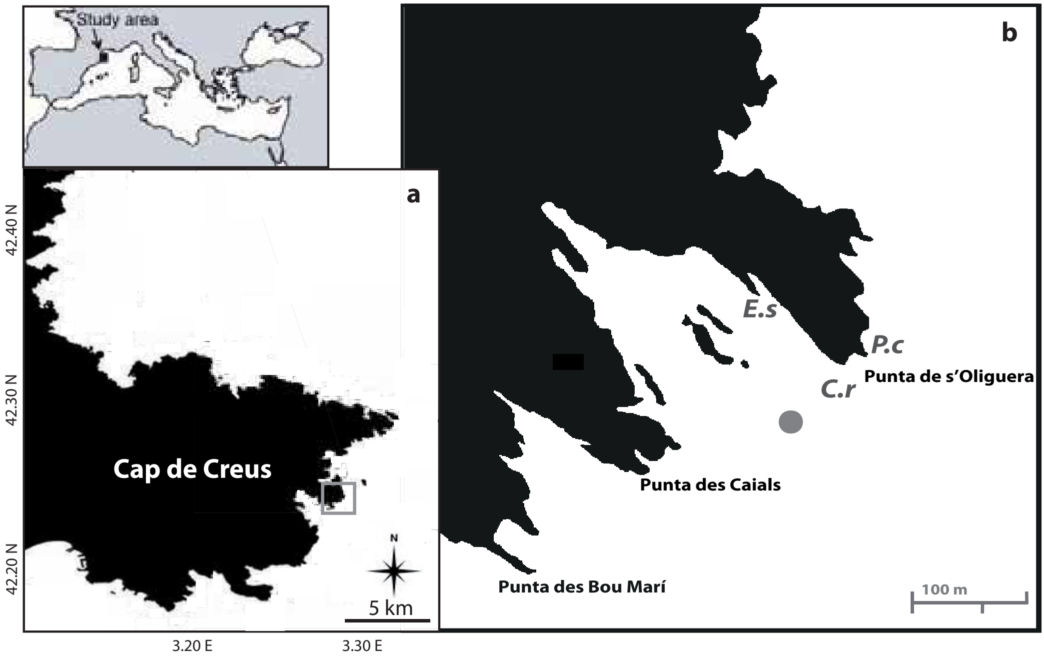


Fig. 1. Map of the study area (a), and location of the sampling site (b). *C.r*, *E.s* and *P.c* indicate the position of the *Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata* populations, respectively, while circle indicate the position where the environmental features were monitored.

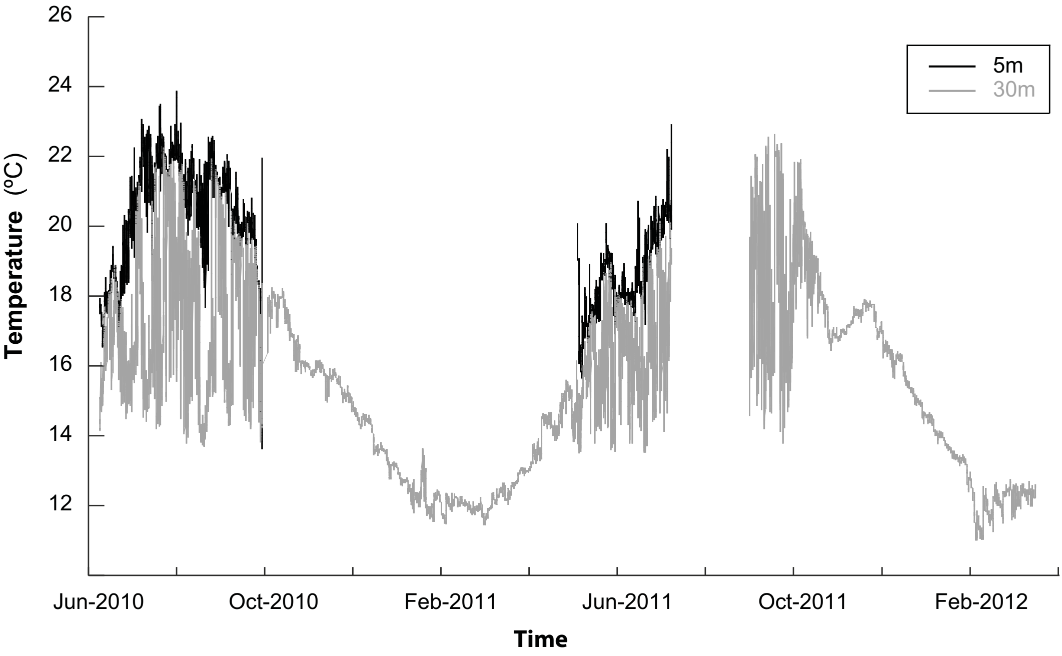


Fig. 2. Water temperature in the sampling site at depths of 5 and 30 m.

cated at 5 and 30 m depth. The results showed that the water temperature in the study area ranged from 13.6℃ to 23.7℃ at 5 m depth, and from 11.0℃ to 22.7℃ at 30 m depth (Fig. 2). The upper layer of the water column (5 m depth) is characterized by a marked seasonality, whereas large and fast variations in temperature (up to 4 ℃ in one hour, and sometimes almost 10℃ within one day) occurred from spring to autumn at 30 m depth. In 2010, the water column stratification began to develop in early June and broke in last September. Conversely, the stratification in 2011 started in May and was broken in middle October, 57 days later than in 2010.

Currents

Passive suspension feeders, such as gorgonians, depend on the water current for food delivery. The effect of flow velocity on feeding abilities has been the focus of several investigations (Best 1988; Okamura 1990; Patterson et al. 1991). In general, current velocity has a significant effect on the capture rates of food particles by passive suspension feeders (Patterson 1984; Best 1988; Harvell and LaBarbera 1985). Consequently, the amount of lipid reserves could change depending on the current intensity. Current speed was measured with RCM9 Aanderaa, situated in the middle of Caials bay at approximately 29 m depth (Fig. 1) with a sampling frequency of 1 hour. However, due to technical problems, current measures were not taken in the same period as the other measurements. The dataset was recorded from May 2012 to January 2013. The results showed that the sampling area is exposed to bidirectional currents during most part of the time (ANNEX 1, p. 97). The dominant currents are from north (Fig. 3), but, due to

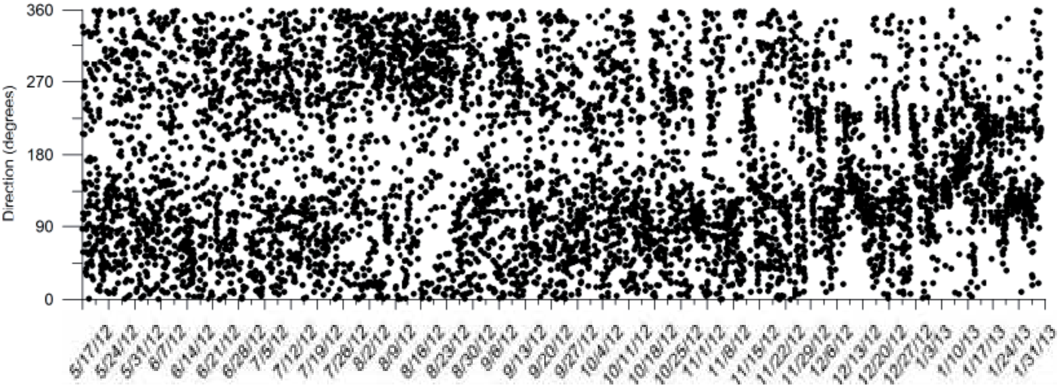


Fig. 3. Annual direction of currents in the sampling site.

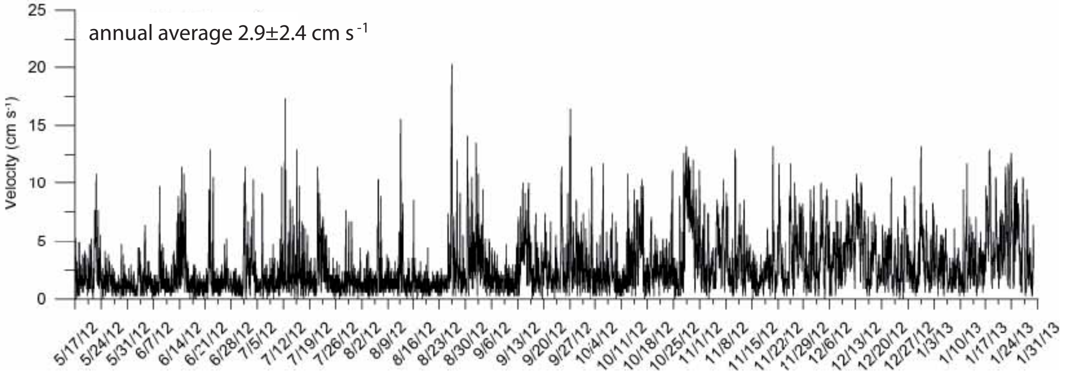


Fig. 4. Annual current velocities in the sampling site.

the geographic position of the bay, the strongest current was from north-east (Fig. 3, Fig. 4). This current is caused by easterly winds, which are rare (<6% of the time) and brief (less than 3 days), so the mean annual velocity of the current is weak (2.9 ± 2.4 cm/s). The current velocities showed a seasonal variation with higher values at the end of the summer and autumn (Fig. 5). The currents in the study area are not influenced by the diurnal or lunar tides (data not shown), as previously observed (Alberola-Lla et al. 1995).

Dissolved oxygen

In marine invertebrates, oxygen availability can influence physiological and behavioural processes (Riedel et al. 2008). In gorgonians, for example, when oxygen availability decreases, polyps reduce their activity (Previati et al. 2010) and consequently, their feeding rates (Coma et al. 2002) and their energy storage capacity. In order to account for these effects, values of dissolved oxygen in the water were recorded from May 2012 to January 2013 with RCM9 Aanderaa, situated in the middle of Caials bay at approximately 29 m depth (Fig. 1). The results of dissolved oxygen showed higher variability in summer and early autumn (Fig. 5), a period which also coincided with the highest variability of seawater temperature. In fact, many authors have demonstrated that high temperatures reduce the oxygen solubility (Carpenter 1966; Truesdale et al. 1955), thus limiting its availability. For gorgonian populations, the optimal rank of dissolved oxygen is 5–6 ml/l (Riedel et al. 2008; Previati et al. 2010). So, the results of dissolved oxygen suggest that the studied gorgonian populations are well oxygenated in the studied area.

Turbidity

Turbidity is defined as the density of suspended particulate matter (SPM) present in the water column. Extremely high turbidity may cause a strong attenuation of light in the water column (Gordon and McCluney 1975; Ivanoff 1975; Morel 1991), thus SPM has an inhibitive effect on primary production. This reduction of primary production causes a decrease in the energy available for gorgonians, although the quantity of available food increases (Grémare et al.

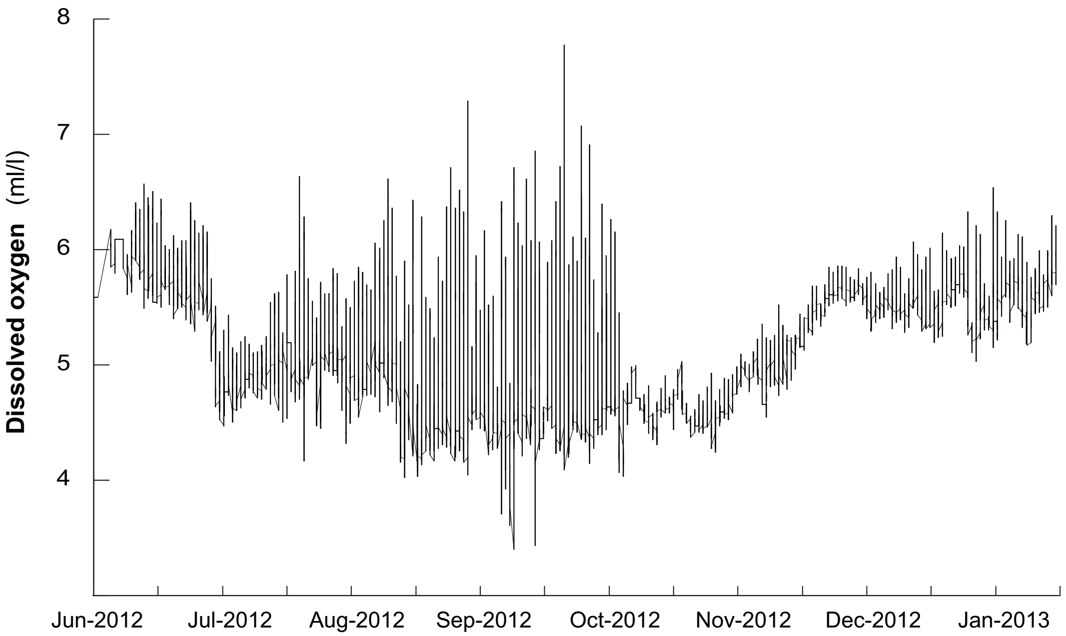


Fig. 5. Annual dissolved oxygen in the water in the sampling site.

1997; Rossi et al. 2003). The values of turbidity were measured from May 2012 to January 2013 with RCM9 Aanderaa, situated in the middle of Caials bay at approximately 29 m depth (Fig. 1). Turbidity values recorded are expressed in nephelometric turbidity units (NTU)(equivalent at the formazin turbidity units (FTU)). The turbidity showed a seasonal variation with maximum values in October and November (3.10 ± 0.85 NTU and 3.51 ± 1.56 NTU, respectively) (Fig. 6, Fig. 7). The period of highest turbidity coincided with the period of maximum current intensity (end of summer and autumn). Considering the absence of any riverine input and taking into account the position of the sensor (1 m over the sea-bottom), these results suggest that the turbidity is mainly due to re-suspended sediment.

Suspended sediment fluxes (SSF)

Sampling of SSF was carried out monthly from May 2010 to November 2011 by means of a sediment trap, following the protocol of Grémare et al. (1997) and Rossi et al. (2003). The sediment trap was moored at 30 m depth on sandy-stone bottoms in the middle of Caials bay (Fig. 1) and it consisted of two polyethylene pipes prolonged by a cone and a collector with an aspect ratio of 4.75. The mouths of the traps were located 3 m above the bottom. Collected samples were centrifuged (4000 rpm, 15 min), frozen (-20°C), and freeze-dried. The collected material was sieved on a 200 µm mesh, and two fractions were weighed to assess the sediment flows: (1) gross sediment >200 µm size, and (2) <200 µm size fraction. The lipid content of the <200 µm fraction was also assessed.

One of the main components of the diet of benthic suspension feeders consists in particulate matter (Ribes et al. 1999; Tsounis et al. 2006; Coma et al. 2015), therefore changes in SSF could affect their nutritional condition. The gross sedimentation flow (GSF) was between 1.96 and 17.85 g dry weight (DW) m^{-2} d^{-1}, while the <200 µm size fraction were between 0.57 and 13.72 g DW m^{-2} d^{-1}. The results showed a marked seasonality, with the highest flow in late spring and autumn (Fig. 7), as previously observed in other coastal areas of the Mediterranean Sea (Grémare et al. 1997; Rossi et al. 2003). The relationship between GSF and <200 µm size fraction was constant during the year, but average GSF and <200 µm size fraction were different between 2010 and 2011, being higher in the second year. Overall, the values measured in the present study were lower than in previous studies in Banyuls sur Mer and in Medes Islands (Grémare et al. 1997; Rossi et al. 2003).

However, SSF is composed by both inorganic and organic matter, and thus not all the SSF constitute suitable food for gorgonians. The OM content in suspended sediment was higher than in previous studies (Grémare et al. 1997; Rossi et al. 2003), but did not show any clear seasonality (Fig. 8).

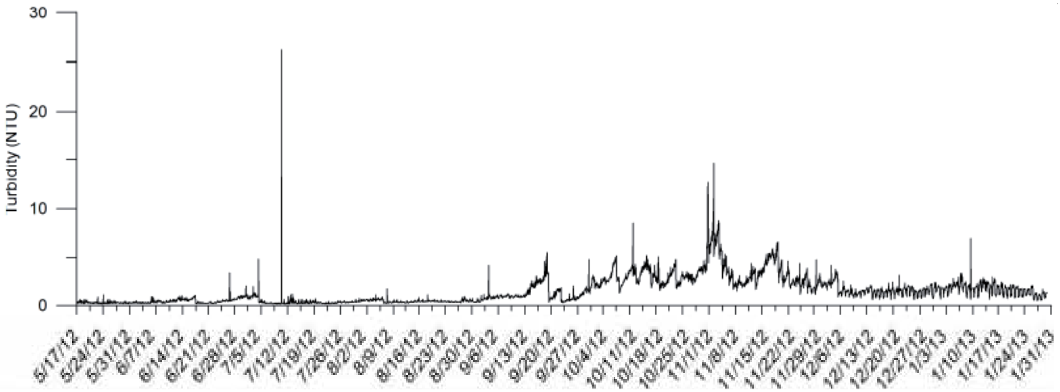


Fig. 6. Annual turbidity values recorded in nephelometric turbidity units (NTU) (equivalent at the formazin turbidity units (FTU)) in the sampling site.

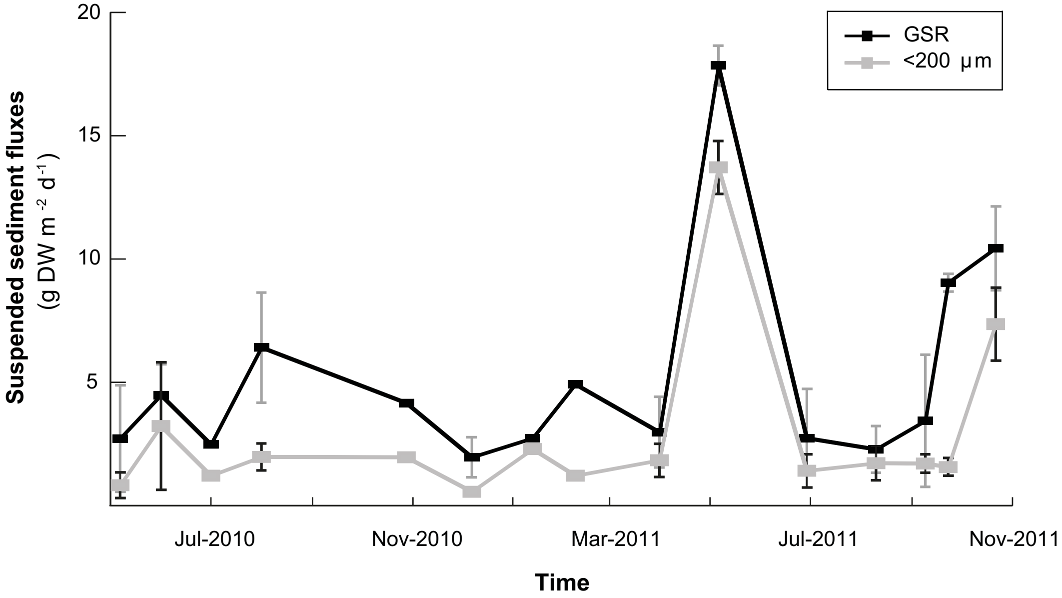


Fig. 7. Suspended sediment flux in the sampling site from May 2010 to November 2011 (mean ± SD). GSF (gross sedimentation flow) is characterized by the fraction of >200 µm size (black line), while <200 µm size represent the fraction of suspended particulate matter available for gorgonians (grey line).

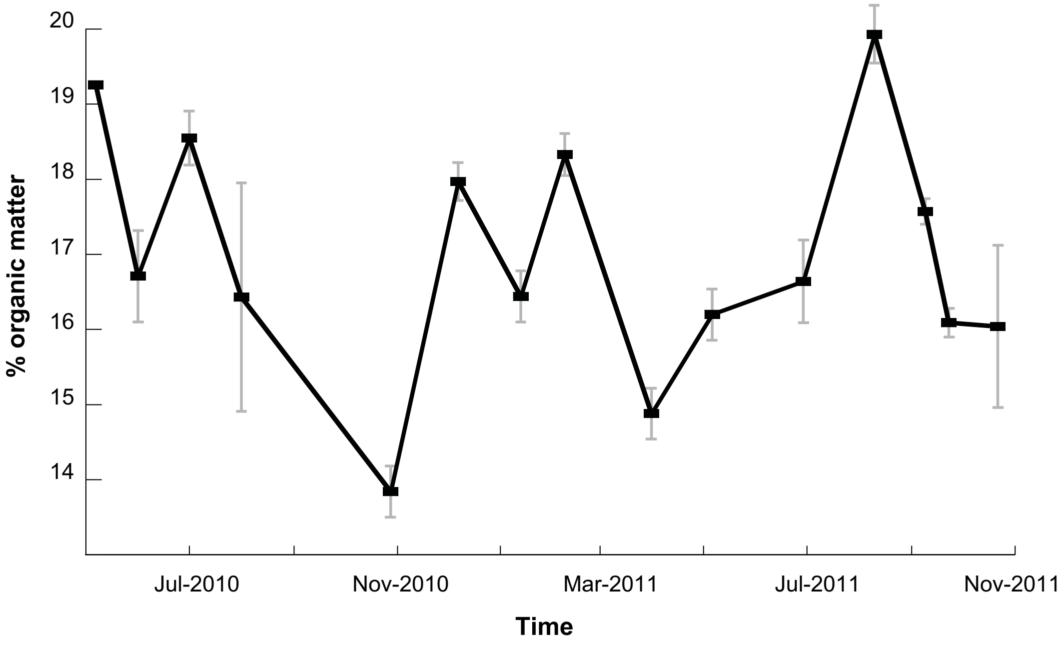


Fig. 8. Percentage of organic matter (mean ± SD) in the suspended particulate matter in the sampling site from May 2010 to November 2011.

Lipid content in SSF

Lipid content in the <200 µm size fraction of SSF ranged from 0.82 (December 7th, 2010) to 24.47, and 20.22 µg mg^{-1} DW (October 28th, 2010 and May 7th, 2010 respectively) (Fig. 9). The average lipid quantity was different between 2010 and 2011. In the first year, results showed 2 minimum peaks, one in late summer and the other in middle winter. In the second year, the lipid quantity showed a high variability with no evident seasonal cycle. The measured lipid concentration was higher than in previous works (Grémare et al. 1997 and Rossi et al. 2003). Therefore, although the suspended matter was less abundant (Fig. 7), its nutritional value was higher than previously reported for other Mediterranean coastal areas (Fig. 9).

Zooplankton

Epibenthic zooplankton abundance was quantified monthly from May 2010 to November 2011 with a 100 µm plankton net (22 cm diameter) dragged by SCUBA divers along 100 m, ~30–50 cm over the gorgonians, as in Coma et al. (1994) and Rossi et al. (2004). Zooplankton was fixed in 6% formaldehyde in seawater and then identified and quantified to the lowest taxonomic level.

Concentration and composition of zooplankton

Zooplankton represents the highest energetic content food source for benthic suspension feeders, and thus its availability can significantly influence the fitness of these species. Results showed a marked seasonality in zooplankton, with the highest concentrations at the end of spring and autumn (Fig. 10). However, the annual concentration of zooplankton in the study area was lower than the concentration found by Coma et al. (1994) and Rossi et al. (2004), suggesting that the study area is generally poor in zooplankton secondary production. The zooplankton was mainly formed of copepods, whose density in the water column during the stratification period was 27.7 ± 54.7 ind m^{-3} in 2010, and 79.5 ± 53.9 ind m^{-3} in 2011. Therefore, zooplankton availability was higher in 2011 than in 2010.

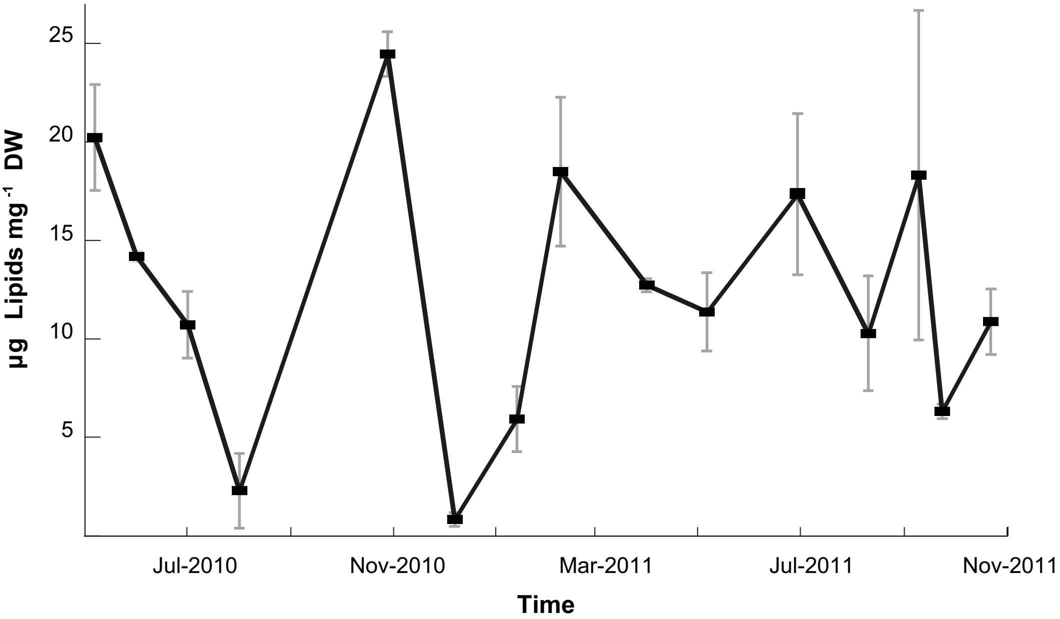


Fig. 9. Lipid content (µg mg^{-1} DW) in the suspended particulate matter in the sampling site from May 2010 to November 2011 (mean ± SD).

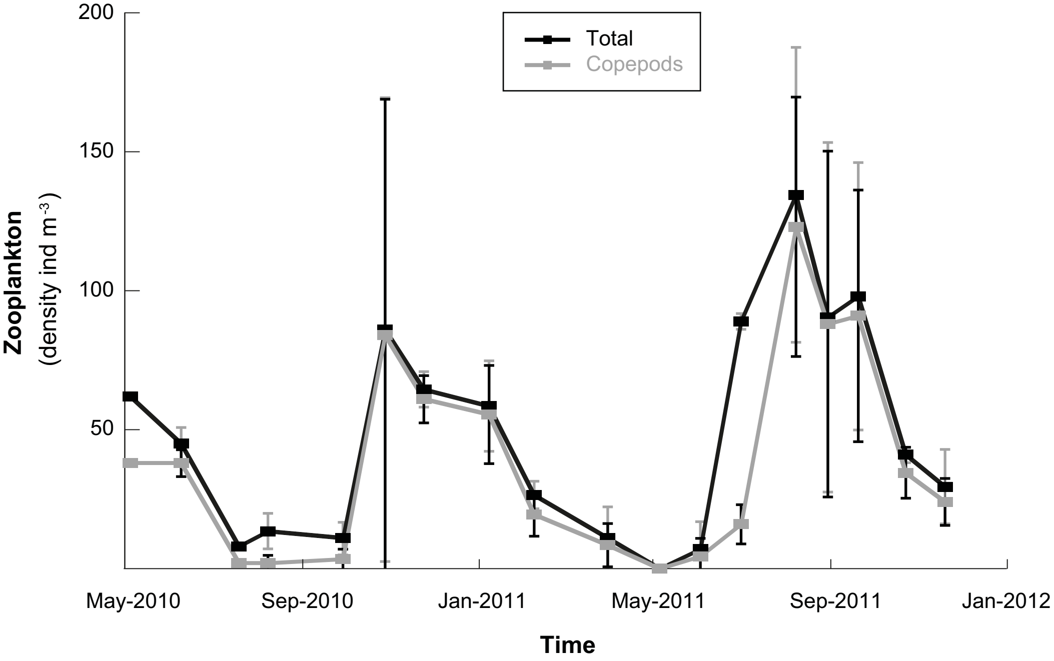


Fig. 10. Density of zooplankton (black line) and copepods (grey line) (ind m^{-3}) in the sampling site from May 2010 to November 2011 (mean ± SD).

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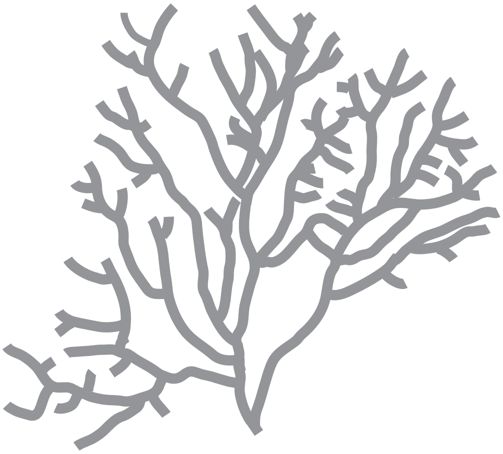
Chapter 1

Energetic resource allocation for reproduction in the temperate octocorals *Corallium rubrum* and *Paramuricea clavata*: Contrasting reproductive strategies of surface brooders *versus* internal brooders



ABSTRACT

The present study investigates the energetic investment in the reproduction of two Mediterranean gorgonians characterized by different reproductive strategies: *Corallium rubrum* (internal brooder) and *Paramuricea clavata* (external brooder). A direct approach counting sexual products was concurrently used along with a biochemical approach (lipid content and free fatty acid content and composition) in order to investigate the parental energetic investment and energetic demands in the reproductive output. The present study has demonstrated for the first time that most part of the energetic cost is due to the reproductive activity (i.e. gametogenesis and spawning), and not to the direct transfer of lipid reserves from mother colonies to the oocytes. Moreover, results also showed that the two species have different life history strategies with *C. rubrum* investing less energy in reproduction than *P. clavata*. Both species are sensitive to environmental conditions, P. clavata being more vulnerable to interannual environmental changes than *C. rubrum*. Different life history strategies may drive to different recovery dynamics after major disturbances, and the present results suggest that *C. rubrum* strategy is focused more on resistance, while *P. clavata* on resilience.

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Coral Reef (submitted)

VILADRICH N (2015) Doctoral Thesis pp 33-52

INTRODUCTION

Resistance and resilience of marine benthic communities are closely related to the life history strategies of the main species that compose them (Stearns 1992; Bramanti and Edmunds 2015). Available energy resources are usually finite and need to be partitioned into different processes such as growth, defence, recovery and reproduction (Giesel 1976; Lawrence 1985; Lawrence and McClintock 1994). In particular, the amount of energy invested into the reproduction of organisms, it presents as challenge of optimising parental survival against offspring fitness (Jablonski and Lutz 1983). This dilemma is represented for example in contrasting life history strategies, which are commonly categorised within two extremes: the r-oriented strategies, associated with high fecundity (i.e. number of sexual products) and high resilience to disturbances, and the k-oriented strategies, associated with a lower investment in the offspring (low fecundity) but a high resistance to disturbances (Stearns 1976).

Parental energy investment into offspring production may play a key role in population dynamics, affecting larval and early settlers' survival (Strathmann 1985). Whereas several studies looked at the consequences of this investment on larval behaviour, longevity and competency (Richmond 1987; Pechenik 1990; Harms 1992; Havenhand 1993; Harii et al. 2002, 2010), there is almost no information about the repercussion of reproductive energetic cost on the parental individuals. Such repercussion on parental organisms can be profound, since reproduction typically entails a high energetic cost (Lawrence and McClintock 1994; Doughty and Shine 1997). Indeed, in many species, gametogenesis is restricted to periods when food intake is high and can support this energetic investment (e.g. MacGinitie and MacGinitie 1949; Giese 1959; Rossi et al. 2006a). Depending on their investment in reproduction, parental individuals will have more or less energy available for their survival and recovery after reproduction.

A useful method of studying energetic implications of reproductive effort on parental organisms is the quantification of lipid content before and after reproduction, since lipids are used as the most efficient energy source in many animal groups (Lehninger 1982). Lipid content in benthic invertebrates is subject to high temporal variability (Raymond et al. 2007) due to fluctuations in the food availability and abiotic factors (Rossi et al. 2006a; Baptista et al. 2012). The total lipid content is the sum of several lipid compounds such as wax esters, phospholipids and free fatty acids (FA; Imbs 2013). The wax esters and phospholipids are considered stable energy reserves, while the FA represent a source of immediate/fast energy with high power efficiency (high ATP/FA molecule) (Sargent et al. 1988). Indeed, FA content can increase under stress situations, such as starvation and thermal stress, in order to compensate for the increment in metabolic demand (Sargent et al. 1999). In addition, FA composition may reflect the nature of metabolic demands (Díaz-Almeyda et al. 2011; Imbs 2013; Viladrich et al. 2015), and the amount of the different kinds of FA (Saturated Fatty Acids (SFA), Mono Unsaturated Fatty Acids (MUFA) and Poly Unsaturated Fatty Acids (PUFA)) may determine the fitness of individuals. In fact, PUFA are highly energetic FA, essential for overcoming stress conditions, since they can be converted into many other FA (selective accumulation) (Müller-Navarra et al. 2000; Wacker and Von Elert 2001), while MUFA, and especially SFA, are mainly used as basic metabolic energy (Sargent et al. 1999; Dalsgaard et al. 2003).

In benthic organisms, octocorals may be particularly useful for studying the effects of energetic resource allocation in reproduction. In octocorals, fitness and survival of adults may often be limited to the energy resource available, as they use a passive feeding mode by capturing prey particles suspended in the water column (Gili and Coma 1998). Passive feeding is a strategy of energy conservation, where the heterotrophic energy supply relies on water currents, and seasonal plankton availability (Sebens 1987). Consequently, these organisms can suffer some temporal limitations on heterotrophic food availability (Sebens et al. 1996; Coma et al. 2000; Rossi et al. 2004). Ecologically, gorgonians are of significance, as they are considered the most conspicuous ecosystem engineering species in many benthic communities around the world (Gili and Coma 1998; Coma and Ribes 2003; Wild et al. 2011), being one of the main three-dimensional constituents of the so-called "animal forests" (Rossi 2013). The life cycle of gorgonians is characterized by an adult sessile stage and a larval dispersal phase that ensures propagation. Three different sexual reproductive strategies have been observed in gorgonians: 1) spawning, where sperm and eggs are released and fertilized in the water column, 2) surface brooding, where the eggs are retained by mucous material, and fertilized on the surface of female colonies, and 3) brooding, where eggs are fertilized inside polyps of female colonies, which retain the zygotes and early embryos inside their polyps. Colonies fecundity may be changed according to environmental conditions, either by seawater temperature (Bayne et al. 1975; Brey 1995) or food availability (Qian and Chia 1992; Gori et al. 2013), due to the nutritional conditions of parental colonies. Individual fitness of parental colonies can also influence on larval and early settlers' survival (Strathmann 1985), as its survival capacity depends, in part, on larval energy reserves that are transferred by the mother colony (e.g. in the form of yolk, Mousseau and Fox 1998; Roff 2002; Maestripieri and Mateo 2009).

Corallium rubrum (Linnaeus, 1758) and *Paramuricea clavata* (Risso, 1828) are characteristic species of gorgonian assemblages in the Mediterranean Sea which thrive on rocky bottoms, with moderate to high current regimes (Ballesteros 2006). Their diet mainly consists of suspended particulate organic matter and zooplankton (Coma et al. 1994; Ribes et al. 1999; Tsounis et al. 2006a). Both species are gonochoric, developing annual gametes, which last between 13-18 months for oocytes, and 6-7 months for spermatic sacs (Vighi 1972; Coma et al. 1995a; Santangelo et al. 2003). *C. rubrum* is an internal brooder with early sexual maturity and low fecundity (Santangelo et al. 2003; Bramanti et al. 2005; Tsounis et al. 2006b). Conversely, *P. clavata* is characterized by late reproductive age, and a surface brooder strategy with high fecundity (Coma et al. 1995b). This different fecundity should result in different energetic changes in parental colonies during the reproductive period. Furthermore, owing to the fact that oocytes are mainly composed of lipids (60–70% dry weight (DW), Arai et al. 1993) while spermatic sacs are mainly composed of proteins (Ferguson 1975), a different energetic change should also be expected between male and female colonies.

The objective of the present study was to test the differences in energetic modifications of parental colonies during the reproductive period in two gorgonian species (*C. rubrum* and *P. clavata*) characterized by different reproductive strategies (internal and surface brooders). The final target was to know if the energy investment could be one of the clues to understanding the resilience and resistance capability of these species. To achieve this objective we quantified the gamete production of the two species (number, size and volume of oocytes and spermaries) and we measured the quantity of organic matter, lipid content, and FA content and composition in the coenenchyma, before and after spawning. Measurements were carried out in different years (2010 and 2011) in order to take into account the temporal variability of environmental conditions, and how these may affect the reproductive output in the same population.

MATERIALS AND METHODS

Sampling procedure

Colonies of *Corallium rubrum* (N= 30) and *Paramuricea clavata* (N= 30) were sampled by SCUBA diving at Punta s'Oliguera in Cap de Creus (42°17'03'' N; 003°17'95'' E, northwestern Mediterranean Sea; Fig. 1). Both populations of *C. rubrum* and *P. clavata* were located on rocky walls (~100 m apart) at 25–30 m depth. Sampled *C. rubrum* colonies were 4–5 cm height (sexually mature according to Tsounis et al. 2006b), with basal diameter larger than 7 mm (minimum fishing legal size, Tsounis et al. 2007), whereas sampled *P. clavata* colonies were higher than 30 cm (sexually mature according to Coma et al. 1995a).

During each sampling, one fragment per primary branch was collected from each colony and divided into two portions. One portion (~1 cm) was fixed in 10% formalin and used for quantifying the production of oocytes and spermaries (Coma et al. 1995a; Rossi and Gili 2009), while the other portion (1–2 cm) was immediately frozen in liquid nitrogen, maintained at -80°C until freeze-drying, for 24 h at -110°C and a pressure of 100 mbar, and finally stored at -20°C pending biochemical analyses (organic matter, lipid and fatty acid contents).

Production of oocytes and spermatic sacs

Oocyte and spermary production was quantified monthly in five female and five male colonies for each species from May to August 2010 and 2011. Sex identification was performed under the dissecting microscope and confirmed under optical microscope (Coma et al. 1995a; Santangelo et al. 2003; Cupido et al. 2012). Six polyps per colony were dissected under a stereomicroscope (Olympus SZ-STS), and all the sexual products found in each polyp were photographed (Moticam 5, 5.0 million pixels). Pictures were then analysed with Macnification 2.01 software (Schols and Lorson 2008) in order to measure the area (A) and circularity of each oocyte or spermary. Circularity, defined as the ratio between the area of the sexual product and the area of a circle with the same perimeter, determines to what extent a shape can approximate a circle. Since circularity was always > 90%, sexual products were considered spheres and their diameter (d) was calculated according to the equation

$$d = 2(A/\pi)^{-1/2} (1)$$

where A is the area, and their volume (V) was calculated according to the equation

$$V = 4/3\pi(d/2)^3 (2)$$

where d is the diameter calculated with equation (1) (Gori et al. 2007).

This section was aimed mainly at determining the exact spawning period of both species and so, at defining the period before and after spawning (see Results section). A total of 480 polyps for each species were examined, and more than 800 and 8,200 sexual products were measured for *C. rubrum* and *P. clavata*, respectively.

Organic matter content

Organic matter (OM) in the coenenchyme of *C. rubrum* and *P. clavata* was quantified in five female

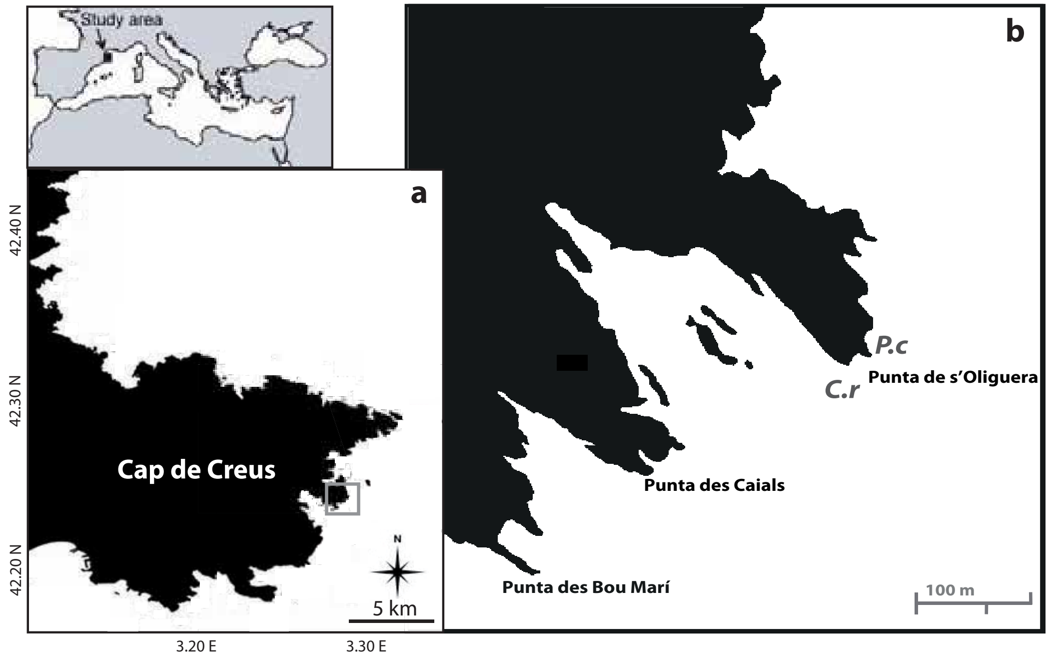


Fig. 1. Map of the study area (a), and location of the sampling site (b). *C.r* and *P.c* indicate the position of the *Corallium rubrum* and *Paramuricea clavata* populations, respectively.

and five male colonies for each species before and after spawning, in 2010 and 2011. Approximately 100 mg (± 0.1 mg) of coenenchyme DW from each sample were reduced to ash during 4 h at 500°C in a muffle furnace (Relp 2H-M9). OM was then calculated as the difference between dry and ash weight (Slattery and McClintock 1995). Results are expressed in percentage with respect to the initial dry weight of the sample. A total of 40 colonies for each species were analysed.

Lipid content

The total lipid content in the organic matter was quantified in five female and five male colonies for each species before and after spawning, in 2010 and 2011, following the colorimetric method of Barnes and Blastock (1973). Approximately 10 mg (± 0.1 mg) of coenenchyme DW from each sample were homogenized in 3 ml of chloroform-methanol (2:1), and total lipids were quantified colorimetrically, with cholesterol as a standard. Results are presented in µg Lipid per mg^{-1} of OM. A total of 40 colonies for each species were analysed.

Free Fatty Acid content and composition

Free FA content and composition was examined in three female and three male colonies for each species before and after spawning, in 2010 and 2011, according to an update of the methodology previously used by Rossi and Fiorillo (2010), Soler-Membrives et al. (2011) and Gori et al. (2012). Previous studies have already shown that three samples are sufficient for statistical comparisons in determinations of FA content and composition (Wheeler and Morrissey 2003; Rossi et al. 2006b). Approximately 10–12 mg (± 0.1 mg) of coenenchyme DW from each sample were dissolved in 3:1 dichloromethane-methanol spiked with an internal standard (2-octyldodecanoic acid and 5β-cholanic acid) in order to estimate recuperation. Microwave-assisted extraction (initial ramp of 2.5 min up to 70°C and then, 5 min at 70°C with a power of 1200W, Kornilova and Rosell-Melé 2003) was performed and, after centrifugation (2000 rpm, 5 min), the extract was dried in a centrifugal vacuum concentrator (Ruiz et al. 2004). Samples were then re-dissolved in 0.5 ml of chloroform and eluted through a 500 mg aminopropyl glass column (previously activated with 4 ml of n-hexane). A first fraction composed of neutral lipids was eluted with 3 ml of chloroform:2-propanol (2:1) and the FA recovered with 8.5 ml of diethyl ether:acetic acid (98:2). The FA fraction was methylated using a solution of 20% Methanol/Boron trifluoride heated at 90°C for 1h. Reaction was quenched with 4 ml of salt-saturated water. Methyl esters of fatty acids were recovered by a triple extraction with 3 ml of n-hexane. The combined extracts were dried, re-dissolved in 1.5 ml of chloroform, and eluted through a glass column filled with sodium sulphate, to remove residual water. After evaporation with nitrogen, the extracted samples were stored at -20°C until analysis. At the moment of injection, samples were re-dissolved in 80 µl of isooctane, and gas chromatography (GC) analysis was performed with an Agilent Technologies 7820A GC system equipped with a flame ionization detector, a splitless injector and a DB-5ms Agilent column (60 m length, 0.25 mm internal diameter and 0.25 µm phase thickness). Hydrogen was used as a carrier gas at 30 mL·min^{-1}. The high compound numbers in the samples and the similarity of retention required a complex method of temperature ramps, with the oven temperature programmed to increase from 50°C to 160°C at 20°C min^{-1}, from 160°C to 188°C at 0.5°C min^{-1}, from 188°C to 229°C at 20°C min^{-1}, from 229°C to 235°C at 2°C min^{-1} and, finally, from 235°C to 300°C at 5°C min^{-1}. The injector and detector temperatures were 300°C and 320°C, respectively. Methyl esters of FA were identified by comparing their retention times with those of standard FA (37 FAME compounds, Supelco® Mix C^4-C^{24}). FA were quantified by integrating areas under peaks in the GC traces (Chromquest 4.1 software), with calibrations derived from standards. Results are presented in µg FA mg^{-1} of OM, and in percentage of Saturated Fatty Acids (SFA), Mono Unsaturated Fatty Acids (MUFA) and Poly Unsaturated Fatty Acids (PUFA) mg^{-1} of OM. A total of 24 colonies for each species were analysed.

Statistical analysis

Differences between years and sex regarding volume, diameter and number of oocytes and spermaries for each species were tested using the non-parametric Wilcoxon–Mann–Whitney test because the data were not normally distributed. The test was performed with the R-language function "wilcox.test" of the R software platform (R Development Core Team 2007). Differences in OM content, lipid content, FA content and composition and in SFA, MUFA and PUFA percentages were tested using a four-way ANOVA, considering year (two levels, 2010 and 2011), species (two levels, *C. rubrum* – *P. clavata*), release period (two levels, before and after spawning), and sex (two levels, male - female) as independent variables. Before performing ANOVAs, normality of data residuals and variance homogeneity were tested with Shapiro-Wilk, and Bartlett test (R-language function "shapiro.test" and "bartlett.test"). When variances were not homogeneous, necessary transformations were applied. ANOVA tests were performed with the R-language function "aov" (Chambers and Hastie 1992), followed, when appropriate, by a Tukey post hoc test (R-language function "TukeyHSD").

Finally, the analysed colonies were ordered based on their FA composition using a principal component analysis (PCA) of transformed data ($p' = \arcsin(p^{1/2})$) with the R-language function "princomp"(Vegan library; Oksanen et al. 2005).

RESULTS

Oocyte and spermatic sac production

Changes in frequency distributions of sexual product diameter in *Corallium rubrum* (Fig. 2a, b) and *Paramuricea clavata* (Fig. 3a, b) colonies allowed to identify the exact spawning moment in 2010 and 2011. In *P. clavata* colonies, the spawning time corresponded to the absence of spermary (Coma et al. 1995a), while in *C. rubrum* colonies, it corresponded to the disappearance of larvae inside the female polyps (Tsounis et al. 2006b). *C. rubrum* spawned between 19thJuly and 8thAugust 2010 (Fig. 2a), and between 29thJune and 4thAugust in 2011(Fig. 2b). On the basis of these observations, samples collected on 9thJune 2010 and 5thJune 2011 were considered as "pre-spawning", and samples collected on 8th and 4th August 2010 and 2011 respectively, were considered "post-spawning". For *P. clavata*, samples collected on 9th June 2010 and 3th June 2011 were considered as "pre-spawning", and samples collected on 19thJuly 2010 and 4thAugust 2011 were considered "post- spawning".

Less volume of spermaries with a larger size and less abundance were observed for *C. rubrum* in 2011 with respect to 2010 (Wilcoxon– Mann–Whitney test, $p<0.001$; Fig. 4). For *P. clavata*, the volume of oocytes and diameter of oocytes and spermaries significantly decreased in 2011 (Wilcoxon– Mann–Whitney test, $p<0.01$; Fig. 4a, b). Conversely, the number of sexual products per polyp did not show significant differences between years and sexes (Wilcoxon– Mann–Whitney test, $p>0.05$; Fig.4c). The volume, diameter and number of sexual products showed significant differences when the two species were compared (Wilcoxon– Mann–Whitney test, $p<0.001$; Fig. 4).

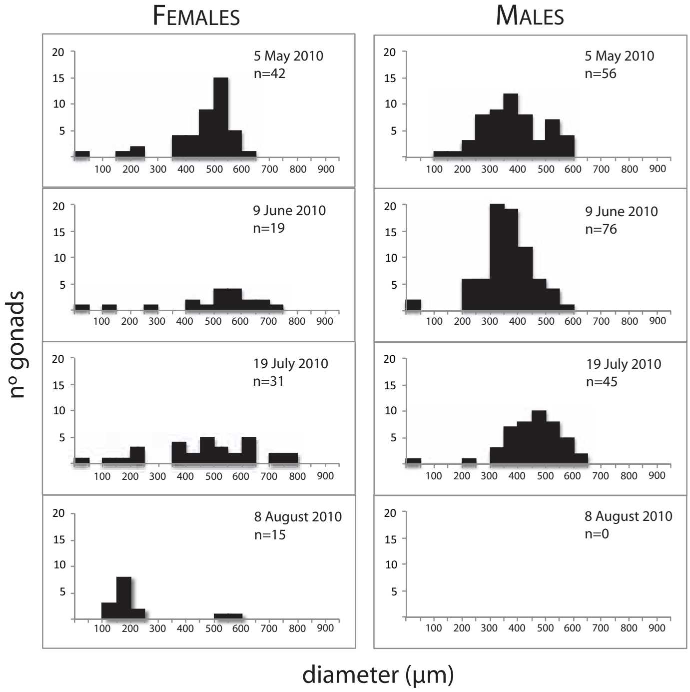
Organic matter content

OM content in the coenenchyma was on average 24.07 ± 6.4 % and 30.2 ± 6.4 % for *C. rubrum* and *P. clavata*, respectively, with no significant differences within each species (between sexes, and spawning times), or between species (ANOVA four-way, $p>0.05$).

Lipid content

Lipid content in *C. rubrum* female colonies significantly decreased after spawning in 2010 (ANOVA four-way, $p<0.001$; Fig. 5a). In *P. clavata* colonies, lipid content significantly decreased after spawning in both sexes in 2010 (ANOVA four-way, $p<0.005$; Fig. 5b). In 2010, lipid content of *P. clavata* colonies was higher in female than in male colonies before spawning (ANOVA four-way, $p<0.001$), but there were no differences between sexes after spawning. In 2011, lipid content in both sexes of *P. clavata* significantly decreased after spawning (ANOVA four-way, $p<0.001$; Fig. 5b), however, without any significant difference between sexes. No differences in lipid content were observed between 2010 and 2011 in both species. When the two species were compared, lipid content was significantly higher only in *P. clavata* female colonies before spawning in 2010 (ANOVA four-way, $p<0.001$; Fig. 5).

a) **2010**



b) **2011**

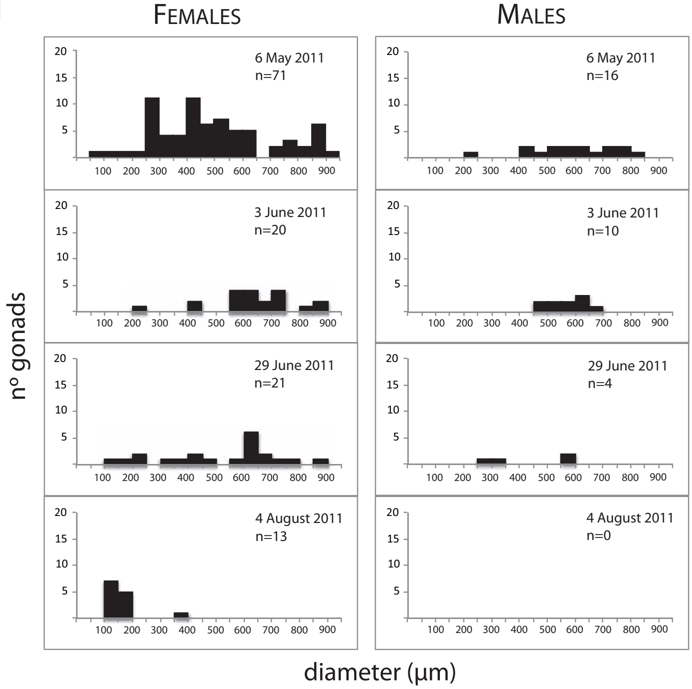
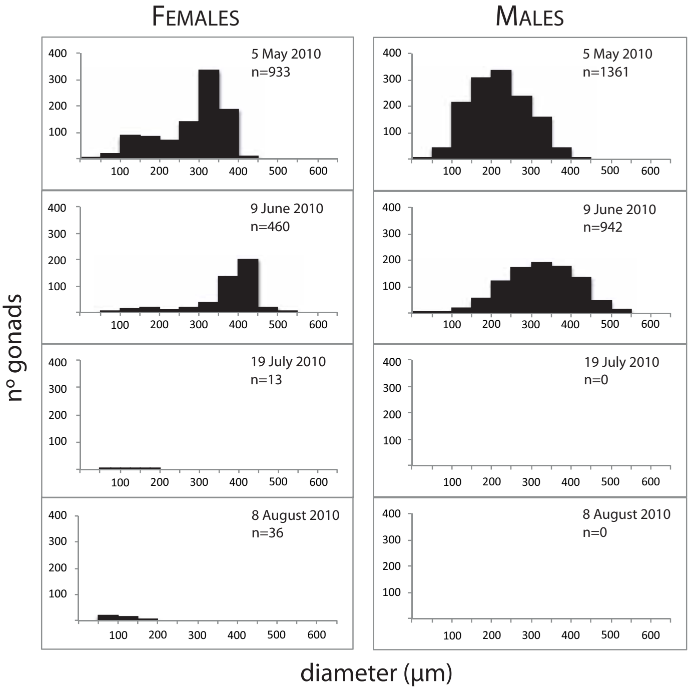


Fig. 2. *Corallium rubrum*. Distribution of gonadal diameter frequency (µm) in 30 female and male polyps; year 2010 (a) and 2011 (b) (n= gonads number).

a) **2010**



b) **2011**

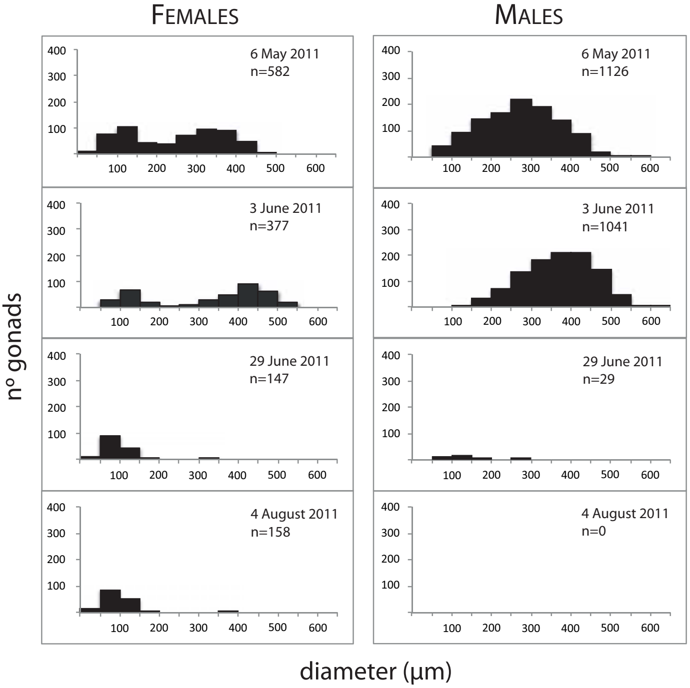


Fig. 3. *Paramuricea clavata*. Distribution of gonadal diameter frequency (µm) in 30 female and male polyps; year 2010 (a) and 2011 (b) (n= gonads number).

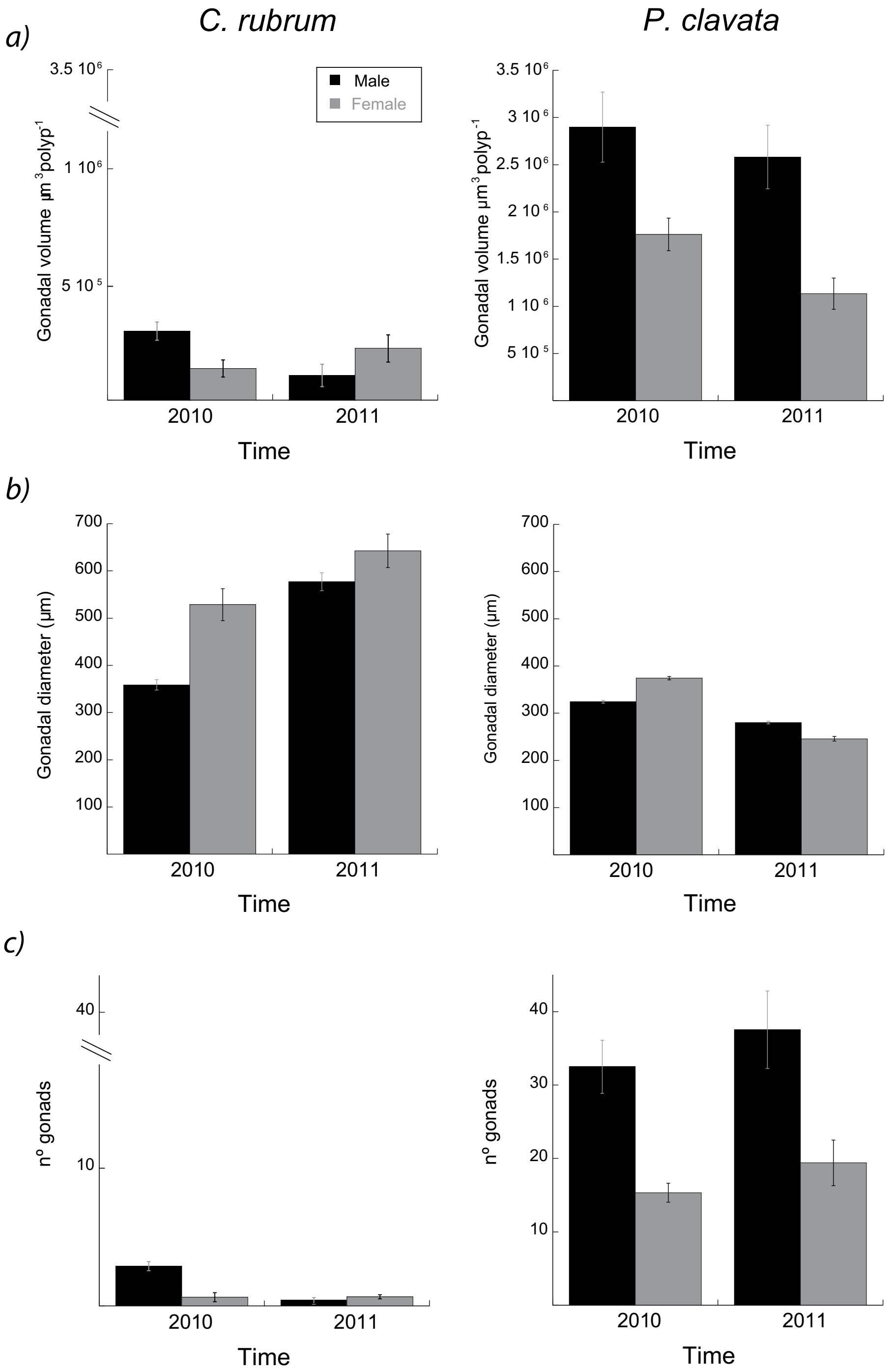


Fig. 4. Gonadal volume (a), gonadal diameter (b) and gonad number (c) per polyp of five male (black bars) and female (grey bars) colonies (mean ± SD) in two years (2010, 2011) for *Corallium rubrum* and *Paramuricea clavata*, respectively.

Fatty acid content and composition

Free FA content in *C. rubrum* colonies significantly increased after spawning only in 2010 (ANOVA four-way, $p<0.01$; Fig. 6a). In 2011, FA content of *C. rubrum* did not differ between sexes or after spawning (ANOVA four-way, $p>0.05$; Fig. 6a). In *C. rubrum* colonies, FA content after spawning was significantly higher in 2010 than in 2011 (ANOVA four-way, $p<0.01$; Fig. 6a). In *P. clavata* colonies, FA content significantly increased after spawning both in 2010 and 2011 (ANOVA four-way, $p<0.001$; Fig. 6a), without differences between sexes. FA content in females after spawning was higher in 2011 than in 2010 (ANOVA four-way, $p<0.05$; Fig. 6a). When the two species were compared, *P. clavata* showed a significantly higher FA content only after spawning in 2011 (ANOVA four-way, $p<0.001$; Fig. 6a).

In *C. rubrum* colonies, SFA decreased and PUFA increased after spawning in 2010 and 2011 (ANOVA four-way, $p<0.001$; Fig. 6b). MUFA were only different before and after spawning in 2011 (ANOVA four-way, $p<0.001$; Fig. 6b). In *P. clavata* colonies, SFA decreased with spawning in males only in 2010 (ANOVA four-way, $p<0.05$; Fig. 6b), whereas MUFA increased after spawning in female colonies in 2010 (ANOVA four-way, $p<0.01$) but decreased in both sexes in 2011 (ANOVA four-way, $p<0.001$; Fig. 6b). PUFA percentage did not show any significant differences between sexes, or related to spawning (ANOVA four-way, $p>0.05$; Fig. 6b). When comparing both years, PUFA were higher in 2011, whereas MUFA were higher in 2010 (ANOVA four-way, $p<0.001$; Fig. 6b).

According to the PCA, there was a clear change in the main FA composition of *C. rubrum* before and after spawning, both in 2010 and 2011 (Fig. 7), with 16:0 and 18:1 (n-9) as main FA before spawning, and 20:5(n-3) and 22:6(n-3) as the most representative after spawning (Annex 2, p. 103). Conversely, colonies of *P. clavata* were mixed in a third weak group (Fig. 7), with 16:0, 18:1(n-9) and 20:4(n-6) as dominant FA before spawning, and, as in *C. rubrum* colonies, the amounts of 20:5(n-3) and 22:6 (n-3) were greater after emission (Annex 2). When the two species were compared, *C. rubrum* colonies showed the highest concentration of 16:0 and 18:1(n-9), whereas 20:4(n-6), 20:5(n-3), 22:6(n-3) and C24PUFA were dominant in *P. clavata* colonies.

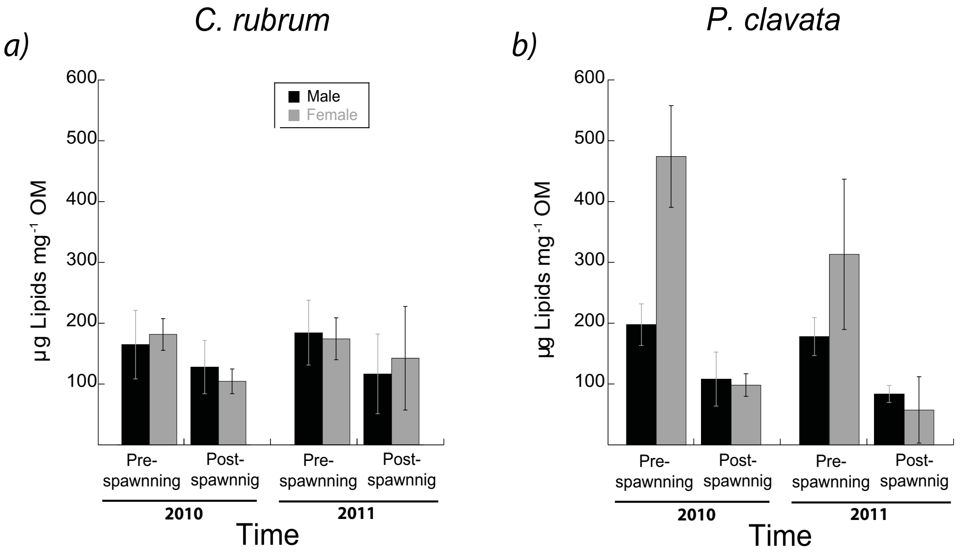


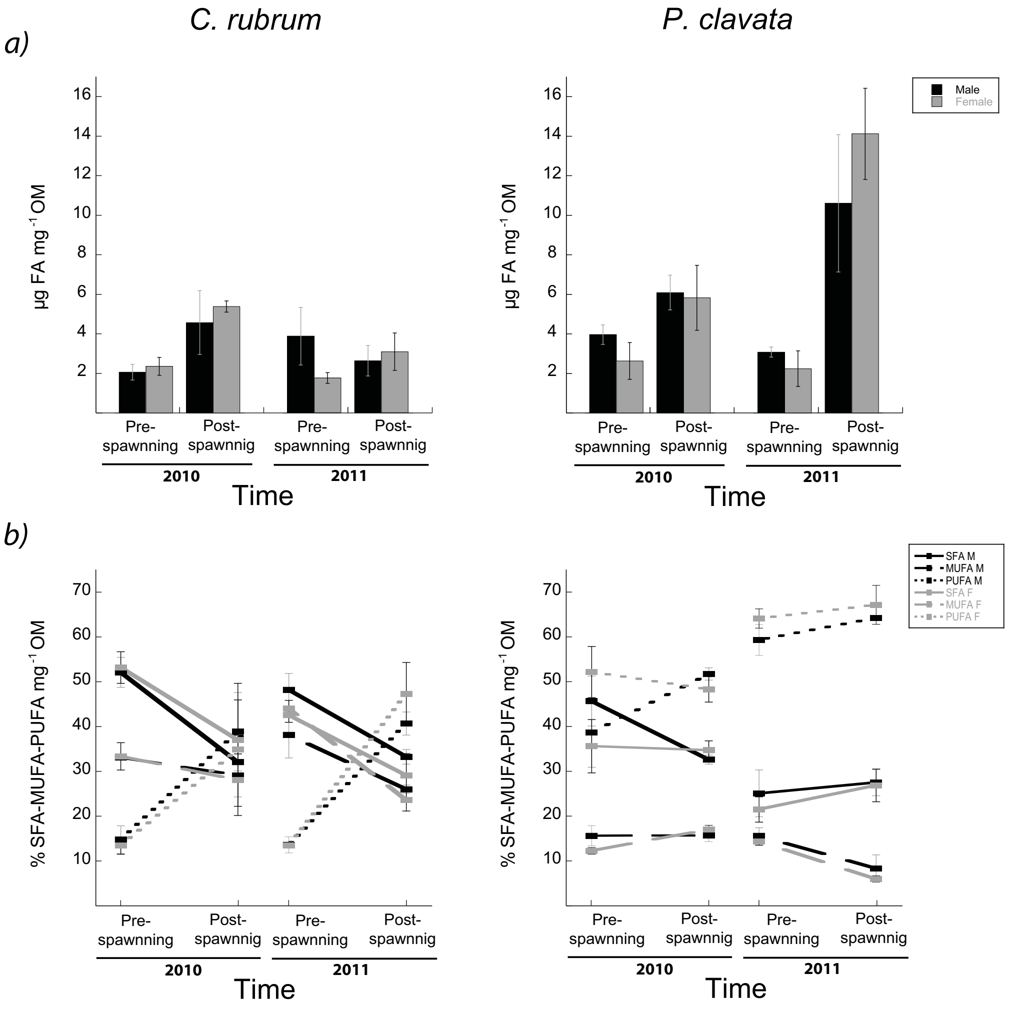
Fig. 5. Lipid content (µg mg^{-1} OM) in tissue of *Corallium rubrum* (a) and *Paramuricea clavata* (b) in male (black bars) and female (grey bars) colonies (N = 5) before and after spawning in years 2010 and 2011.

DISCUSSION

This study compares, for the first time, changes in energy reserves during reproduction (i.e. gametogenesis and spawning) in parental colonies of two octocoral species characterized by different reproductive strategies: *Corallium rubrum* (internal brooder) and *Paramuricea clavata* (external brooder). Results of gonadal output, lipid content, fatty acid content and composition suggest that *C. rubrum* has a lower energetic investment in reproduction than *P. clavata*.

Energetic cost of reproduction according to reproductive strategy

Results about gametes production showed a lower reproductive effort in *C. rubrum* compared to *P. clavata*. Indeed, despite the larger size of sexual products (i.e. oocytes and spermaries) in *C. rubrum* colonies, the total volume per polyp was significantly lower (one order of magnitude) than in *P. clavata* (Fig. 4). This pattern agrees with previous results: low number and large size of sexual products in internal


C. rubrum

P. clavata

Fig. 6. Fatty acid content (a) (µg mg^{-1} OM) and percentage of fatty acid functionality composition (SFA, MUFA, PUFA) (b) in male (black bars) and female (grey bars) colonies of *Corallium rubrum* and *Paramuricea clavata* (N = 3) before and after spawning in years 2010 and 2011.

brooders (e.g. *Ainigmaptilon antarcticum*, Orejas et al. 2002; *Eunicella singularis*, Ribes et al. 2007), and high number and small size of sexual products in broadcast and surface brooders (e.g. *Alcyonium acaule,* Fiorillo et al. 2013; *Pseudopterogorgia elisabethae*, Gutierrez-Rodriguez and Lasker 2004).

The high reproductive effort of *P. clavata* coincides with a severe reduction in its lipid content after spawning, pointing to the existence of a relationship between reproductive effort and reproductive cost (Fig. 5). Lipid content of coral tissue may change due to the energetic cost of metabolism, cellular reposition, and also as a consequence of reproduction (Ward 1995; Yamashiro et al. 2001; 2005; Grotolli et al. 2004; Tsounis et al. 2012). Therefore, a clear decrease in lipid content after spawning is expected in broadcast and surface brooder species with high fecundity, such as *P. clavata* (Rinkevich and Loya 1979; Leuzinger et al. 2003; Villinski et al. 2003; Rossi et al. 2006a). Conversely, lipid content did not change during reproduction in *C. rubrum* (Rossi and Tsounis 2007), as previously observed in internal brooder species with low fecundity (Cantin et al. 2007), since energetic cost of reproduction is probably negligible compared to the high lipid content in coral tissue (Stimson 1987; Ward 1995).

Besides these interspecies differences, both species also showed an interannual variability in their gamete production. Several studies demonstrated that changes in environmental conditions may affect the volume, size and number of sexual products in corals and gorgonians (Barnes and Barnes 1965; Bayne et al. 1975, 1978; Brambilla 1982; Qian and Chia 1991; Brey 1995; Gori et al. 2013). Nevertheless, the clear differences observed in temperature and food availability in the water column between 2010 and 2011 (see pre-chapter) cannot be used to explain the interannual differences in gamete production observed due to the lack of environmental data about the spring of 2009, when the formation and development of sexual products of year 2010 started (Vighi 1972; Coma et al. 1995b; Santangelo et al. 2003; Gori et al. 2013).

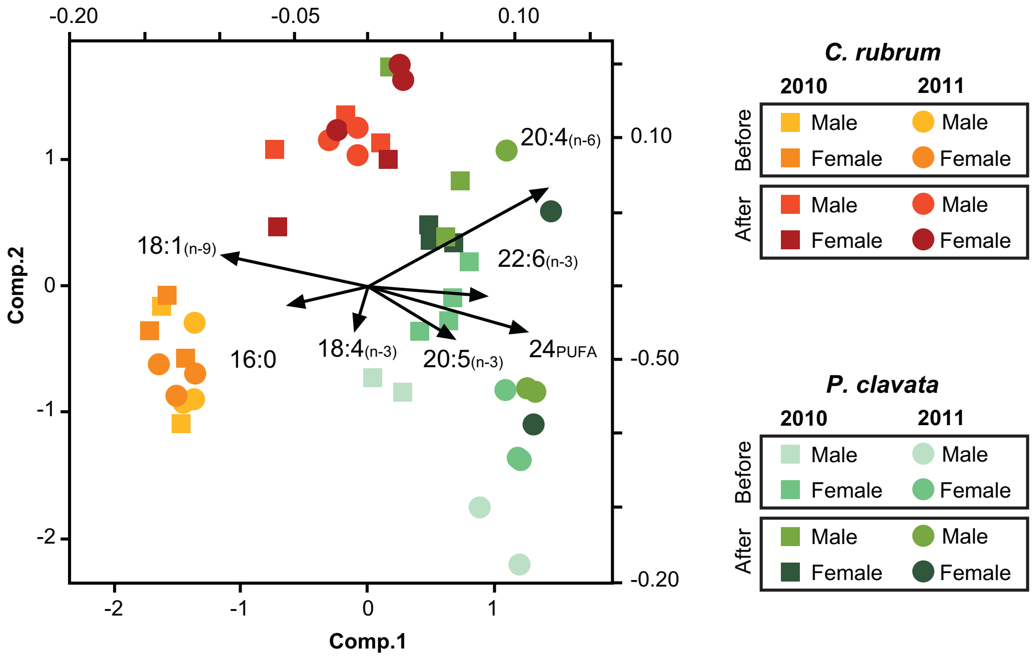


Fig. 7. Principal component analysis (PCA) biplot illustrating the ordering of the studied colonies with regard to their fatty acid composition, and the roles of the first seven fatty acids classified according to the variance.

Energy investment in reproduction according to colony sex

So far, it has been generally assumed that reproductive investment is higher in female than in male colonies, since oocytes are mainly composed of lipids (60–70% DW, Arai et al. 1993), whereas spermatic sacs are made up of proteins (Ferguson 1975). However, our results showed that when *P. clavata* displayed the same number and volume of oocytes and spermaries (summer 2011), its lipid content significantly decreased in the same way in both male and female colonies. Therefore, this has demonstrated for the first time that most part of the energetic cost is due to the reproductive activity (i.e. gametogenesis and spawning), and not to the direct transfer of lipid reserves from mother colonies to oocytes.

Energetic demands and requirements during and after reproduction

Besides the differences observed in the energetic cost of reproduction (as indicated by the lipid content) between *C. rubrum* and *P. clavata*, the two species also showed clear differences in their free FA content. Since FA represents a source of immediate/fast energy, its content is proportional to the metabolic demand of an organism (Sargent et al. 1988). According to Sargent et al. (1999), the FA content can increase under stress situations, such as pathogen exposure, starvation or thermal stress, since metabolic demands are met by obtaining FA from lipid reserves (Imbs 2013). Therefore, the increase of FA content after spawning observed in both studied species is probably a mechanism to overcome reproductive stress as well as the elevated temperature and reduced food availability, characteristic of Mediterranean summers (Coma et al. 2000). However, whilst in *P. clavata* this increase occurred in both years, in *C. rubrum* FA content only increased after spawning in 2010 (Fig. 6a). The summer of 2010 was characterized by lower food availability in the water column compared to 2011, whereas elevated seawater temperatures lasted longer in 2011 than in 2010 (see pre-chapter). Prolonged elevated temperatures may stress gorgonians because of the resulting increased respiration and decreased polyp activity (Previati et al. 2010). Therefore, our results suggest that, while *C. rubrum* is mainly affected by food availability, *P.clavata* is also affected by increased temperatures. This stronger repercussion of environmental conditions on *P. clavata* is probably related to the high energetic cost that reproduction involves for this species, making *P. clavata* more vulnerable to summer starvation than *C. rubrum*. In fact, it has been observed that during a mass mortality event, *P. clavata* suffered higher mortality than *C. rubrum* (Cerrano et al. 2000; Garrabou et al. 2002, 2009).

Free FA compounds may also reflect the nature of metabolic demands, because they can be synthesized by complex metabolic reactions to satisfy the specific demands (Díaz-Almeyda et al. 2011; Imbs 2013; Viladrich et al. 2015). Therefore, knowledge on their quality (SFA, MUFA, PUFA) and composition (FA components) may give information on the metabolic changes related to reproductive activity and/or starvation period in the studied species.

Before spawning, FA content was similar in both species. However, its composition in terms of functional types (SFA, MUFA, PUFA) (Fig. 6b) and single FA (Fig. 7) was very different. *C. rubrum* showed a high concentration of 16:0 and 18:1(n-9) with [SFA]>[MUFA]>[PUFA], as commonly observed in reef-building scleractinians (Meyer et al. 1977; Al-Lihaibi et al. 1998). Conversely, *P. clavata*, as well as octocorals in general, are characterized by [PUFA]>[SFA]>[MUFA] and the presence of 24PUFA (Imbs et al. 2010; Baptist et al. 2012). High concentration of 16:0 in *C. rubrum* before spawning could be related to its axial skeleton, which is developed from inorganic $CaCO_3$ and an organic matrix (Allemand et al. 2011;Debreuil et al. 2011) mainly composed by ester cetyl palmitate (precursor of 16:0) (Young et al. 1971), which favours the accumulation/precipitation of $CaCO_3$ (Ennever et al. 1971). The proportion of 16:0 significantly decreases in *C. rubrum* after spawning, when several Mediterranean marine invertebrates drastically reduce their growth rates as a consequence of the summer reduction in food availability (Turon and Becerro 1992; Coma et al. 1998). Therefore, *C. rubrum* could be investing more in growth than in reproduction, suggesting that its life history strategy is mainly oriented to the maintenance of adult colonies. In *C. rubrum* colonies, a decrease after spawning also occurs for 18:1(n-9) and 18:4(n-3), which can be related to their role in the maturation of sexual products (Pérez et al. 2007). In *P. clavata* colonies, the importance of 20:5(n-3) together with 18:4(n-3) before spawning may confirm the high energetic investment of this species in reproductive activity, since these FA have been related to increased fecundity, fertility and egg quality (Fernández et al. 1995; Izquierdo 2001). 20:5(n-3) is typically present in the gonadal tissue of the jellyfish *Pelagia noctiluca* (Milisenda et al. *in preparation*), probably being involved in the development of the nervous system of larvae (Chapelle 1986; Sorbera et al. 1998; Mazorra et al. 2003). On the other hand, after spawning, both *C. rubrum* and *P. clavata* increased their 20:4(n-6) content, which has been related to the production of biologically active eicosanoids under stress or unfavourable conditions (Sargent et al. 1999) since they support immune system functioning and osmoregulation (Chapelle 1986; Mazorra et al. 2003).

Finally, while FA composition of *C. rubrum* did not show difference between 2010 and 2011, *P. clavata* presented a higher percentage of 22:6(n-3), 20:5(n-3) and 24PUFA in 2011 than 2010. This interannual variability in *P. clavata* might confirm that this species is more affected by environmental conditions, such as food availability and temperature, than *C. rubrum*, as previously mentioned. Indeed, the increase in percentage of these FA can be related to stress period suffered due to prolonged elevated temperature in the summer of 2011 (see pre-chapter), since FA omega-3 (n-3) has been previously related to an improvement in the stability of cellular membrane as a response to changes in seawater temperature (Klinger et al. 1996).

CONCLUSIONS

This study revealed that energy invested (lipid content) in reproduction, mainly entailed by reproductive activity, and energy demands (FA content) for survival after spawning are higher in the surface than in the internal brooder species, being positively correlated with the volume of oocytes and spermaries produced. Our results also showed that *P. clavata* is more sensitive to environmental conditions, probably due to its high energetic investment in reproductive activity. The implications of this sensitivity could explain the extreme vulnerability of *P. clavata* to prolonged elevated seawater temperatures resulting in extended mass mortalities (Cerrano et al. 2000; Pérez et al. 2000; Garrabou et al. 2009). On the other hand, the low investment in reproductive activity observed in *C. rubrum* could result in an increased resistance of adult colonies to thermal stress. However, the high fecundity of *P. clavata* can result in extremely higher recruitments after mass mortality events compared to *C. rubrum* (Santangelo et al. 2015), suggesting that brooder species could be more resistant to thermal stress but less resilient to mass mortality events.

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Chapter 2

Energetic resource allocation for reproduction in temperate octocorals *Corallium rubrum* and *Eunicella singularis*: Contrasting mixotrophic and heterotrophic strategies

ABSTRACT
The present study investigates the energetic investment during the reproductive activity of two Mediterranean gorgonians characterized by different trophic strategies: *Eunicella singularis* (mixotrophic, partly autotrophic and partly heterotrophic) and *Corallium rubrum* (heterotrophic). Both are internal brooders, releasing their larvae in summer. A biochemical approach analysing lipid content and free fatty acid (FA) concentration and composition were applied in combination with the quantification of oocyte and spermatic sac production. Lipid and FA content were higher in *E. singularis* than in *C. rubrum*, the heterotrophic species. However, the results showed that both species invest a small amount of energy in reproduction, probably due to their low reproductive output as a consequence of low fecundity. The highest FA content in *E. singularis* could be explained by the higher metabolic demand and the exchange of metabolites due to the presence of symbiotic algae. The higher inter-annual variability found in lipid and free FA content in *C. rubrum* suggested that this species is more sensitive to environmental changes and inter-annual seasonal constraints than *E. singularis*. The mixotrophic species may be less affected by the environmental and biological features of the water column because of its autotrophic energy input, especially in spring and summer. These differences could partly explain the different demographic distribution of the two species, as well as their resistance/resilience capability against short-term disturbances.

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VILADRICH N (2015) Doctoral Thesis pp 53-74

INTRODUCTION

The resistance and resilience of organisms in front of different stressors are partly related to their trophic strategy (Grottoli et al. 2006; Viladrich et al. 2015) as well as to the amount of their reserves stored in the form of lipids (Cejas et al. 2004; Rossi et al. 2006a; Seemann et al. 2013). Several trophic strategies exist, however, to simplify matters, it has been considered that a species can be either monophagous or polyfagous. Monophagous species are characterized by one specific diet type, and thus, their capacity to accumulate lipid reserves depends on the availability of specific sources of food. Conversely, polyfagous species can adapt their diet according to food availability, the lipid accumulated by these species being related to the type, quantity and quality of the food. The different accumulation of lipid reserves derived from a differential type of trophic strategy will influence the individual nutritional condition, survival, reproduction and growth (Szmant and Gassman 1990; Anthony and Connolly 2004; Grimsditch and Salm 2006). Despite the importance of the trophic strategy for the resistance and resilience of organisms, their study may often be complex due to the interaction with other factors, such as the different search and capture feeding strategies (MacArthur and Pianka 1966). In this sense, species with a passive feeding mode, like corals and gorgonians (Gili and Coma 1998), may be particularly useful for minimising the effects of these interactions and thus, for studying the direct consequences of trophic strategies on the amount of energy available to organisms.

In general, the energy supply in passive suspension feeders is derived from the capture of zooplankton and particulate organic matter suspended in the surrounding water column (Gili and Coma 1998), and in some cases also from the uptake of dissolved organic matter (Al-Moghrabi et al. 1993). However, although heterotrophic nutrition has also been highlighted as very important for the energy supply in several symbiotic corals and gorgonians (e.g. Goreau et al. 1971; Sebens et al. 1996; Ferrier-Pagès et al. 2003; Houlbrèque et al. 2004; Palardy et al. 2008), in these species, the autotrophic carbon produced by the symbionts may cover most of their metabolic needs (Muscatine et al. 1981, 1984; Tremblay et al. 2014). Combining autotrophy and heterotrophy (mixotrophy) is a way to maximize nutrient acquisition and ecological success in environments where light and plankton concentration can be very variable and often limiting (Muller-Parker and Davy 2001; Grottoli et al. 2006). Indeed, food availability may occasionally become a constraining factor for non-symbiotic corals and gorgonians (Gili and Ros 1985; Coma and Ribes 2003; Rossi et al. 2004, 2006a; Tsounis et al. 2006a), while the trophic plasticity of mixotrophic species would allow increased energy acquisition through obtaining an energy surplus with respect to non-symbiotic species (Goreau et al. 1971; Anthony and Fabricius 2000; Gori et al. 2012).

Nutritional conditions of parental colonies may affect the survival of future generations, as it determines the number and survival of offspring (Strathmann 1985; Simpson 2009; Gori et al. 2013). Even so, to my knowledge, the implications and consequences of nutritional state on the reproductive activity have never been compared among different species of passive suspension feeders. It would be expected that mixotrophic species contain higher or more constant energy reserves, which should result in a higher or more constant energetic investment in reproduction. These data on the amount of energy allocated to reproduction by different species can be crucial for determining the survival capacity of parental colonies and offspring, as well as for the understanding of their distribution patterns (Cocito et al. 2013).

Reproductive investment in corals and gorgonians has mainly been estimated by quantifying fecundity and oocyte volume (e.g. Hall and Hughes 1996). However, this approach does not account for the energetic cost caused by gamete production or by tissue repair associated with spawning. This fact potentially understates reproductive investment and, therefore, does not take into account the possible energetic implications for the survival of parental colonies and larvae (Calow 1979). A more precise estimate of the energetic reproductive investment can be obtained by quantifying the lipid content change in their tissues before and after larval release (Stimson 1978; Ward 1995; Leuzinger et al. 2003).

The total lipid content is the sum of several lipid compounds such as wax esters, phospholipids, triacylglycerols and free fatty acids (FA; Imbs 2013). The wax esters and triacylglycerols are considered stable energy reserves that can be oxidized to provide energy in form of free FA (Gurr et al. 2002). The free FA represent the source of immediately available energy with high efficiency (high ATP/FA molecule) that is used by individuals (Sargent et al. 1988). In this sense, the free FA content can highlight how the colonies' metabolic demands vary according to the reproductive period or their trophic strategy (Díaz-Almeyda et al. 2011; Imbs 2013; Viladrich et al. 2015). Indeed, the free FA content can increase due to stress situations in order to compensate for the increment of metabolic demand (Sargent et al. 1999). On the other hand, the FA composition may reflect the dietary input, and thus it can be used as a natural biomarker to trace and quantify the main food sources (Harland et al. 1993; Dalsgaard et al. 2003; Zhukova and Titlyanov 2003). It has been shown that the presence of lipid sources provided by symbiotic dinoflagellates leads to a significant difference in FA compositions between symbiotic and non-symbiotic octocorals (Imbs et al. 2007; Gori et al. 2012). Species with symbiotic algae typically possess the Poly Unsaturated Fatty Acid (PUFA) biomarkers 18:3(n-6) and 18:4(n-3), macromolecules that they cannot synthesize by themselves and are transferred from the symbiotic algae (Papina et al. 2003). PUFA, in particular n-3 and n-6, play important metabolic roles in the regulation of metabolism, affecting growth, respiration, energy generation, enzyme activity, as well as the production and development of eggs and planula larvae (Rinkevich 1989; Harland et al. 1992; Arai et al. 1993; Ward 1995). It is now accepted that its selective concentration may affect resilience and resistance, and consequently, the dynamics of populations (Rossi et al. 2006b).

In the Mediterranean Sea, *Eunicella singularis* and *Corallium rubrum* are among the most common octocorals in coastal areas (Ballesteros 2006). Whereas *C. rubrum* only feed on suspended particulate matter and zooplankton (Tsounis et al. 2006a), *E. singularis* is the only gorgonian hosting the dinoflagellate *Symbiodinium* sp. symbiont (Forcioli et al. 2011), having a mixotrophic trophic strategy (Coma et al. 2015; Ferrier-Pagès et al. 2015). Even if it acquires 2–3 times less autotrophic carbon from symbionts than scleractinian corals, *E. singularis* maintain a positive carbon budget due to a very efficient light utilization (Ferrier-Pagès et al. 2015). Nutrient acquisition by heterotrophic feeding in this gorgonian is also particularly important (Coma et al. 2015), since it dwells habitats with lower light levels, colder waters, and higher nutrient concentrations compared to tropical areas (Gori et al. 2012). Thus, *E. singularis* is able to use heterotrophic and autotrophic energy to sustain its basic metabolism (Ezzat et al. 2013) and optimize its nutrient input (Gori et al. 2012; Ferrier-Pagès et al. 2015). The relationship between autotrophic and heterotrophic feeding in *E. singularis* has recently been studied (Cocito et al. 2013; Ezzat el al. 2013; Ferrier-Pagès et al. 2015; Coma et al. 2015), but, to my knowledge, the effects of its high nutritional plasticity on reproduction have never been analysed.

E. singularis and *C. rubrum* are long-lived gonochoric species with annual reproduction occurring in summer (Santangelo et al. 2003; Bramanti et al. 2005; Tsounis et al. 2006b; Ribes et al. 2007; Gori et al. 2007). The reproductive output is based on an internal brooding reproductive strategy, releasing planula larvae, which have a pelagic larval duration (PLD) of approximately one month (Theodor 1967; Weinberg and Weinberg 1979; Martinez-Quintana et al. 2014).

E. singularis and *C. rubrum* represent a perfect case study in order to understand how passive suspension feeders having similar reproductive features but differing in their trophic strategies invest their stored energy in the offspring. Thus, the objective of the present study was to investigate the possible differences in the energetic investment during reproduction in two Mediterranean gorgonian species characterized by different trophic strategies. To achieve this objective, oocyte and spermary sac production (number, size and volume), content of organic matter, lipids and FA, together with FA composition were quantified in *E. singularis* and *C. rubrum* before and after spawning, as a proxy for parental investment. The analyses were carried out in different years (2010 and 2011) in order to assess the temporal variability entailed by environmental conditions, and how it may affect the reproductive output and energetic condition of the same population. The results of this study may help to understand the functioning of gorgonian populations, their distribution patterns as well as their capability to face punctual or recurrent perturbations.

MATERIALS AND METHODS

Sampling procedure

Corallium rubrum (N=30) and *Eunicella singularis* (N=30) colonies were sampled by SCUBA diving at Punta s'Oliguera in Cap de Creus (42° 17'03'' N; 003° 17'95'' E, northwestern Mediterranean Sea; Fig. 1). Populations of both species were located in the same rocky wall at different depths (25–30 m for *C. rubrum* and 13–16 m for *E. singularis). C. rubrum* colonies larger than 4–5 cm height (sexually mature according to Tsounis et al. 2006b) were sampled haphazardly, due to the small size of the colonies. For *E. singularis*, colonies >20 cm height (sexually mature colonies according to Ribes et al. 2007) were tagged and sampled.

During each sampling (see the following paragraphs), one fragment of primary branch was collected from each colony and divided into two portions. The first portion (~1 cm, base) was fixed in 10% formalin for the study of sexual product development and output (Coma et al. 1995; Rossi and Gili 2009). The second portion (1–2 cm, top) was frozen in liquid nitrogen and transported to the laboratory for biochemical analysis. This second fragment was stored at −80°C, freeze-dried for 24 h at −110°C and a pressure of 100 mbar, and stored at −20°C pending analysis (organic matter, stable isotopes, total lipids and free fatty acid content).

Stable Isotopes

The stable isotope (SI) ($\delta^{13}C$ and $\delta^{15}N$) composition in gorgonian tissue was assessed from seasonal samples (May, August, November, January) of 3 colonies for each species (*C. rubrum* and *E. singularis*) to identify its trophic position (Gori et al. 2012; Cocito et al. 2013; Elias-Piera et al. 2013). One first portion

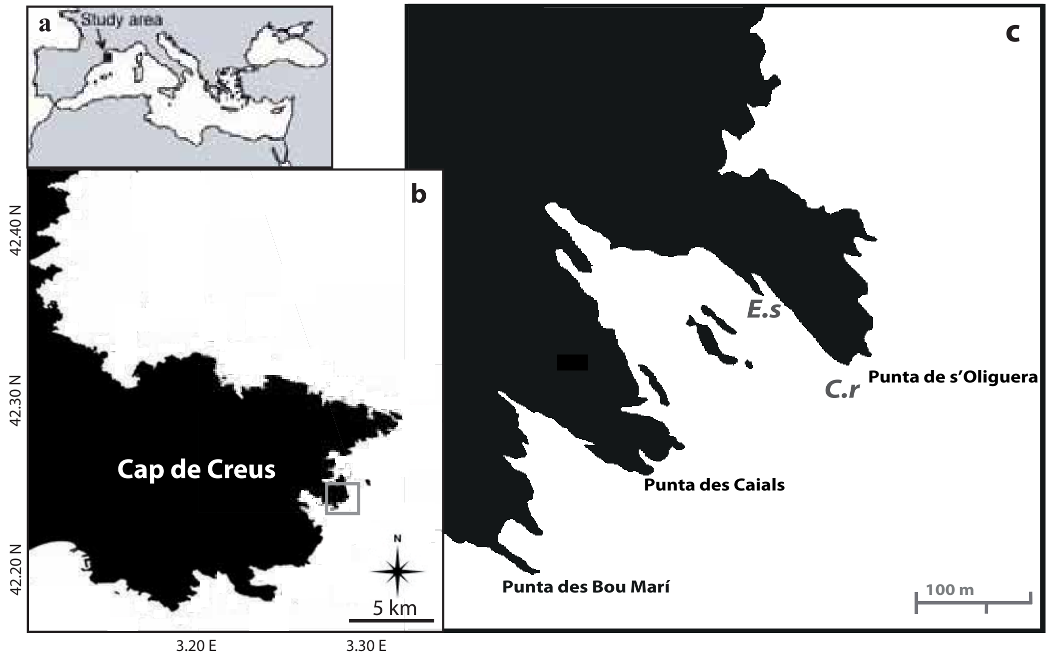

Fig. 1. Map of the study area (a, b), and location of the sampling site (c). *C.r* and *E.s* indicate the position of the *Corallium rubrum* and *Eunicella singularis* populations, respectively.

of 2 mg (± 0.001 mg) of tissue Dry Weight (DW) (dried at 80°C during 24 hours and weighed using a balance Mettler Toledo model XS3DU) was taken from each sample, and analysed for nitrogen ($\delta^{15}N$). Another portion of 2 mg (± 0.001 mg) of tissue DW from each sample was fumed with concentrated HCl during 48 h to eliminate the inorganic fraction and, then analysed for carbon ($\delta^{13}C$). $\delta^{13}C$ and $\delta^{15}N$ were determined by using a Thermo Flash EA 1112 analyser and a Thermo Delta V Advantage spectrometer following the same methodology as Gori et al. (2012) and Elias-Piera et al. (2013). Isotope ratios are expressed as parts per thousand (‰) (different from a standard reference material) according to the following equation:

$$\delta^{X} = [(R_{sample}/R_{standard}) - 1] \cdot 10^{3} \quad (1)$$

where “X” is ^{13}C or ^{15}N and “R” is the corresponding ratio C^{13}/C^{12} or N^{15}/N^{14}. R standard for C^{13} and N^{15} are from PeeDee Belemnite (PDB) and atmospheric N^{2}, respectively.

Production of oocytes and spermatic sacs

Oocyte and spermary production of *C. rubrum* and *E. singularis* were followed from May to August 2010, and from April to August 2011 to describe the sexual product development and to determine the exact moment of larval release in both years (see Results section). For each species, five colonies of each sex were identified, and six polyps per colony were dissected following the methodology described in Chapter 1. Sex identification was performed under the dissecting microscope and confirmed under optical microscope (Santangelo et al. 2003; Ribes et al. 2007). A total of 480 polyps from each gorgonian population were examined, and more than 800 and 1700 gonads were measured for *C. rubrum* and *E. singularis*, respectively.

Organic matter content

Organic matter (OM) in the coenenchyme of *C. rubrum* and *E. singularis* was quantified before and after larval release in 2010 and 2011, using five colonies for each sex and species. Approximately 100 mg (± 0.1 mg) of coenenchyme DW from each sample was weighed, combusted for 4 hours at 500°C in a muffle furnace (Relp 2H-M9) and weighed again. OM was then calculated as the difference between dry and ash weight (Slattery and McClintock 1995). Results are expressed in percentage with respect to the initial dry weight of the sample. A total of 40 colonies for each species were analysed.

Lipid content

The total lipid content in the OM was quantified in five female and five male colonies for each species (*C. rubrum* and *E. singularis*) before and after larval release in 2010 and 2011, following the colorimetric method of Barnes and Blastock (1973). Approximately 10 mg (± 0.1 mg) of coenenchyme DW from each colony were homogenized in 3 ml of chloroform-methanol (2:1), and total lipids were quantified colorimetrically, with cholesterol as a standard. Results are presented in μg Lipid per mg^{-1} of OM. A total of 40 colonies for each species were analysed.

Free fatty acid content and composition

Free FA content and composition was assessed in three female and three male colonies for each species (*C. rubrum* and *E. singularis*) before and after larval release in 2010 and 2011, according to the methodology described in Chapter 1. Briefly, a total amount of 10–12 mg (± 0.1 mg) of coenenchyme DW from each sample was analysed. FA were identified and quantified with gas chromatography (GC) analysis performed with an Agilent Technologies 7820A GC system instrument equipped with a DB-5ms Agilent column (60 m length, 0.25 mm internal diameter and 0.25 μm phase thickness). Methyl esters of FA were identified by comparing their retention times with those of standard FA (37 FAME compounds, Supelco® Mix C^{4}-C^{24}) and were quantified by integrating areas under peaks in the GC traces (Chromquest 4.1 software), with calibrations derived from standards. The results are presented in μg FA mg^{-1} of OM, and in percentage of Saturated Fatty Acids (SFA), Mono Unsaturated Fatty Acids (MUFA) and Poly Unsaturated Fatty Acids (PUFA) (see Chapter 1). A total of 24 colonies for each species were analysed.

Statistical treatment

Differences in volume, diameter and number of sexual products between years, sexes and species were tested using the non-parametric Wilcoxon–Mann–Whitney since the data were not normally distributed. The test was performed with the R-language function "wilcox.test" of the R software platform (R Development Core Team 2008). Differences in SI were tested using a two-way ANOVA with season (four levels, spring, summer, autumn and winter) and species (two levels, *C. rubrum* and *E. singularis*) as independent variables. Differences in OM content, lipid content, FA content and composition in SFA, MUFA and PUFA were tested using a four-way ANOVA with year (two levels, 2010 and 2011), species (two levels, *C. rubrum* and *E. singularis*), release period (two levels, before and after larval release) and sex (two levels, male and female) as independent variables. Before performing ANOVAs, normality of data residuals and variance homogeneity were tested with Shapiro-Wilk, and Bartlett test (R-language function "shapiro.test" and "bartlett.test", respectively). When variances were not homogeneous, necessary transformations were applied. ANOVA tests were performed with the R-language function "aov" (Chambers and Hastie 1992), followed, when appropriate, by a Tukey post hoc test (R-language function "tukeyHSD").

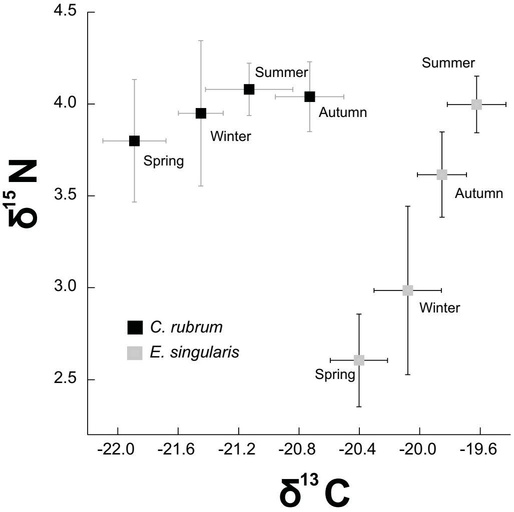

Fig. 2. Stable isotope (δ15N and δ13C) composition in Corallium rubrum (black squares) and Eunicella singularis (grey squares) (n=3 for each point) (mean ± SD).

Finally, the analysed colonies were arranged on the basis of their FA composition, using a principal component analysis (PCA) of transformed data ($p' = \arcsin(p^{1/2})$) with the R-language function "princomp" (Vegan library; Oksanen et al. 2005).

RESULTS

Stable Isotope

No differences between seasons were found in the stable isotope composition for *Corallium rubrum* (ANOVA one-way, $p > 0.05$; Fig. 2), whereas *Eunicella singularis* showed significant differences in δ^{13}C between spring and summer, and in δ^{15}N between spring, summer and autumn (ANOVA one-way, $p<0.01$; Fig. 2). Significant differences in SI proportion (δ^{13}C and δ^{15}N) were found between both species. While δ^{13}C showed significant differences between species in all the seasons (ANOVA two-way, $p<0.001$; Fig. 2), δ^{15}N was significantly different only in spring (ANOVA two-way, $p<0.01$; Fig. 2).

Production of oocytes and spermatic sacs

Changes in monthly frequency distribution of sexual products in *C. rubrum* (Fig. 3a, b) and *E. singularis* (Fig. 4a, b) colonies allowed to identify the exact spawning moment in 2010 and 2011. The release time corresponded to the disappearance of larvae inside the female polyps. Larvae of *C. rubrum* were released between 19th July and 8th August 2010 (Fig. 3a), and between 29th June and 4th August 2011 (Fig. 3b). On the basis of these observations, samples collected on 9th and 5th June 2010 and 2011 respectively, were considered as "pre-spawning", and samples collected on 8th and 4th August 2010 and 2011 respectively, were considered "post-spawning".

For *E. singularis*, the spawning period was between the 9th June and 8th August in 2010 (Fig. 4a), and between 5th June and 4th August in 2011 (Fig. 4b). Thus, samples of 9th and 5th June 2010 and 2011,

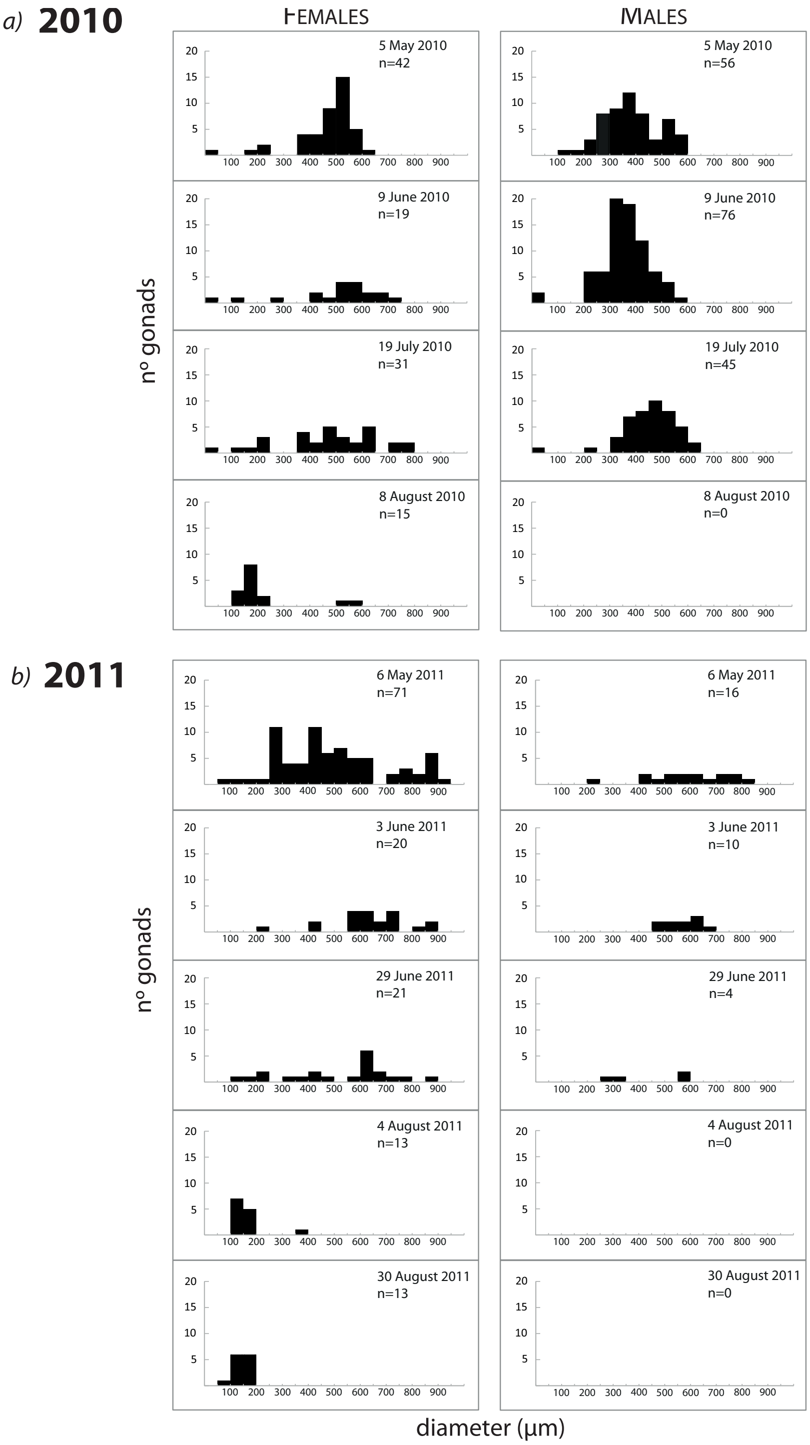

Fig. 3. *Corallium rubrum.* Distribution of gonadal diameter frequency (µm) in 30 female and male polyps; year 2010 (a) and 2011 (b) (n= sexual product number).

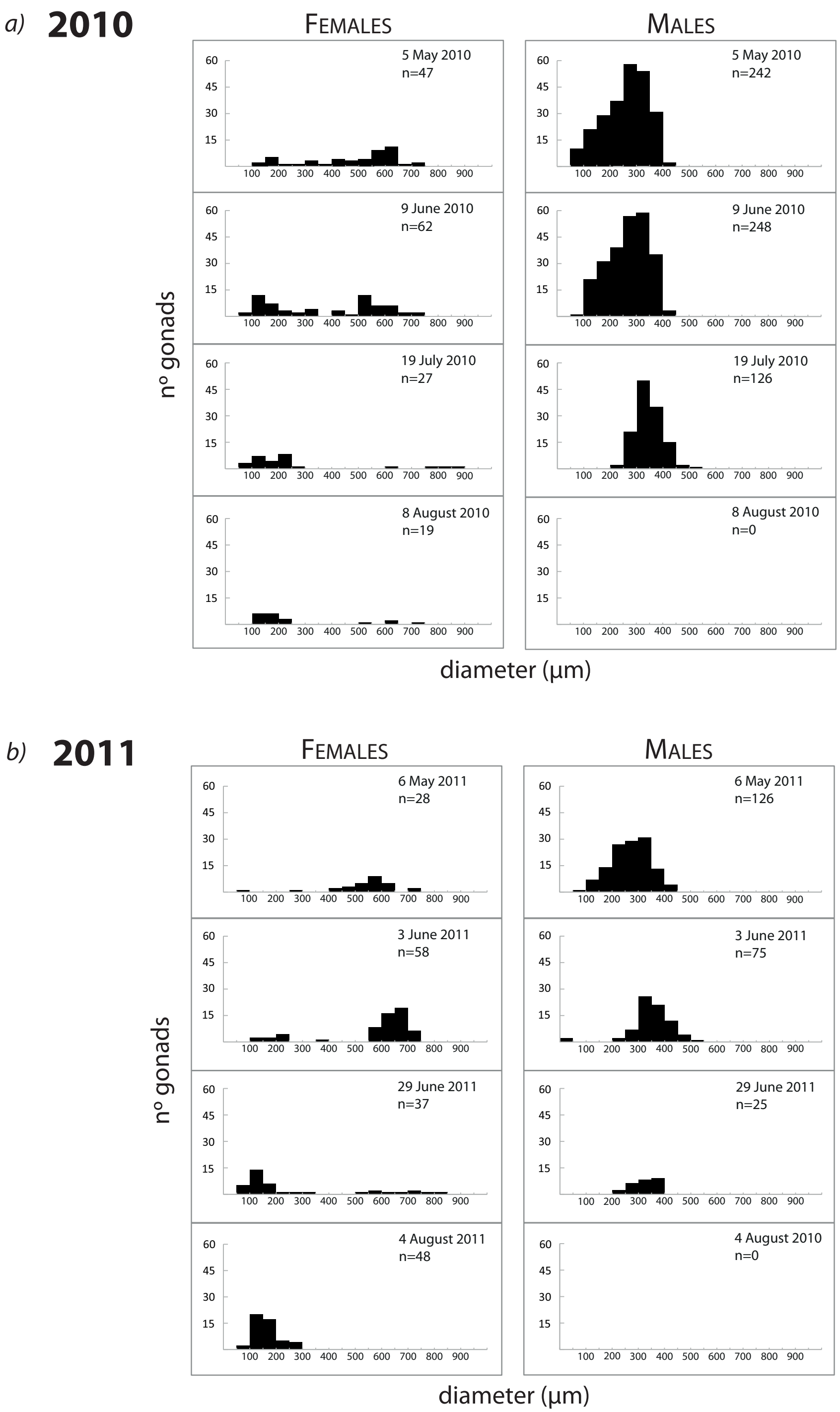

Fig. 4. *Eunicella singularis*. Distribution of gonadal diameter frequency (µm) in 30 female and male polyps; year 2010 (a) and 2011 (b) (n= sexual product number).

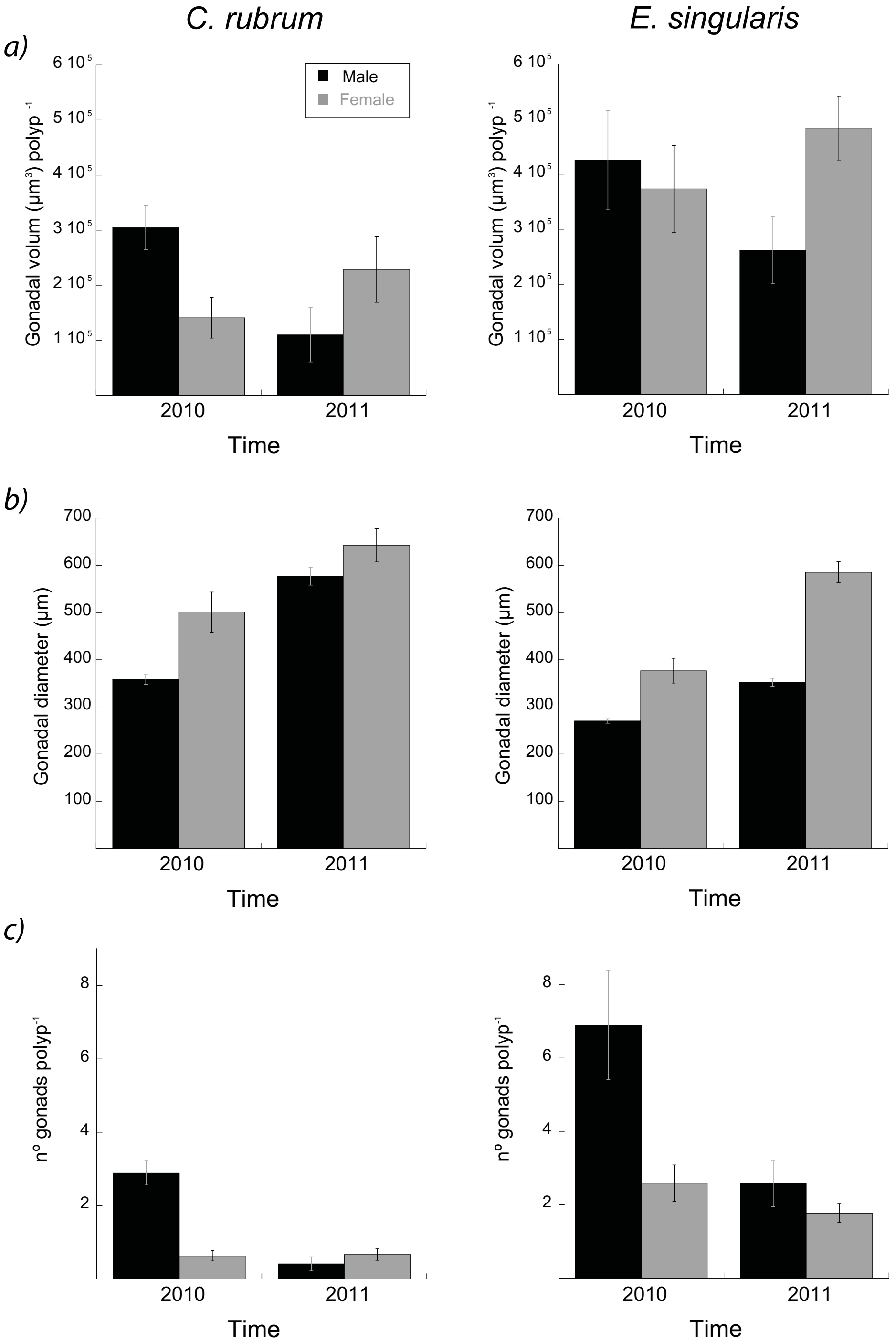

Fig. 5. Volume (a), diameter (b) and number (c) of sexual products per polyp of five male (black bars) and female (grey bars) colonies (mean ± SE) in two years (2010, 2011) for *Corallium rubrum* and *Eunicella singularis*, respectively.

respectively, were considered as "pre-spawning", and samples of 8th and 4th August 2010 and 2011, respectively, were categorised as "post-spawning".

In *C. rubrum*, the oocytes did not show significant differences in size, while spermatic sacs in 2011 showed a larger size, minor number and smaller total volume with respect to 2010 (Wilcoxon– Mann–Whitney test, $p<0.001$; Fig. 5). In *E. singularis*, the diameter of oocytes and spermatic sacs significantly increased in 2011 (Wilcoxon– Mann–Whitney test, $p<0.001$; Fig. 5b). Conversely, the number and volume of sexual products per polyp did not show significant differences between years and sexes (Wilcoxon– Mann–Whitney test, $p>0.05$; Fig. 5a, c). The volume and number of oocytes in both years, and spermatic sacs only in 2011, were different between the two species (Wilcoxon– Mann–Whitney test, $p<0.001$; Fig. 5a, c), being higher in *E. singularis* than in *C. rubrum*. The diameter of sexual products was higher in *C. rubrum* than in *E. singularis* (Wilcoxon– Mann–Whitney test, $p<0.01$; Fig. 5b), except between female colonies in 2011, when no differences were found (Wilcoxon– Mann–Whitney test, $p>0.05$; Fig. 5b).

Organic matter content

OM content in the coenenchyma was on average 24.07 ± 6.4 % and 24.38 ± 5.8 % for *C. rubrum* and *E. singularis*, respectively. The OM content did not show any significant differences either within species, considering the sex and sampling periods (ANOVA four-way $p>0.05$), or between species (ANOVA four-way, $p>0.05$).

Lipid content

Lipid content in *C. rubrum* female colonies significantly decreased after larval release in 2010 (ANOVA four-way, $p<0.001$; Fig. 6a). *E. singularis* did not show any significant difference in two factors considered (sexes and sampling periods) (ANOVA four-way $p>0.05$, Fig. 6b). When the two species were compared, lipid content was significantly higher in *E. singularis* female colonies in 2010 (ANOVA four-way $p<0.05$, Fig. 6) and male colonies after the larval release in both years (ANOVA four-way $p<0.01$, Fig. 6).

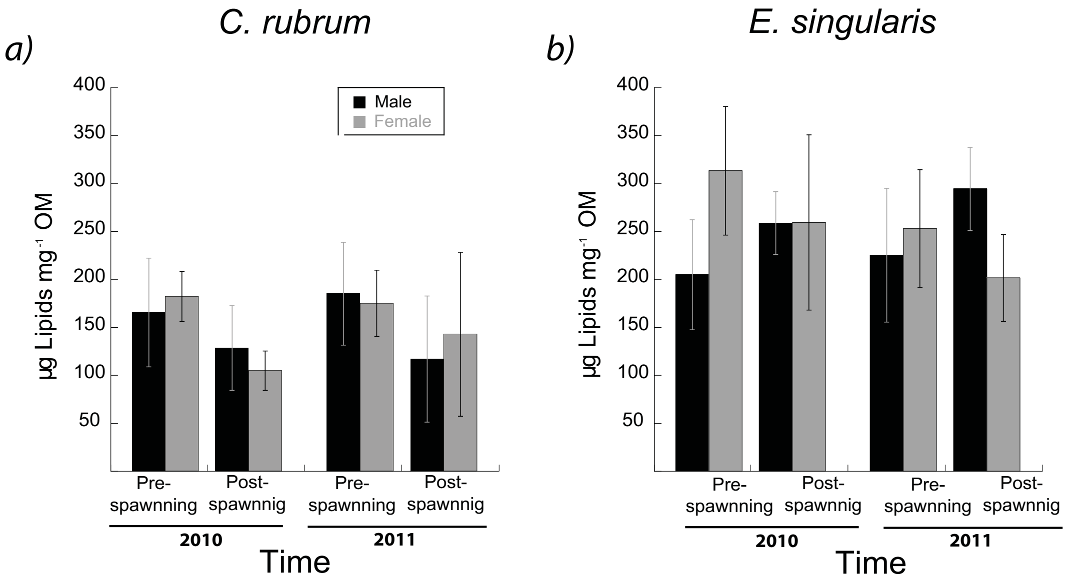

Fig. 6. Lipid content (µg mg-1 OM) (mean ± SD) in tissue of *Corallium rubrum* (a) and *Eunicella singularis* (b) in male (black bars) and female (grey bars) colonies (N = 5) before and after spawning in 2010 and 2011.

Free fatty acid content and composition

Free FA content in *C. rubrum* colonies significantly increased after larvae release only in 2010 (ANOVA four-way, p<0.05; Fig. 7a), whereas in *C. rubrum*, free FA content after larval release was significantly higher in 2010 than in 2011 (ANOVA four-way, p<0.05; Fig. 7a). Free FA content in *E. singularis* only displayed significant differences on male colonies in 2010 (ANOVA four-way, p<0.05; Fig. 7a), being more concentrated after the larval release. The comparison between species showed a higher free FA content in *E. singularis* than in *C. rubrum* (ANOVA four-way, p<0.001; Fig. 7a).

In *C. rubrum*, SFA decreased and PUFA increased after larval release in 2010 and 2011 (ANOVA four-way, p<0.001; Fig. 7b). MUFA were only different before and after release in 2011 (ANOVA four-way, p<0.01; Fig. 7b). Conversely, in *E. singularis,* the FA class (SFA, MUFA, PUFA) did not show significant differences among sexes, sampling periods and years (ANOVA four-way, p>0.05; Fig. 7b). However, the results of 2011 showed a higher variability (i.e. standard deviation) than 2010. When the two species were compared, *E. singularis* showed significantly lower SFA percentages in female colonies and higher PUFA percentages in both sexes only before larval release in 2010 (ANOVA four-way, p<0.05;

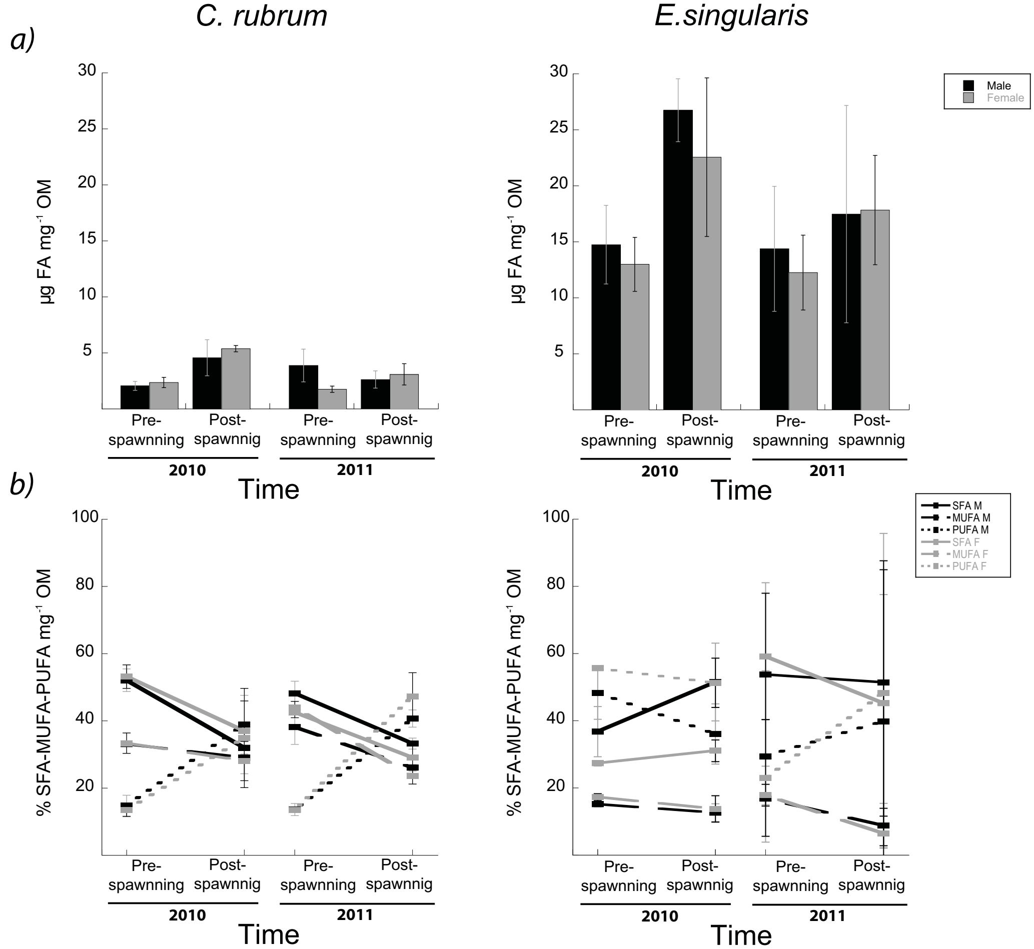

Fig. 7. Fatty acid content (a) (μg mg-1 OM) and percentage of fatty acid functionality composition (SFA, MUFA, PUFA) (b) in male (black bars) and female (grey bars) colonies of *Corallium rubrum* and *Eunicella singularis* (N = 3) before and after larval release in 2010 and 2011 (mean ± SD).

Fig. 7b). MUFA percentages were always higher in *C. rubrum* than *E. singularis* (ANOVA four-way, p<0.01; Fig. 7b). Interestingly, one of the greatest differences between both species was the high variability of *E. singularis* with respect to *C. rubrum*.

According to the PCA, there was a clear change in the main FA composition of *C. rubrum* colonies before and after larval release, both in 2010 and 2011 (Fig. 8), with 16:0 as main FA before spawning, and 20:4(n-6), as the most representative FA after spawning (Annex 3, p. 107). Conversely, colonies of *E. singularis* showed changes in the FA composition between years but not before and after release (Fig. 8). In this case, the 18:1(n-7) and 18:2(n-6) were dominant FA in 2010, while the amounts of 16:0, 20:4(n-6) and 24PUFA were greater in 2011 (Annex 3). Comparing both species, *C. rubrum* colonies showed the highest concentration of 18:1(n-9), whereas 18:1(n-7) and 18:2(n-6) were dominant in *E. singularis* colonies.

DISCUSSION

This study analysed the variation of energetic reserves during the reproductive period (i.e. gametogenesis and spawning) of adult colonies in two gorgonian species with different trophic strategies: *Corallium rubrum* (heterotrophic energy input) and *Eunicella singularis* (mixotrophic energy input). The complementary methodologies used (i.e. stable isotope, gonadal output, lipid content and free FA content and composition) showed that the energetic allocation during the reproductive period (pre and post-spawning) is similar between the two species, but the nutritional condition of *E. singularis* had a higher energy content and a lower inter-annual variability, probably due to the presence of symbionts, which could result in a buffer in front of the potential environmental condition variability.

Trophic strategies confirmed by SI analysis

The isotopic values measured in both species are within the expected range of passive suspension

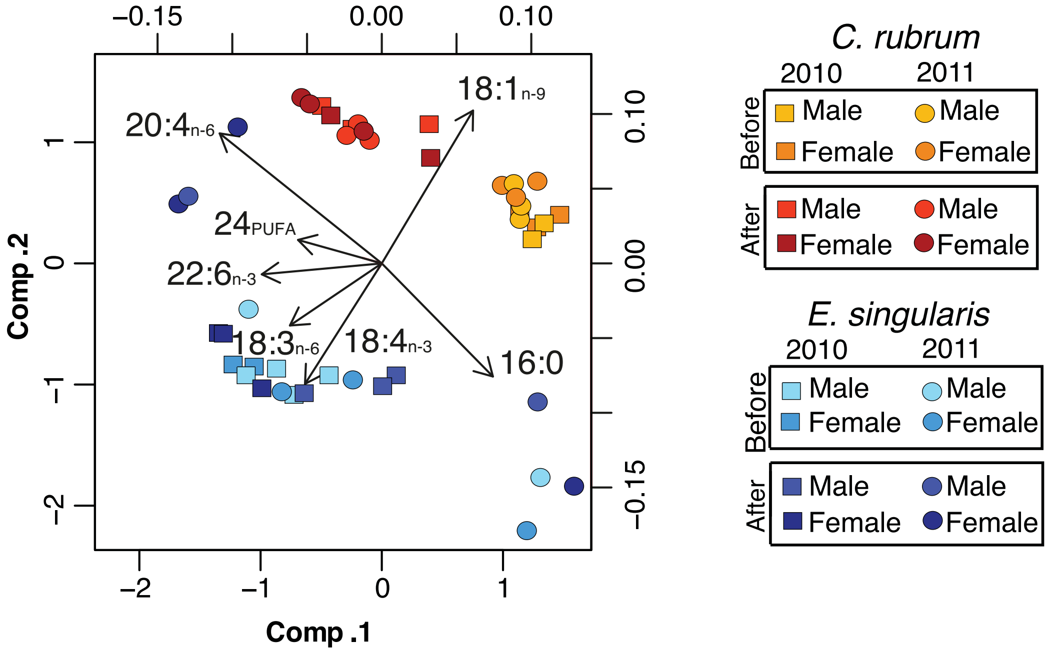

Fig. 8. Principal component analysis (PCA) biplot illustrating the ordering of the studied colonies with regard to their fatty acid composition, and the roles of the first seven fatty acids classified according to variance.

feeders (Fig. 2) (Carlier et al. 2007; Cocito et al. 2013). The $\delta^{13}C$ and $\delta^{15}N$ values found in *C. rubrum* suggest that the diet of this species is mainly based on suspended particulate organic matter (POM) (Darnaude et al. 2004 a, b; Carlier et al. 2007; Elias-Piera et al. 2013). This energetic input coming from the POM is in line with previous feeding experiments, especially because part of the re-suspended organic matter available to the colonies is an essential part of the energy input (Tsounis et al. 2006a). On the other hand, *E. singularis* displayed a high seasonal trophic plasticity, as has been previously observed in symbiotic populations of this species (Gori et al. 2012). In symbiotic corals, the nitrogen isotopic signature can be confusing, since it does not follow the general pattern of augment in function of increasing trophic level (Minagawa and Wada 1984; Post 2002; Carlier et al. 2007). This fact is due to the nitrogen reabsorption by the symbionts, a process that can also increase the nitrogen isotopic signature (Reynaud et al. 2009; Ferrier-Pagès et al. 2011; Cocito et al. 2013). For this reason, only the carbon isotopic signature may be used as indicator of the trophic level of symbiotic species (Muscatine et al. 1989; Risk et al. 1994; Reynaud et al. 2002). It has been demonstrated that autotrophic carbon acquisition causes a more positive ${}^{13}C$ signature, because part of the translocated carbon comes directly from the algae (Land et al. 1975; Muscatine et al. 1989; Reynaud et al. 2002; Swart et al. 2005; Tremblay et al. 2014). So, the $\delta^{13}C$ values in *E. singularis* suggest that this species is more autotrophic in summer, as has been previously reported (Gori et al. 2012; Cocito et al. 2013; Ezzat et al. 2013), and more heterotrophic in winter and spring, when the light conditions are not at the optimum and the available organic matter is higher (see pre-chapter; Calbet et al. 2001; Rossi and Gili 2005; Ferrier-Pagès et al. 2015). Interestingly, a recent study on prey capture rates of *E. singularis* found the greatest number and size of prey per polyp in spring (Coma et al. 2015), confirming the highest heterotrophic carbon acquisition in this mixotrophic gorgonian.

Energy investment in reproduction

Number and volume of oocytes and spermatic sacs per polyp were higher in *E. singularis*, but *C. rubrum* showed a larger size of sexual products (Fig. 5). Previously, it has been observed that the reproductive output can vary according to the quantity and quality of available food (Qian and Chia 1992; Gori et al. 2013). It would be expected then that mixotrophic species should present a high reproductive output, as well as a higher number and volume of sexual products. The present results of *E. singularis* and *C. rubrum* support this conclusion. Bleached corals show a lower number and volume of sexual products than healthy colonies (Guzman and Holst 1993; Mendes and Woodley 2002), suggesting the importance of the autotrophic energetic surplus for these mixotrophic species. A recent study on the reproductive cycle in *E. singularis* found the same differences when comparing symbiotic (shallow) with deep (60 m) asymbiotic populations, the latter having a lower volume of sexual products, as well as a lower amount of lipids (Gori et al. 2012).

In addition to the differences in sexual products, both species showed an inter-annual variability in their gamete production (Fig. 5). The volume, size and number of sexual products per polyp may change according to environmental conditions (Barnes and Barnes 1965; Bayne et al. 1975, 1978; Brambilla 1982; Qian and Chia 1991; Brey 1995; Tsounis et al. 2006b; Gori et al. 2007; Gori et al. 2013). In the present study, temperature and food availability in the water column were show differences during the study period between 2010 and 2011 (see pre-chapter). However, the data on environmental conditions only cover the period from May 2010 to November 2011. This limitation of the dataset does not allow it to explain the inter-annual variability observed in the sexual products due to the lack of data about spring 2009, during which the formation and development of sexual products of year 2010 started. More precisely, sexual products of 2010 underwent their maximum size increase rate approximately between March-April of the same year (Vighi 1972; Santangelo et al. 2003; Tsounis et al. 2006b; Gori et al. 2007; Ribes et al. 2007), while the number of sexual products per polyp had already been determined in May-June 2009 (Gori et al. 2013).

Results regarding the lipid content for both species are in line with previous studies (Fig. 6) (*C. rubrum*:

Rossi and Tsounis 2007; Bramanti et al. 2013; *E. singularis*: Gori et al. 2007; Gori et al. 2012). Lipid content values in *E. singularis* found in this study coincide with those of other symbiotic corals (Harland et al. 1993; Yamashiro et al. 1999; Grottoli et al. 2004; Shirur et al. 2014), while values in *C. rubrum* are comparable with other non-symbiotic octocorals (Rossi et al. 2012; Rossi et al. 2006a; Hamoutene et al. 2008; Tsounis et al. 2012). Therefore, the higher values found in *E. singularis* with respect to *C. rubrum* are probably due to the energy surplus obtained by this species from its symbionts. Interestingly, energetic reserves in *E. singularis*, despite the high values, did not show a statistically significant reduction after larval release. The initial hypothesis was that mixotrophy could result in a higher energetic investment in reproduction due to the high parental nutritional state (Bayne et al. 1975, 1978; Mckillup and Buther 1979; Thompson 1983; George 1994). However, the results suggest that there is not a direct relationship between energetic reserves and parental investment in reproduction in this mixotrophic species and, therefore, the parental investment could be determined mainly by the reproductive output, as also observed in Chapter 1.

Male colonies displayed higher lipid content in *E. singularis* than in *C. rubrum* after spawning. The presence of a high amount of energetic reserves could give a better resistance to unfavourable conditions (i.e. high seawater temperature and/or starvation) and a faster recuperation capability after disturbances (Grottoli et al. 2006). After the release of sexual products, colonies can require a higher energetic cost to repair the processes associated with spawning (Calow 1979). It is therefore possible that these differences could only be observed in male colonies due to their lower investment in reproduction (Ferguson 1975; Arai et al. 1993), furthermore implying that they have a faster recovery than female colonies.

In 2010, the lipid content in female colonies was lower in *C. rubrum* than *E. singularis*. During summer, the Mediterranean Sea is characterised by strong water stratification, with limited food availability and high temperature in shallow waters (Turon and Becerro 1992; Coma et al. 2000; Rossi and Gili 2005), representing a critical period for benthic suspension feeders. As a result, benthic suspension feeders experience a low energy input period that lasts approximately from mid summer to early winter (Rossi et al. 2006a; Rossi and Tsounis 2007; Rossi et al. 2012). The summer of 2010 was characterized by lower food availability in the water column with respect to 2011 (see pre-chapter), being a potential cause of decrease in feeding rates in *C. rubrum* and *E. singularis* (Tsounis et al. 2006a; Coma et al. 2015). Differently from *C. rubrum*, *E. singularis* can compensate the decreased feeding rate with the energy input from its symbiotic algae, and better face the summer starvation period (Previati et al. 2010; Cocito et al. 2013). Indeed, Sbrescia et al. (2008) observed that *E. singularis* might quickly recover after a mass mortality event, demonstrating the high resistance in front of environmental conditions of this species. Besides, the wide distribution of *E. singularis* in comparison to *C. rubrum* (Rossi et al. 2008; Gori et al. 2011; Angiolillo et al. *in press*) could also be related to the higher resistance afforded by its facultative feeding mode (Grottoli et al. 2006), but it also necessary to consider that the tremendous harvesting pressure on *C. rubrum* populations may distort the natural presence of this precious coral (Tsounis et al. 2010).

The potential role of fatty acids

Besides the differences observed in lipid content (i.e. energetic reserves), the two species also showed different energetic requirements (free FA concentration) probably due to the necessity of satisfying their metabolic demands. FA are essential constituents of cell membrane lipids, precursors of bioactive metabolites and, therefore, reflect specific cellular physiological functions and physiological states of species (Sargent et al. 1990, 1999). In *E. singularis*, the free FA content was higher than in *C. rubrum* (Fig. 7), suggesting that *E. singularis* has a higher metabolic demand, probably due to the presence of symbiotic algae. The energetic costs associated to the presence of symbiotic algae include mechanisms to cope with high oxygen tension (activation and increase in levels of antioxidant enzymes) and possible regulation of the algae growth rates (Muller-Parker and D'Elia 1997). Indeed, symbiont loss at high water temperatures seem to be due to elevated energetic costs for keeping the symbiotic algae by gorgonian species (Ezzat et al. 2013). In addition, faster-growing species, such as *E. singularis*, are generally assumed to have higher respiration rates, and thus higher metabolic demands, than the slower-growing species, like *C. rubrum* (Weinberg and Weinberg 1979; Gates and Edmunds 1999; Marschal et al. 2004). Finally, the variability (i.e., standard deviation) in FA content among samples of *E. singularis* may be partly explained by an uneven distribution of symbiotic algae in anthozoan tissue (Bachok et al. 2006), resulting in differences in photosynthetic rates and in cellular energy demand on coral tissue (Oku et al. 2002).

On the other hand, the free FA content can increase during stress conditions, due to the presence of pathogens or as a consequence of starvation (Sargent et al. 1999), since the synthesis of FA from lipid reserves can satisfy the metabolic requirements (Imbs 2013). In this context, the results showed that while in *E. singularis* no significant differences were found before and after larval release and between years, in *C. rubrum*, the FA content was higher after spawning in 2010 (Fig. 6a), suggesting that *C. rubrum* suffered stress conditions. The constant values of organic matter content obtained allow to exclude that the observed effect is due to prolonged stress (Bramanti et al. 2013). In 2010, the summer period was characterized by lower POM and zooplankton availability in the water column with respect to 2011 (see prechapter), which is the principal food source for *C. rubrum* (Tsounis et al. 2006a). Therefore, while *C. rubrum* is affected by food availability, *E. singularis* does not experience the same impact, probably due to its trophic plasticity (Grottoli et al. 2006).

Certain FA can reflect dietary input and, thus, can be used as natural biomarkers in order to trace and quantify the diet types (Harland et al. 1993; Dalsgaard et al. 2003; Zhukova and Titlyanov 2003). Knowledge of their quantity (FA content), quality (% SFA, MUFA, PUFA) and composition (FA components) may give information about the relative importance of autotrophy versus heterotrophy (Figueiredo et al. 2012), the main source of heterotrophic organic matter inputs, and their implication in the reproductive activity and/or starvation period.

The results regarding FA composition in both species studied are in line with the previous literature related to heterotrophic and autotrophic behaviour (Imbs et al. 2010). While *C. rubrum* presented the highest concentration of 18:1(n-9), *E. singularis* was characterized by 18:3(n-6) and 18:4(n-3) (Fig.8). The FA 18:1(n-9) has been associated to detrital matter (Schultz and Quinn 1973; Fahl and Kattner 1993), which is the principal food source for *C. rubrum* (Tsounis et al. 2006a). On the other hand, a higher percentage of 18:3(n-6) and 18:4(n-3), together with a higher PUFA n-3 and n-6 proportion, has been related to autotrophic feeding (Bachok et al. 2006; Imbs et al. 2007, 2009). When both species were compared, the higher proportion of PUFA n-3 and n-6 in *E. singularis* with respect to *C. rubrum* was only observed before larval release (Annex 3), because *C. rubrum* increased these PUFA proportions after release. This fact can be interpreted in the light of what I have shown in Chapter 1, where I concluded that the PUFA increase after larval release could be due to a stress period caused by summer starvation. Therefore, the study of feeding by trophic biomarkers should be carefully interpreted, due to the fact that FA composition and concentration can change in response to stress.

The main metabolic demands of *C. rubrum* may be due to growth (16:0, before release) and to stress situation caused by a starvation period (20:4(n-6), after larvae release), which occurs during the summer in the Mediterranean Sea (Coma et al. 2000) (see Chapter 1). Conversely, the metabolic demands of *E. singularis* did not seem related to the reproductive activity or the summer starvation period, but with a more autotrophic or heterotrophic feeding behaviour. Interestingly, the biomarkers related to autotrophic feeding (18:3(n-6) and 18:4(n-3)) showed the highest proportion in 2010, coinciding with the lowest food availability in the water column (see pre-chapter). This may indicate a possible feeding strategy shift of the species in the critical period of spring-early summer with a more autotrophic carbon input, when food availability is scarce. In 2011, the FA composition in *E. singularis* displayed a high variability between their components and between colonies which can be related to the presence of the two nutrition modes (autotrophic and heterotrophic) in parallel to optimize its energetic input (Gori et al. 2012; Ferrier-Pagès et al. 2015).

CONCLUSIONS

The present study revealed that the lipid content of octocorals increases with the presence of symbiotic algae, but the energy invested (lipid) in the reproductive activity is not related to the amount of reserves in parental colonies. Again, the results showed that the energy required (FA) for metabolism seems also correlated with the presence of symbiotic algae, due to the cost involved in their maintenance (Muller-Parker and D'Elia 1997; Ezzat et al. 2013). However, the results also showed that shallow populations of *E. singularis* are potentially more resistant to environmental feature variation due to the energy surplus obtained by their symbiotic algae. This fact is corroborated by the study of Sbrescia et al. (2008), who observed that *E. singularis* might quickly recover after a mass mortality event. The implications of this sensitivity to environmental conditions in the light of global climate change affecting shallow waters (above 40 meters depth) needs further evaluation, but could result in a phase shift of mixotrophic and heterotrophic coral communities. Future studies on the reproductive physiology of octocorals (including lipid and FA composition) might reveal a risk of recruitment failure in species employing certain feeding strategies, and help to understand the consequences of climate change on octocoral communities.

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Chapter 3

Differential energy allocation between maternal colonies and larvae in the octocorals *Corallium rubrum, Eunicella singularis* and *Paramuricea clavata*

ABSTRACT

The present study investigates the potential energetic resource allocation from parental colonies to their larvae in three Mediterranean gorgonian species (*Corallium rubrum, Eunicella singularis* and *Paramuricea clavata*), as well as the differences in energy storage patterns between their larvae, depending on the trophic and reproductive ecology of octocorals. *C. rubum* and *E. singularis* are both internal brooders, but the first is non-symbiotic (heterotrophic) while the second is symbiotic (mixotrophic energy input). *P. clavata* is a non-symbiotic gorgonian with a surface brooding reproductive strategy. In order to investigate the quality of energetic reserves transferred from maternal colonies to larvae, the composition of free fatty acids (FA) was compared between parental colonies and offspring (i.e. larvae). *C. rubrum* showed a similar FA composition in maternal colonies and in their larvae, probably due to a non-selective transfer of energy. Conversely, *E. singularis* displayed a possibly selective transfer of symbiotic algae from maternal colony to larvae, whereas *P. clavata* colonies generally transfer low amounts of energy to their oocytes. Moreover, the present study proposes the quantification of FA content and the analysis of its composition as a new approach in order to obtain more information about the activity rates and to understand the dispersal and recruitment capability of species, since FA are strictly related to the metabolic needs of an individual (part of the organic matter available for activity and settlement). The results of the present study are in line with previous literature, suggesting that E. singularis larvae have the highest capacity of dispersal and/or crawling behaviour compared to *C. rubrum* and *P. clavata*. The energy allocation and the activity of the *P. clavata* larvae suggest they settle close to the parental colonies. The different FA composition in the larvae of the three species may suggest that the energetic strategy of octocorals differs and may be part of species-specific settlement and recovery strategy in front of environmental impact.

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VILADRICH N (2015) Doctoral Thesis pp 75-90

INTRODUCTION

Life histories of marine sessile invertebrates are highly diverse, involving a broad array of larval forms and developments (Jacobs and Podolsky 2010). The majority of marine sessile invertebrates are characterized by a complex life cycle with a sedentary adult phase and a mobile larval phase. For such species, the free-swimming larva is the only way of dispersal and, consequently, has a paramount role in the spatial and genetic population structure, as well as in its resilience and colonization capabilities (Palumbi 1994; Heller and Zavaleta 2009; Hart and Marko 2010). Understanding the reproductive biology, larval behaviour and connectivity among populations is critical for studies of population dynamics, management purposes as well as for the design of effective marine protected areas (Cowen et al. 2000).

As in most marine organisms, marine invertebrate larvae can be broadly classified into two categories, lecithotrophic or planktotrophic, depending on their source of nutrition during development. Planktotrophic larvae require external food sources to complete development, whereas lecithotrophic larvae are capable of completing development based solely on maternal provisions (Thorson 1950; Pechenik 1990; Morgan 1995). In the latter case, the energy allocation per larva is higher than in the planktotrophic ones (Jablonski and Lutz 1983; Villinski et al. 2002). Maternal investment in larvae derives mainly from the lipid reserve transfer (Richmond 1987; Arai et al. 1993), such as wax esters, triacylglycerols, phospholipids and free fatty acids (FA; Imbs 2013). The composition of FA transferred to larvae can just reflect the composition of FA in mother organisms (non selective transfer) (Dalsgaard et al. 2003), or can be significantly different due to the transfer of only some of the FA (selective transfer). In addition, some symbiotic species with lecithotrophic larvae can also transfer *Symbiodinium* to their larvae, which should provide additional energy to offspring during development (Richmond 1987). These differences in the energetic allocation from maternal colonies to larvae, or in the capacity to obtain energy during the planktonic phase, may influence longevity and competence periods, i.e. dispersal capacity (Richmond 1987, 1989; Pechenik 1990; Qian et al. 1990; Harms 1992; Jaeckle and Manahan 1992; Havenhand 1993; Ben-David-Zaslow and Benayahu 1998; Harii et al. 2010).

For spatially structured populations, the dispersal of larvae, but also the site where the larval settle, underpin the distribution patterns (Hughes and Jackson 1985; Smith 1992; Babcock and Mundy 1996). Some larvae of sessile invertebrates have the capacity to choose their settlement site, either based on the presence of conspecifics (Crisp 1974) or as response to chemical cues (Baird and Morse 2004; Gleason et al. 2009). This larval behaviour can influence population dynamics due to the reduction of post-settlement mortality (Todd 1979). However, due to the microscopic size, the behaviour of larvae during settlement site selection is virtually impossible to study *in situ* for most sessile invertebrates.

It has been demonstrated that an active search for a settlement site, as well as active swimming, induces a high metabolic demand (Okubo et al. 2008; Rivest and Hofmann 2014). Indeed, the survival and behaviour of larvae depend largely on the level of energy metabolized to sustain embryonic, larval and post-larval development (Holland and Spencer 1973; Gallager and Mann 1986). Therefore, studies on larval metabolic demands may be performed to better understand their dispersal and recruitment capacities. Up to now, studies on larval metabolic demands have been based on the quantification of oxygen consumption by larvae, but their values could be overestimated due to technical limitations (Hoegh-Golderberg and Manahan 1995; Graham et al. 2013). In the present study, the free FA content in the larval stage was analysed as a measure of their metabolic demands, as free FA are the principal source of energy which ensures ATP in larvae. This approach has been thoroughly used in the fish culture industry. For example, it has been demonstrated that a deficiency in free FA delays growth and reduces swimming activity and survival of fish (Izquierdo 1996; Copeman et al. 2002; Bransden et al. 2005), leading to a low recruitment into adult stocks (Bell and Sargent 1996). Studying the free FA composition may help to understand the nature of metabolic demands (i.e. energetic requirements), as previously observed in Chapters 1 and 2. The different kind of FA (Saturated Fatty Acids (SFA), Mono Unsaturated Fatty Acids (MUFA) and Poly Unsaturated Fatty Acids (PUFA)) may be a good proxy to study the fitness of larvae. In general, when FA are catabolized, SFA and MUFA are preferentially consumed and PUFA are selectively retained (Rainuzzo et al. 1994; Tocher 2003). Therefore, the availability of large amounts of some PUFA is considered essential for larval development and fitness (DeMott and Muller-Navarra 1997; Wen et al. 2002).

Among the above mentioned benthic sessile invertebrates, gorgonians are of great significance, as they are conspicuous ecosystem engineering species in many benthic communities around the world (Gili and Coma 1998; Coma and Ribes 2003; Wild et al. 2011), being one of the main three-dimensional constituents of the "animal forests" (Rossi 2013). Gorgonians exhibit three different strategies of sexual reproduction: 1) broadcast spawning, where sperm and oocytes are released in water column, 2) surface brooding, where the eggs are retained by mucous material on the surface of the female bodies, and 3) internal brooding, where female colonies retain the zygotes and embryos within their body to protect the larvae during their development (Ribes et al. 2007).

In the Mediterranean Sea, the non-symbiotic octocorals *Corallium rubrum*, and *Paramuricea clavata*, together with the symbiotic *Eunicella singularis* are characteristic species of gorgonian assemblages in the coastal areas. They are gonochoric, releasing lecithotrophic larvae annually during summer (Coma et al. 1995; Santangelo et al. 2003; Ribes et al. 2007). *C. rubrum* and *E. singularis* are internal brooder species, whereas *P. clavata* is a surface brooder (Coma et al. 1995; Santangelo et al. 2003; Ribes et al. 2007). While both *C. rubrum* and *P. clavata* release non-symbiotic ciliated larvae (planulae), it has been hypothesized that *E. singularis* larvae could present *Symbiodinium* algae (Weinberg 1979). Finally, the trophic ecology of the three species is different, as well as its capability to store energy in front of the seasonal cycle (Coma et al. 1994; Ribes et al. 1999; Rossi et al. 2004; Tsounis et al. 2006; Picciano and Ferrier-Pagès 2007; Gori et al. 2007; Rossi and Tsounis 2007). This fact, together with the reproductive strategy, could be essential for understanding the capability of each species to overcome the challenges of environmental change both at local and global scale.

The main objective of the present study was to test the differences in energetic resource allocation from parental colonies to their larvae and compare the metabolic demands of larvae of the three Mediterranean gorgonians *C. rubrum*, *P. clavata* and *E. singularis*. The final objective is to understand the role of energy allocation from parental colonies to larvae, the role of the amount of energy consumed by the larvae on their dispersal and recruitment capacities, as well as the resilience of the species and their capability to face short term perturbations taking into account some energetic variables that may partially explain recruitment processes. To achieve these objectives, the free FA content and composition of the larvae of *C. rubrum*, *E. singularis* and *P. clavata* were measured and compared with respect to their mother colonies. To our knowledge, the present study is the first to test the viability and dispersal of invertebrates' larvae using data on energetic demands (i.e. amount of free FA).

MATERIALS AND METHODS

Sampling procedure

Larvae of *Paramuricea clavata* were collected by SCUBA diving off the Spanish coast at Punta s'Oliguera in Cap de Creus on 10th June of 2011 (42° 17'03'' N; 003° 17'95'' E, northwestern Mediterranean Sea; Fig. 1). Larvae of internal brooder species (*Corallium rubrum* and *Eunicella singularis*) are difficult to obtain *in situ*. For this reason, five female colonies were collected for each of the two species and maintained in aquarium during a few days until larval release. Larvae of *E. singularis* were collected on 19th July of 2011, whereas the larvae of *C. rubrum* were obtained on 27th July of 2012.

During sampling, three replicates of 30 larvae for each species were cold shocked with liquid nitrogen and stored at −80°C, freeze-dried for 24 h at −110°C and a pressure of 100 mbar. For further analysis, the dry material was stored at −20°C.

Free fatty acid content and composition

Free FA content and composition was assessed in three replicates for each species according to the methodology described in Chapter 1. The FA of the 30 larvae for each replicate were identified and quantified with gas chromatography (GC) analysis performed with an Agilent Technologies 7820A GC system instrument equipped with a DB-5ms Agilent column (60 m length, 0.25 mm internal diameter and 0.25 m phase thickness). Methyl esters of FA were identified by comparing their retention times with those of standard FA (37 FAME compounds, Supelco® Mix C^4-C^{24}) and were quantified by integrating areas under peaks in the GC traces (Chromquest 4.1 software), with calibrations derived from standards. The results are presented in µg FA larvae^{-1}, and in percentage of Saturated Fatty Acids (SFA), Mono Unsaturated Fatty Acids (MUFA) and Poly Unsaturated Fatty Acids (PUFA), besides each FA component percentage (see Chapter 1). A total of 15 samples, with 30 larvae each, were analysed.

Statistical treatment

Differences regarding FA composition and SFA, MUFA and PUFA amount between parental colonies and their larvae were tested using a one-way ANOVA. Differences in FA content, composition and SFA, MUFA and PUFA between larvae of the different species were also tested using a one-way ANOVA. Before performing ANOVAs, normality of data residuals and variance homogeneity were tested using the Shapiro-Wilk, and Bartlett test (R-language function "shapiro.test" and "bartlett.test", respectively). When variances were not homogeneous, necessary transformations were applied. ANOVA tests were performed with the R-language function "aov" (Chambers and Hastie 1992), followed, when appropriate, by a Tukey post hoc test (R-language function "tukeyHSD").

The parental colonies analysed in Chapter 1 and 2, together with their larvae analysed in this chapter, were ordered based on their FA composition using a principal component analysis (PCA) per-

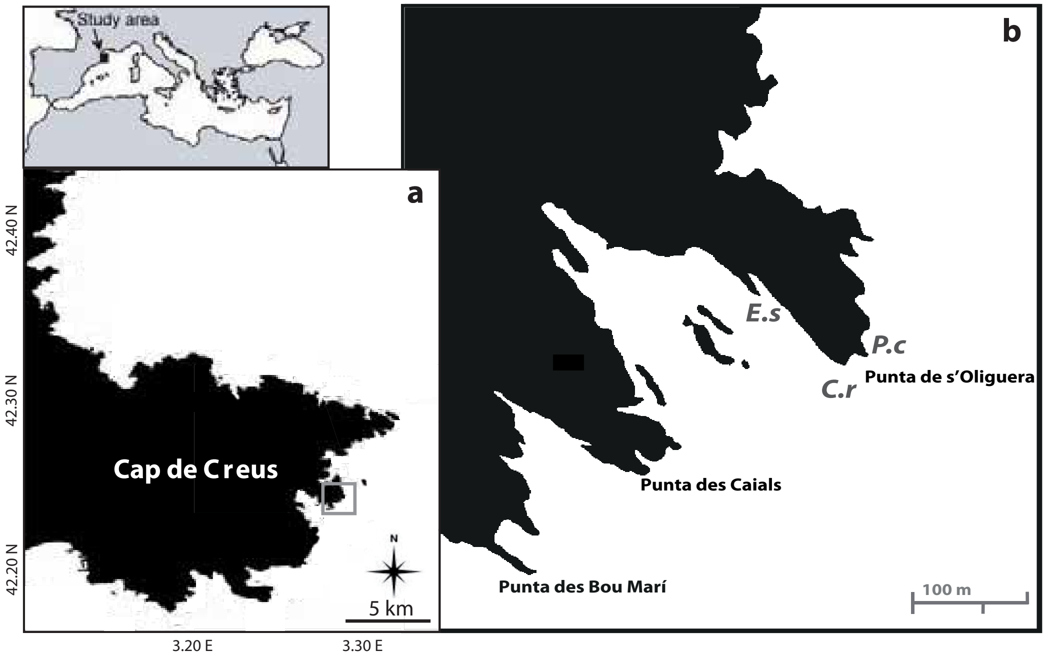


Fig. 1. Map of the study area (a), and location of the sampling site (b). *C.r*, *E.s* and *P.c* indicate the position of the *Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata* populations, respectively.

formed on transformed data ($p' = \arcsin(p^{1/2})$) with the R-language function "princomp" (Vegan library; Oksanen et al. 2005). Finally, another PCA was obtained to compare the FA composition of each larval species.

RESULTS

Fatty acid composition in maternal colonies and their larvae

C. rubrum larvae showed a lower SFA and a higher MUFA percentage than parental colonies (ANOVA one-way, $p<0.01$; Fig. 2a), while no significant differences in PUFA percentage were found (ANOVA one-way, $p>0.05$; Fig. 2a). Parental colonies and larvae of *E. singularis* only showed significant differences in PUFA percentage (ANOVA one-way, $p<0.05$; Fig. 2b), which was higher in larvae. *P. clavata* larvae showed a higher SFA and a lower MUFA and PUFA percentage than parental colonies (ANOVA one-way, $p<0.01$; Fig. 2c).

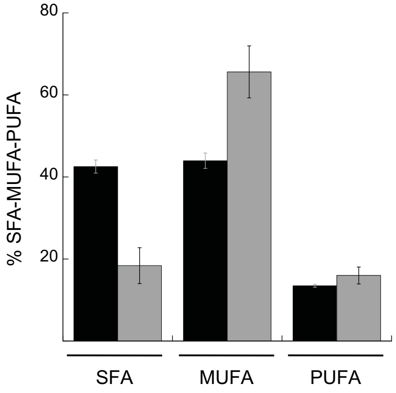
According to the PCA, the main differences in FA composition in larvae with respect to their parental colonies was 18:1(n-9), 18:3(n-3) in *C. rubrum*, 18:3(n-3), 18:4(n-3) in *E. singularis* and 18:2(n-6), 18:3(n-3) in *P. clavata* (Fig. 3).

Fatty acid content and composition in larvae

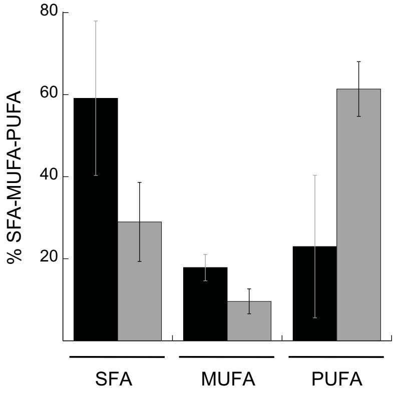
The comparison between larvae of different species showed significant differences between *C. rubrum* and *P. clavata*, and between *E. singularis* and *P. clavata*, being always lower in *P. clavata* (ANOVA one-way, $p<0.001$; Fig. 4a). The percentage of MUFA was higher in *C. rubrum* than in *P. clavata* larvae (ANOVA one-way, $p<0.05$; Fig. 4b), while PUFA percentage was higher in *E. singularis* and *P. clavata* than in *C. rubrum* larvae (ANOVA one-way, $p<0.05$; Fig. 4b).

According to the PCA, the main FA composition in *C. rubrum* larvae was 18:1(n-9), in *E. singularis* larvae it was 18:3(n-3) and 18:4(n-3), and in *P. clavata* larvae it was 18:2(n-4) (Fig. 5).

a) C. rubrum



b) E. singularis



c) P. clavata

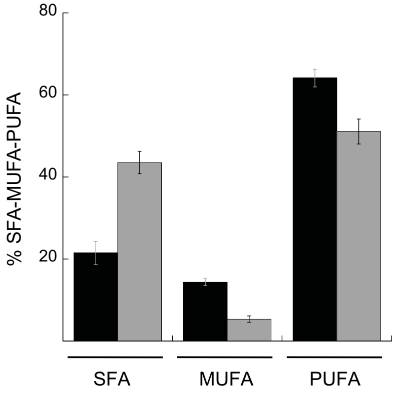


Fig. 2. Percentage of fatty acids functionality composition (SFA, MUFA, PUFA) in parental colonies (black bars) and their larvae (grey bars) for *Corallium rubrum* (a), *Eunicella singularis* (b), and *Paramuricea clavata* (c) (N = 3)(mean ± SD).

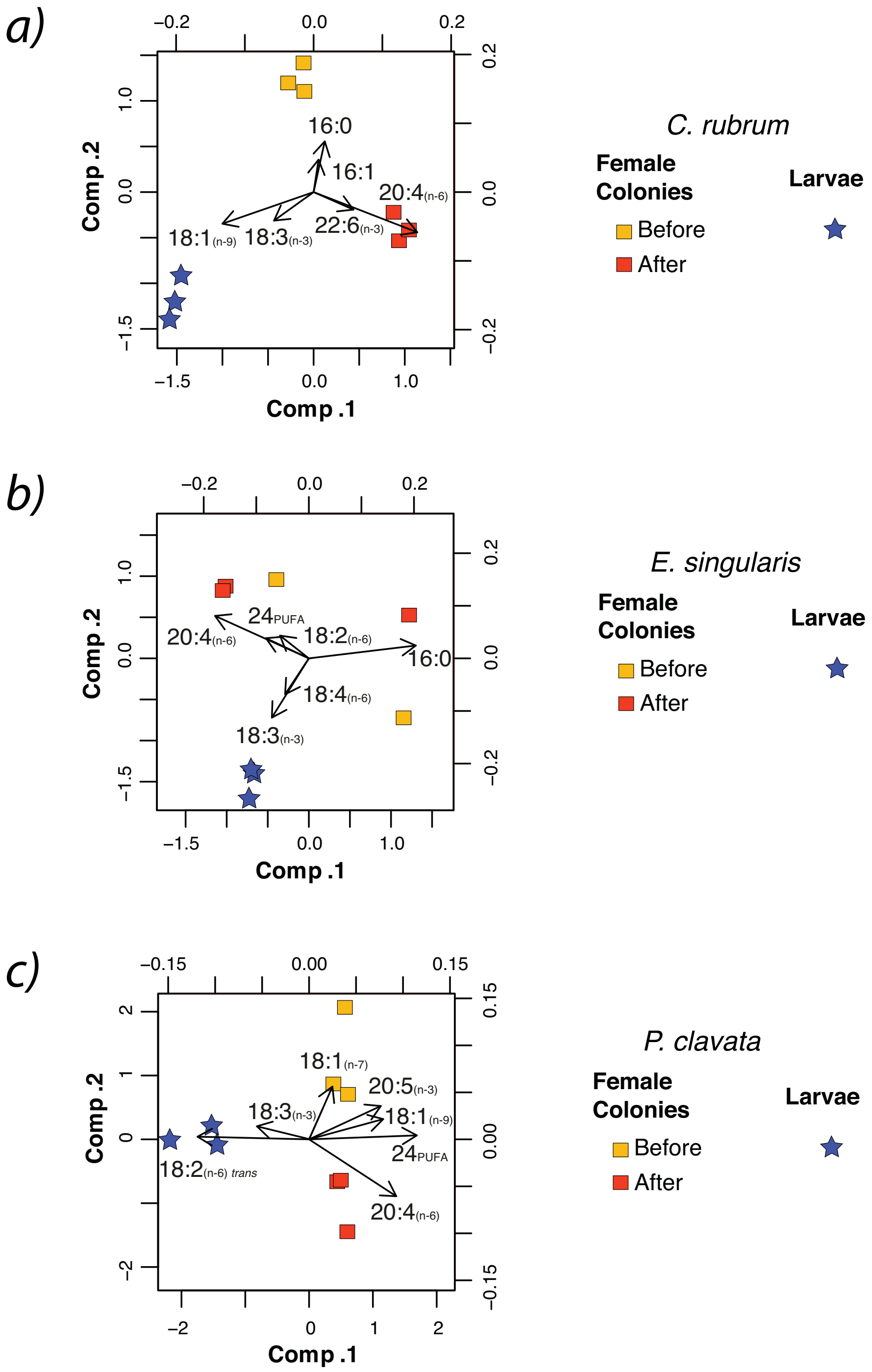


Fig. 3. Principal component analysis (PCA) biplot illustrating the ordering of the studied colonies and larvae for *Corallium rubrum* (a), *Eunicella singularis* (b), and *Paramuricea clavata* (c) with regard to their fatty acid composition, and the roles of the main fatty acids classified according to the variance.

DISCUSSION

Fatty acid allocation from maternal colonies to the larvae

The transfer of SFA, MUFA and PUFA from maternal colonies to oocytes, and consequently to larvae, was very different in the three species (Fig. 2). The quantity and quality of energy allocated by maternal colonies to offspring may profoundly influence growth and survival of their progeny (Reznick 1991; Roff 1992). In general, SFA and MUFA are mainly used for basic metabolic processes, whereas PUFA are mainly devoted to growth and resistance to stress conditions, thus enhancing larval development and survival (Bell and Sargent 1996; Pond et al. 1996; Sargent et al. 1997; Albessard et al. 2001; Rossi et al. 2006). It has been suggested that PUFA content in larvae is related to the nutritional condition of the adults (García 2009), which may explain the same proportion of PUFA observed in *C. rubrum* colonies and their larvae. However, the results suggest that the transfer of energy to the larvae strategy might also depend on the reproductive effort of maternal colonies. Indeed, in *P. clavata,* the PUFA content in larvae is lower than in maternal colonies, probably due to the very high reproductive effort of this species (see Chapter 1), which prevents the transfer of a high amount of PUFA to each offspring. On the other hand, colonies of *E. singularis* seem to invest a large amount of energy in their larvae, since the PUFA content is higher in larvae than in maternal colonies.

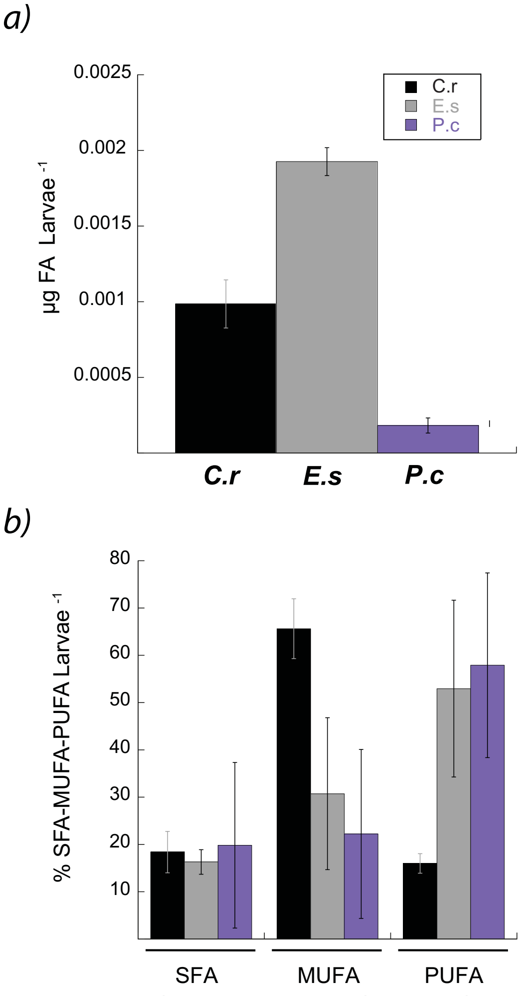


Fig. 4. Fatty acid content (a) (µg mg-1 larvae) and percentage of fatty acids functionality composition (SFA, MUFA, PUFA) (b) in *Corallium rubrum* larvae (black bars), *Eunicella singularis* larvae (grey bars), and *Paramuricea clavata* larvae (violet bars) (N = 3) (mean ± SD).

To better understand the implications and consequences of energy transferred from maternal colonies to larvae, the FA composition in maternal colonies and larvae was examined. In *C. rubrum* there were no differences in the main FA composition between maternal colonies and larvae, supporting the hypothesis that the feeding of the adults may be reflected in their larvae due to a non-selective transfer of the most conspicuous FA, as previously suggested for different marine organisms (Qian and Chia 1991; Harland et al. 1993; Dalsgaard et al. 2003; Figuereido et al. 2012). In fact, the 18:1(n-9) is the principal component of both larvae and maternal colonies of *C. rubrum* (Chapter 1), and it has been associated to detrital matter (Schultz and Quinn 1973; Fahl and Kettner 1993), which is the main food source for this species (Tsounis et al. 2006).

When the larvae of the other internal brooder species (*E. singularis*) were analysed, the results showed a high proportion of the FA 18:4(n-3), that is synthetized by symbiotic algae (Bachok et al. 2006; Imbs et al. 2007, 2009; see Chapter 2). This FA did not decrease in *E. singularis* maternal colonies after larval release and thus it is possible that the transfer of this FA is not due to direct allocation, but to a transfer of *Symbiodinium* from maternal colonies to larvae, as previously proposed by Weinberg (1979). Besides, it has been suggested that coral species characterized by one single clade of *Symbiodinium*, such as *E. singularis* (Forcioli et al. 2011), acquire their symbionts by direct transfer from maternal colonies, whereas species showing different clades tend to acquire symbiotic algae from the medium (Baker 2003). The possibility of a *Symbiodinium* infection from the maternal colony to the offspring is relevant with a view to understand the trophic capacity, as it has been demonstrated that *E. singularis* species, for example, may be facultative symbiotic depending on the depth and light conditions (Gori et al. 2012). It will be a key factor to understanding the successful distribution and recovery in front of short-term stressful conditions.

In the larvae of *P. clavata* FA 18:2(n-6) *trans* and *cis* were found, whereas only 18:2(n-6) *cis* was present in the maternal colonies, highlighting the active synthesis of this FA in the larvae. This could be an adaptive mechanism that allows larvae to cope with low presence of PUFA transferred by maternal colonies in this species (see below). Finally, the larvae of the three species showed a selective incorporation of 18:3(n-3), which is scarce in the mother colonies. It has been suggested that this FA could have beneficial effects for larval development, enhancing for example growth and settlement in bivalve larvae (Jonsson et al. 1999; da Costa et al. 2011).

Metabolic demands of the larvae: potential capacity of dispersal and successful settlement

The analysis of free FA content in the larvae is a measure of their energetic demands, since free FA are their principal source of energy to ensure ATP. Consequently, content and composition of free FA in larvae may directly determine their dispersal and recruitment capacities. The free FA contents observed in the studied larvae are in line with the activity frequency of the three species (i.e. percentage of time during which active swimming or crawling behaviour is displayed by larvae). The activity frequency is 81% in *E. singularis* (Viladrich et al. *in preparation*), 77% in *C. rubrum* (Martinez-Quintana et al. 2015), and much lower in *P. clavata* (Linares et al. 2008; N. Vi-

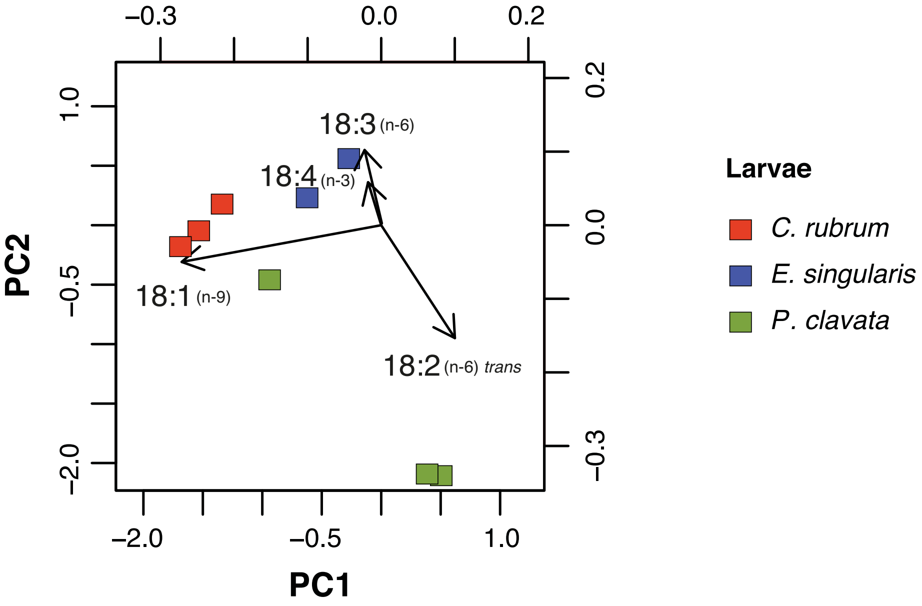


Fig. 5. Principal component analysis (PCA) biplot illustrating the ordering of the studied larvae with regard to their fatty acid composition, and the roles of the first four fatty acids classified according to the variance.

ladrich personal observation). Therefore, in view of these results, the comparison of free FA contents in coral larvae may be a good approximation of their activity rate. Knowledge of the activity rate is key to understanding the potential of larval dispersal and crawling behaviour, since a high activity frequency can be related to long dispersal and active search of substratum (Guizien et al. 2006; Martinez-Quintana et al. 2015). In this sense, the present results suggest that *E. singularis* larvae have the highest dispersal capacity and/or crawling behaviour, followed by larvae of *C. rubrum*, whereas *P. clavata* dispersal capacity should be lower, determining a settlement near the parental colonies. This agrees with recent studies on larval dispersal of *E. singularis* and *C. rubrum* that suggest a high dispersal potential in these species, with a high capacity of their larvae to escape from the bottom boundary layer (Martinez-Quintana et al. 2015; Viladrich et al. *in preparation*). Conversely, *P. clavata* larvae generally settle very close to the maternal colonies (Coma et al. 1995; Linares et al. 2008; Mokhtar-Jamai et al. 2011) possibly due to: (1) its reproductive strategy, i.e. surface brooding, which retains the eggs on the surface of the female bodies (Gutiérrez-Rodríguez and Lasker 2004), (2) the short swimming period of the larvae (Coma et al. 1995; Linares et al. 2008), and (3) the low energetic demands of the larvae, which could indicate a low motility capacity (present study).

High crawling capacity can be related to substratum selection behaviour, which could increase the recruitment rates (Sebens 1983; Mundy and Babcock 1998; Vermeij and Bak 2002). Studies on larvae have demonstrated that *E. singularis* planulae own a higher exploratory behaviour than *P. clavata* (Weinberg and Weinberg 1979; Linares et al. 2008). This agrees with the high activity frequency (Viladrich et al. *in preparation*) and energetic demands of *E. singularis* larvae (present study), and can possibly explain their high recruitment rates observed (Coma et al. 1995; Linares et al. 2008; Gori et al. 2011). In addition, it has been suggested that a deficiency in FA may delay growth and reduce survival (Izquierdo 1996; Copeman et al. 2002; Bransden et al. 2005), leading to high post-settlement mortality (Martinez and Abelson 2013). This fact could explain why *P. clavata* shows a reduced recruitment and slower growth of the primary polyp than *E. singularis* (Weinberg and Weinberg 1979; Ribes et al. 2007; Linares et al. 2008).

In view of the present results, the free FA content in larvae could be a useful proxy to understand the potential dispersal and successful settlement capabilities in different coral and sessile invertebrate species. The early-stage of larval dispersal and recruitment are virtually impossible to detect and quantify in situ for many corals, due to the microscopic size of larval and primary polyps (Miller 2014). Indirect tools are needed to better understand potential recruitment of next generations. In *C. rubrum*, for example, the settlement success is poorly known, since the recruitment rates are obtained one year after settlement (Garrabou and Harmelin 2002; Bramanti et al. 2003) and thus, it is impossible to detect the mortality during the early stage of recruitment (induced by larval stage) or during the juvenile stage.

A detailed study on the different FA composition in larvae from different species may also help to understand the mechanisms underlying different capacities of dispersal and recruitment between species. In *E. singularis*, for example, the high levels of 18:3(n-6) and 18:4(n-3) in larvae could indicate the presence of symbiotic algae (mentioned above), and thus a high capacity of dispersal and recruitment. Indeed, the presence of symbionts may supply a surplus energy to larvae (Richmond 1989) increasing their survival and competency (Ben-David-Zaslow and Benayahu 1998; Harii et al. 2010; Figuereido et al. 2012). On the other hand, the high variability of recruitment observed in *C. rubrum* (Garrabou and Harmelin 2002) could be related, among other things, to the low PUFA content of its larvae that makes them more vulnerable to stress conditions. In addition, the observed non-selective transfer of FA from maternal colonies to larvae in *C. rubrum* may result in a strong dependence of recruitment on the nutritional conditions of the populations (Lasker 1990; Yoshioka 1996; Dunstan and Johnson 1998), upon changes in fitness of adult colonies over time or among habitats (Brazeau and Lasker 1992; Yoshioka 1994; Weinbauer and Velimirov 1996).

Interestingly, some FA may be the key to understanding potential adaptation of new recruits after a stressful episode. Previous studies found that the recruitment rates after a mass mortality event is higher in *P. clavata* than in *C. rubrum* and *E. singularis* (Coma et al. 2006; Cupido et al. 2009; Santangelo et al. 2015). This difference has been attributed to the high reproductive output of *P. clavata*. However, it is possible that such high recruitment rates in *P. clavata* could also be favoured by the presence of 18:2(n-6) *trans*, which is involved in the maintenance of membrane fluidity under increased temperatures (Okuyama et al. 1990, 1991). It is worth noticing that an increase in 20:4(n-6) can also promote resistance to thermal stress (Sargent et al. 1999) but a high energetic input and a high proportion of PUFA are necessary, which can inhibit some secondary metabolic processes such as growth (Heipieper et al. 2003). Conversely, 18:2(n-6) *trans* can be obtained by direct isomerization of 18:2(n-6) *cis* (Diefenbach and Keweloh 1994), which is a reaction requiring low energy (Heipieper et al. 2003). This line of research is indeed relevant with a view to understand how different species cope with stress and how the new settlers (which are the pool needed to maintain a healthy population) may be or may not be successful.

CONCLUSIONS

This study revealed that the energy transferred from maternal colonies to larvae might depend on reproductive effort and the trophic constraints of maternal colonies. The energy used during larval development (i.e. free FA content), could be related to larval dispersal and recruitment capacities. Understanding the dispersal potential and substratum selection behaviour of coral larvae is critical for understanding their spatial distribution and population dynamics (Heck and McCoy 1978; Gerrodette 1981). Indeed, the wide distribution of *E. singularis* and *C. rubrum* with respect to *P. clavata* (Rossi et al. 2008; Linares et al. 2008; Gori et al. 2011; Angiolillo et al. *in press*) could be due to the high dispersal and recruitment capacity of their larvae, whereas the greater recovery rates after a mass mortality event of *P. clavata* with respect to *C. rubrum* and *E. singularis* (Coma et al. 2006; Cupido et al. 2009; Santangelo et al. 2015), could be partly due to their high reproductive output (Santangelo et al. 2015) and an adaptation mechanism of the larvae to environmental stress. These results might then suggest that *P. clavata* strategy is mainly focussed on resilience, linked to the recovery capacity after mortality events.

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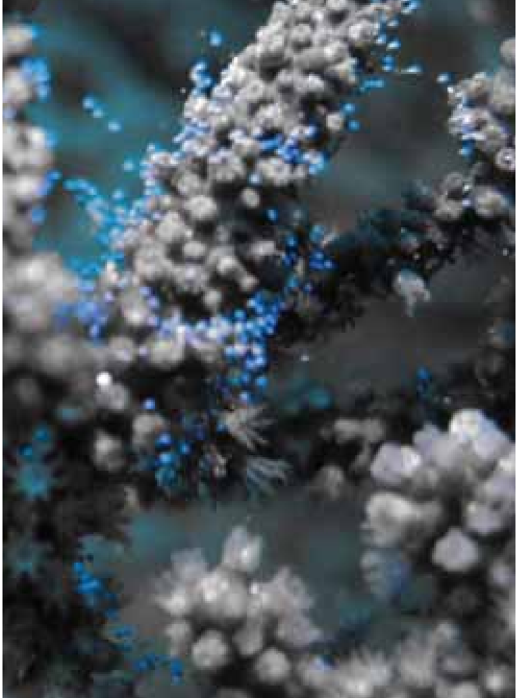
General Discussion



GENERAL DISCUSSION

The present thesis focuses on the energetic investment in reproduction, the effects of this investment on parental colonies and energy transfer to larvae in three of the most abundant gorgonian species in the coastal areas of the Mediterranean Sea (*Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata*). It is important to emphasize that these three Mediterranean gorgonians represent study cases that can be easily extrapolated to other environments, as the three species are representative of different trophic and reproductive strategies.

In order to study the effects of the reproductive process, the gamete production (number, size and volume of oocytes and spermaries) was quantified in the three species, and the quantity of organic matter, lipid content, and free fatty acid (FA) content and composition were measured in their coenenchyma before and after spawning. Moreover, for the purpose of also taking into account the temporal variability of the main environmental features of the water column, an environmental characterization of the study area was carried out during the studied period (2010–2011). The free FA content and composition in larvae and parental colonies were compared, with the aim of understanding the investment of parental colonies on the offspring. Finally, the quantification of the FA content and composition in larvae is proposed as a new approach in order to understand their activity rates and to gain an insight into their dispersal and recruitment capability, since free FA are related to the main metabolic needs of an individual. In this section, the main results obtained in the present study are summarized, and the links among chapters highlighted.

The energetic cost of reproduction according to the reproductive and trophic strategy

In order to study the energetic cost of reproduction according to the reproductive and trophic strategy, the lipid content in parental colonies before and after reproduction was quantified, since these macromolecules are used as the most efficient energy source in most of the animal taxa (Lehninger 1982). The reduction in lipid content after spawning was more severe in *P. clavata* than in *C. rubrum* and *E. singularis* (Chapter 1 and 2), suggesting that the amount of energy invested in reproduction could be related to the reproductive output, being closely linked to the reproductive strategy. On the other hand, the trophic strategy may influence nutritional conditions of colonies that, at the same time, may determine the reproductive output (Strathmann 1985; Simpson 2009; Gori et al. 2013; Chapter 2). However, despite the increase of the reproductive output according to nutritional conditions, the lipid content may not decrease after spawning. This fact may suggest that the trophic strategy can influence the offspring survival, but the reproductive strategy may be essential for understanding the amount of energy invested and thus, the survival of parental colonies.

Energy investment in reproduction according to colony sex

It has been generally assumed that the reproductive investment is higher in female than in male colonies, since oocytes are mainly composed of lipids (60–70% dry weight, Arai et al. 1993), whereas spermatic sacs are made up of proteins (Ferguson 1975). However, the results showed that, although male colonies have a faster recovery than female ones (Chapter 2), the main part of the energetic cost related to reproduction is due to the reproductive activity (i.e. gametogenesis and spawning), and not to the direct transfer of lipid reserves from maternal colonies to oocytes (Chapter 1).

Energetic demands and requirements during and after reproduction

In order to study the energetic demands and requirements linked to the reproductive process, the free FA content and composition in parental colonies before and after reproduction were quantified. The FA represent a source of immediate/fast energy with high power efficiency (high ATP/FA molecule), and thus its content is proportional to the metabolic demand of the organism (Sargent et al. 1988). In addition, free FA compounds may also reflect the nature of metabolic demands, because they can be synthesized by complex metabolic reactions to satisfy the specific demands (Díaz-Almeyda et al. 2011; Imbs 2013; Viladrich et al. 2015). Therefore, knowledge regarding the FA composition may give information on the metabolic changes related to the reproductive activity and/or starvation period in the studied species.

Results about the FA composition confirmed the high energetic investment of *P. clavata* colonies in the reproductive activity (Chapter 1), since the main FA components observed before the spawning have been related to the increase of fecundity, fertility and oocyte quality (Fernández et al. 1995; Izquierdo 2001). Conversely, internal brooder species, characterized by a lower energetic investment in reproduction, could invest more in growth (case of *C. rubrum*; Chapter 1) or in the maintenance of the trophic plasticity (case of *E. singularis*; Chapter 2) than in reproduction, suggesting that life history strategies are mainly oriented to the maintenance of adult colonies.

In general, reproductive mechanisms entail an energetic cost (Calow 1979), which may involve that after spawning, mature organisms are more vulnerable to stress conditions, such as exposure to pathogens, starvation or thermal stress (Bayne et al. 1975; Bayne et al. 1978). The increase in FA content after spawning observed in *C. rubrum* and *P. clavata* is probably a mechanism to overcome this reproductive stress, coupled to the elevated temperature and low food availability that characterize the Mediterranean summer (Coma et al. 2000). Indeed, both species increased their 20:4(n-6) content, which has been related to the production of biologically active eicosanoids under stress or unfavourable conditions (Sargent et al. 1999), since they support immune system functioning and osmoregulation (Chapelle 1986; Mazorra et al. 2003). However, the results suggest that the influence of environmental conditions on the parental colonies is probably related to the reproductive energetic cost, and thus to the reproductive strategy (Chapter 1). On the other hand, mixotrophic species can probably buffer the effects of environmental stress (Chapter 2), due to the good nutritional condition and trophic plasticity (Grottoli et al. 2006). In fact, this trophic plasticity is highlighted by the inter-annual variability observed in the FA composition of *E. singularis*, which indicates a preponderant contribution of the autotrophic input in 2010 compared to the predominance of heterotrophic feeding in 2011, according to environmental conditions (Chapter 2).

Fatty acid allocation from maternal colonies to the larvae

The quantity and quality of energy allocated by maternal colonies to the offspring may profoundly influence the growth and survival of their progeny (Reznick 1991; Roff 1992). In general, SFA and MUFA are mainly used for basic metabolic processes, whereas PUFA are mainly devoted to growth and resistance to stress conditions, thus enhancing larval development and survival (Bell and Sargent 1996; Pond et al. 1996; Sargent et al. 1997; Albessard et al. 2001; Rossi et al. 2006). It has been suggested that in larvae, the PUFA content, as well as FA components are related to the nutritional condition of adults (García 2009). However, the present results suggest that the quality of energy transferred to the larvae might also depend on the reproductive effort or on the direct transfer of symbiotic algae (Chapter 3).

Metabolic demands of the larvae: potential capacity of dispersal and successful settlement

In chapter 3 of the present thesis, the study of the FA content and composition was proposed as a new approach in order to understand the dispersal and recruitment capability of species, since free FA are related to the metabolic needs of an organism (Sargent et al. 1988). The present results are in line with previous literature, suggesting that *E. singularis* larvae have the highest capacity of dispersal and/or crawling behaviour compared to the other two species (Weinberg and Weinberg 1979; Viladrich et al. *in preparation*). The energy allocation and the low metabolic demands of *P. clavata* larvae suggest that the settlement should be close to the parental colonies, as previously reported (Coma et al. 1995; Linares et al. 2008; Mokhtar-Jamai et al. 2011). On the other hand, the results also suggest that the higher recruitment rates after mass mortality event of *P. clavata* with respect to *C. rubrum* and *E. singularis* (Coma et al. 2006; Cupido et al. 2009; Santangelo et al. 2015), could be favoured by the high reproductive output of this species (Santangelo et al.

2015), but also by an adaptation mechanism of the larvae to overcome environmental stress. Finally, the high variability of recruitment observed in *C. rubrum* (Garrabou and Harmelin 2002) could be related, among other things, to the low PUFA content of the larvae and to the non-selective transfer of FA from maternal colonies which makes them more vulnerable to stress conditions and dependent upon the temporal variability in the main environmental conditions (Lasker 1990; Yoshioka 1996; Dunstan and Johnson 1998). The different FA composition in larvae of each species may suggest a species-specific energetic strategy among the studied species, which may influence settlement and recovery rates in front of environmental impacts.

CONCLUDING REMARKS AND FURTHER PROGRESS

This thesis revealed that the species with high energetic investment in reproduction might be more sensitive to environmental conditions. However, these species are normally characterized by a high fecundity that may result in high recruitment. Therefore, these species show a life history strategy more focused on maximizing colonization processes. Conversely, the life history strategy of species with low investment in reproduction is mainly oriented to maximizing the survival of adult colonies in front of adverse environmental conditions. Finally, a high trophic plasticity may increase the resistance of adult organisms to stress conditions and, at the same time, increase offspring survival. This may suggest that species with trophic plasticity may be considered both resistant and resilient.

In the present thesis, classical methods were combined with new biochemical approaches to better understand how the energetic cost of reproduction influences both larvae and parental colonies. However, this work has also generated many new questions to be addressed in future studies. Some of the questions that remain currently unresolved, as well as a few aspects that could enhance our understanding about gorgonian ecology are:

Considering that nutritional condition of animals depends on the trophic resources available, it would be important to study how the energetic cost of reproduction may change according to different populations (spatial comparison) in order to better understand their potential success depending on the place in which they settle and grow.

The implications of sensitivity to environmental conditions in the light of global climate change affecting shallow waters (above 40 meters depth) need further evaluation, as it could finally result in increased fitness of mixotrophic compared to heterotrophic species.

However, one of the key points that need further research is to understand whether the trophic plasticity of mixotrophic species depends on depth. This is particularly important because the mesophotic populations of these species may constitute a deep refuge and play an important role in the recovery of shallow populations, as their populations are more abundant and less affected by environmental stressful conditions.

Another point that needs further research is the clarification of the role of 16:0 in the calcification process, as the amount of this FA could be related to the calcification rates of corals with hard skeleton, and could be the key to understanding the spatial variability in growth rates observed among populations.

To better understand the larval dispersal and recruitment capacities, it would be useful to study the amount of energy consumed and the metabolic demands during the larval stage. This point will be essential for studying the relationship of the available energy with the activity of the larvae and the potential capability to overcome the first settlement stages.

Although scientific research often leads to fewer answers than questions, the results of this thesis may serve as an impulse for future studies focused on the reproductive process of different organisms.

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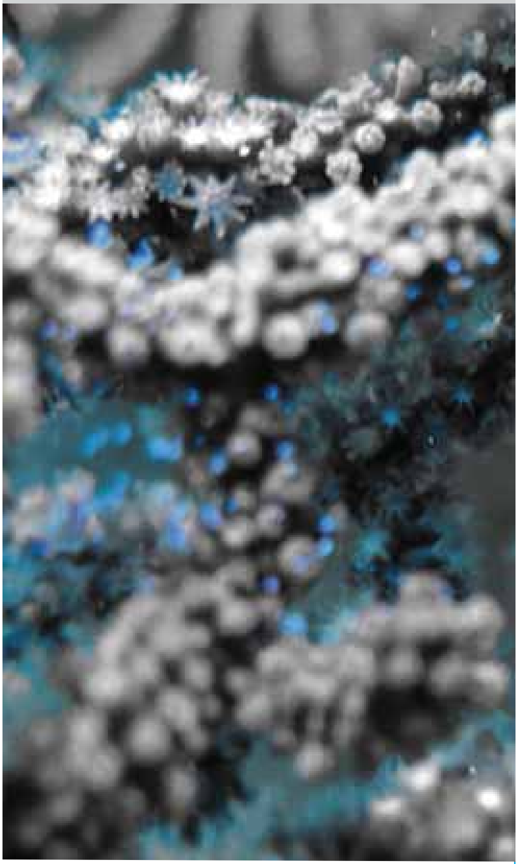
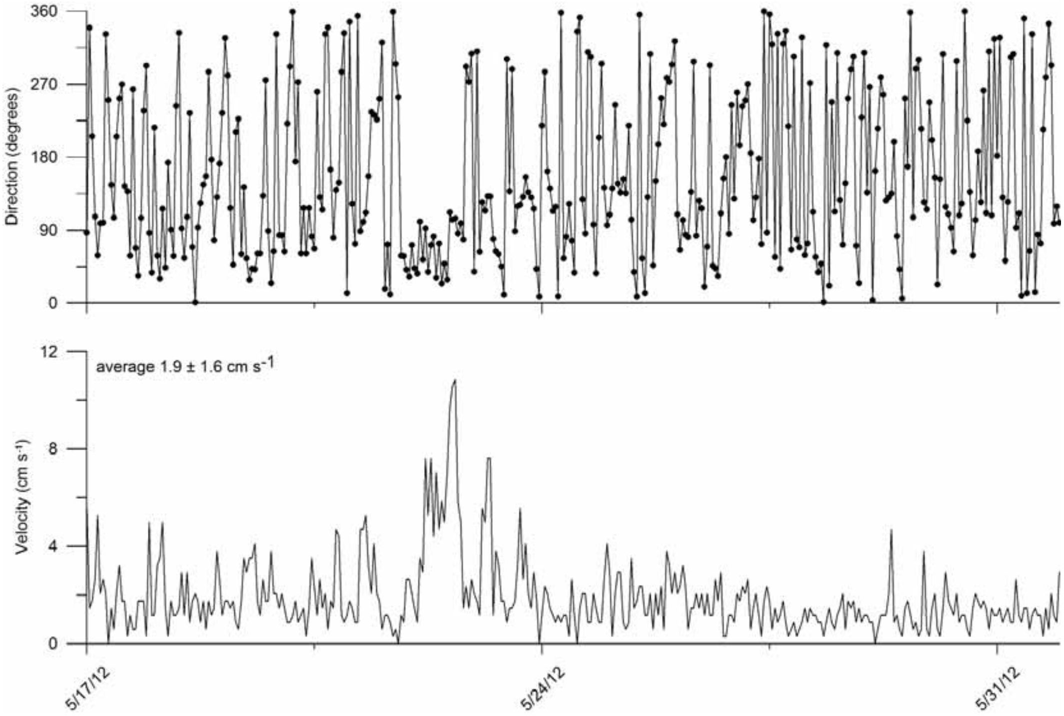
Annex 1

Currents



VILADRICH N (2015) Doctoral Thesis pp 97-102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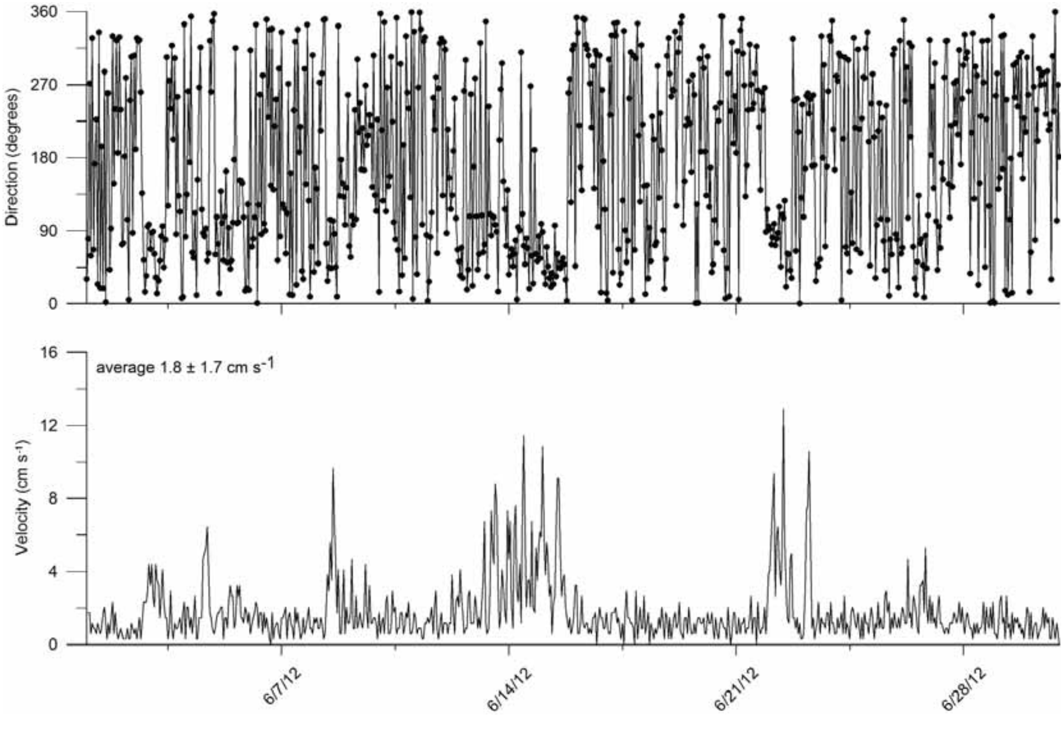
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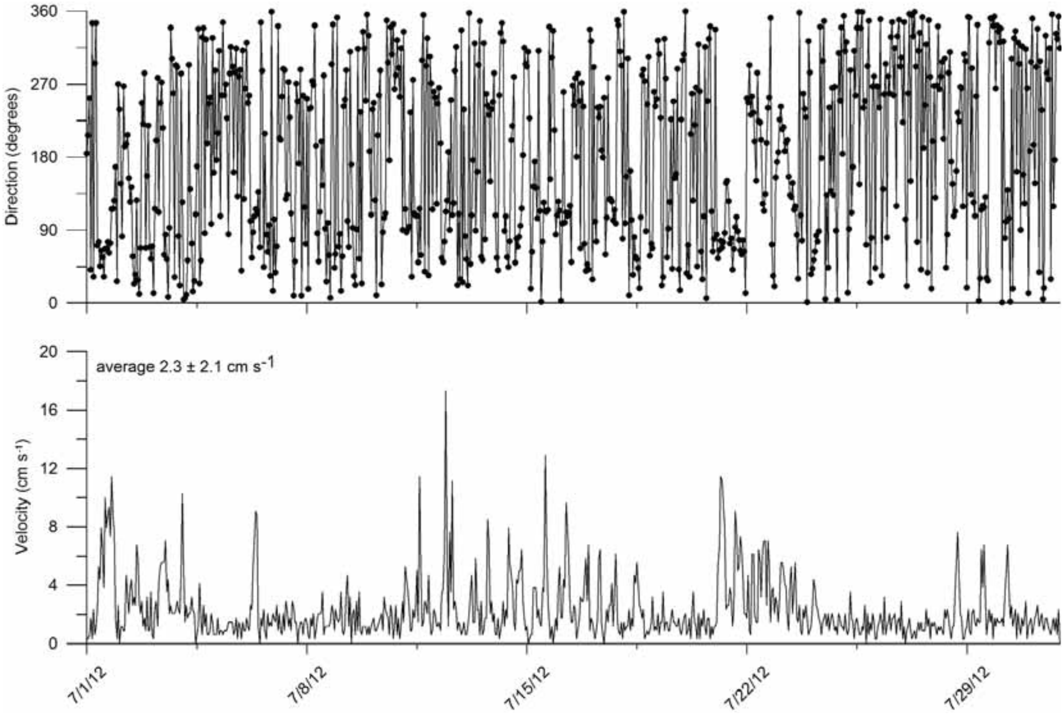
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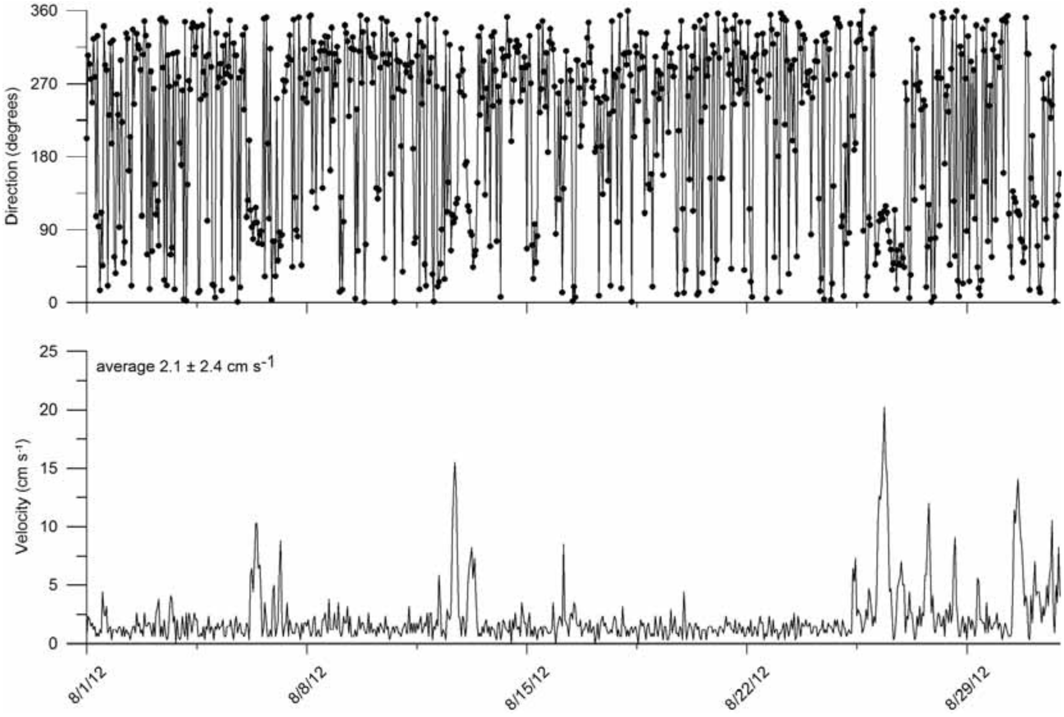
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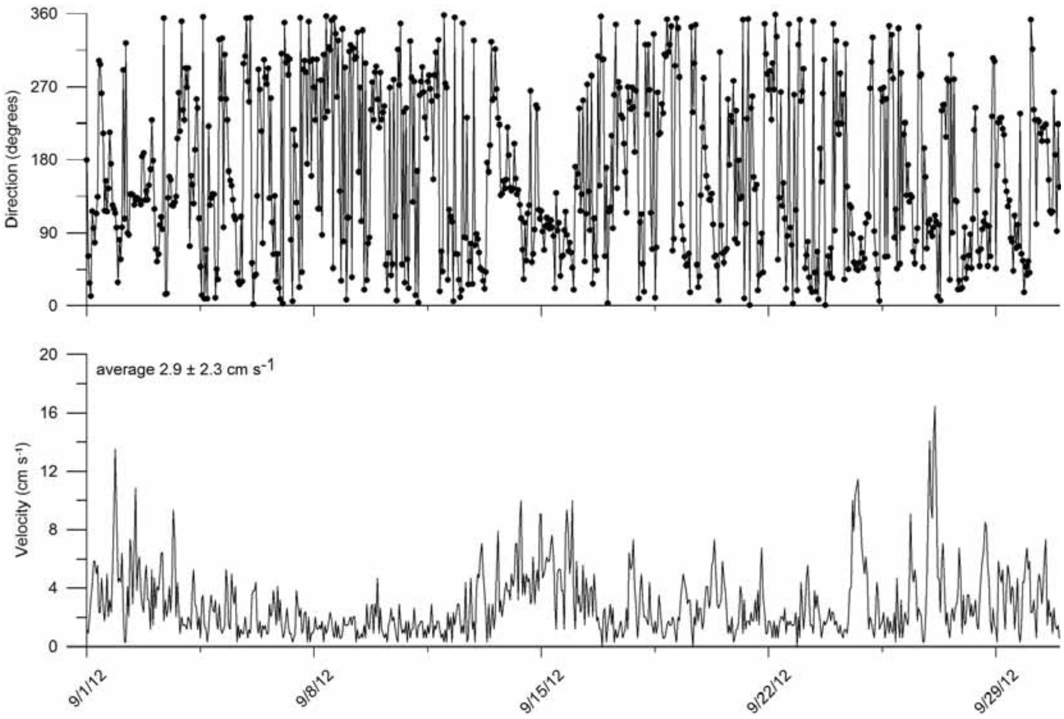
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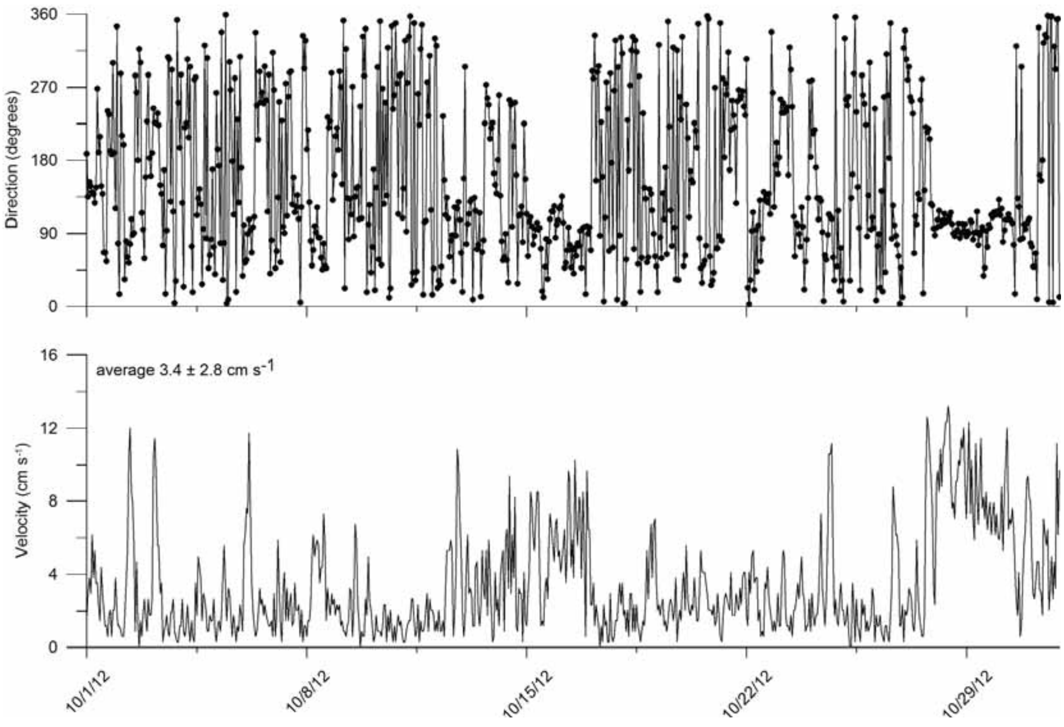
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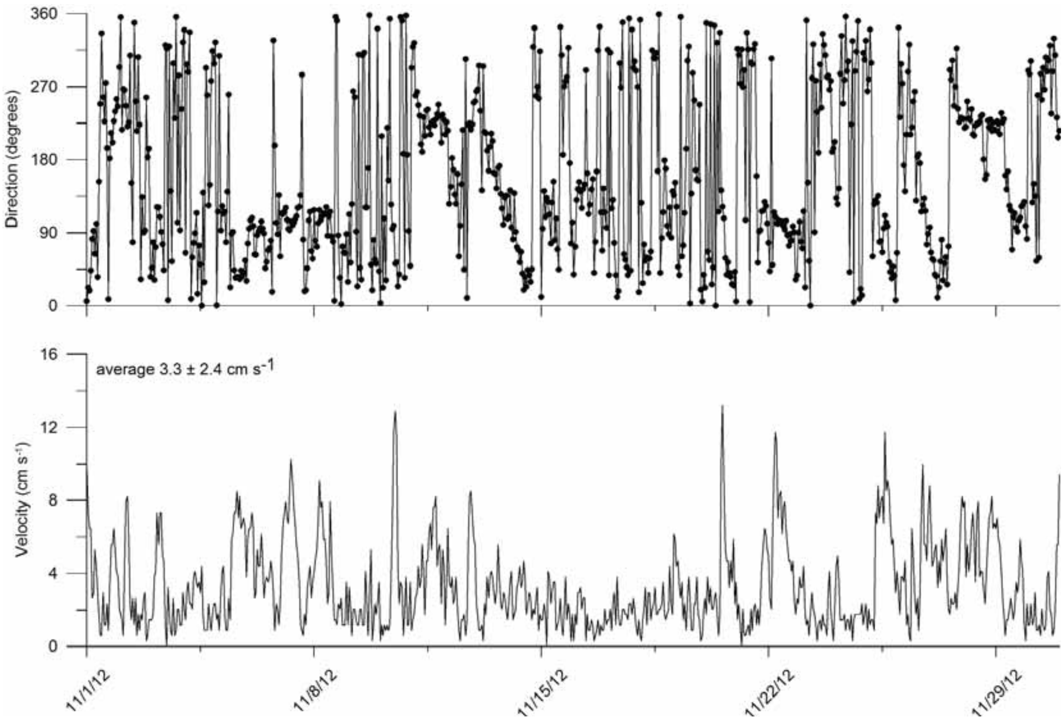
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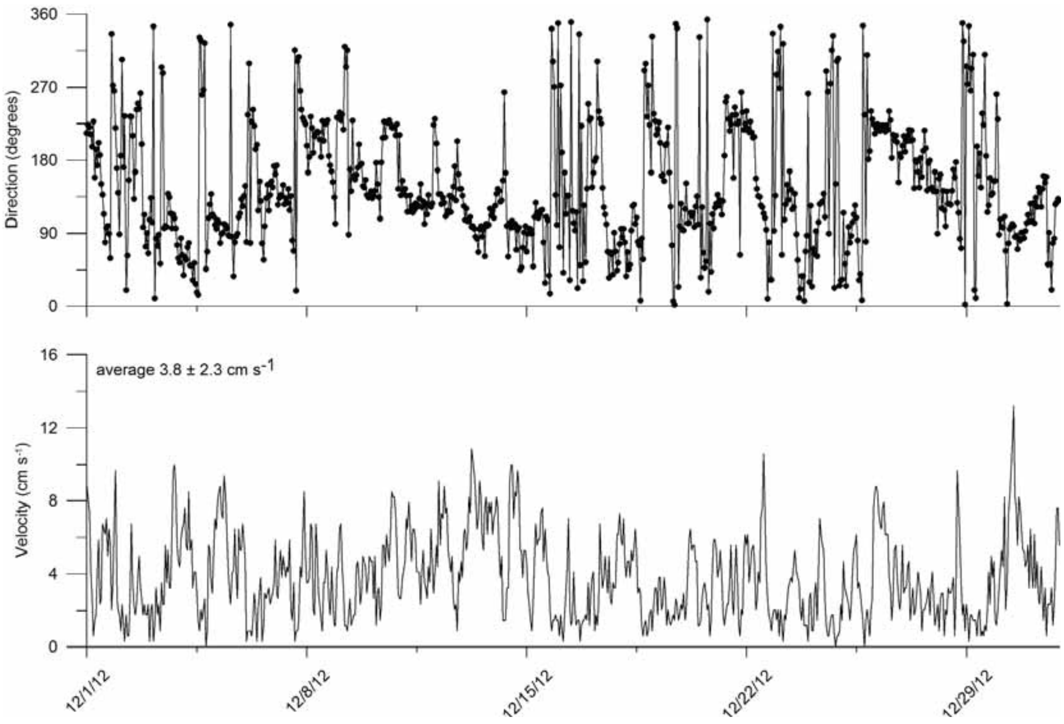
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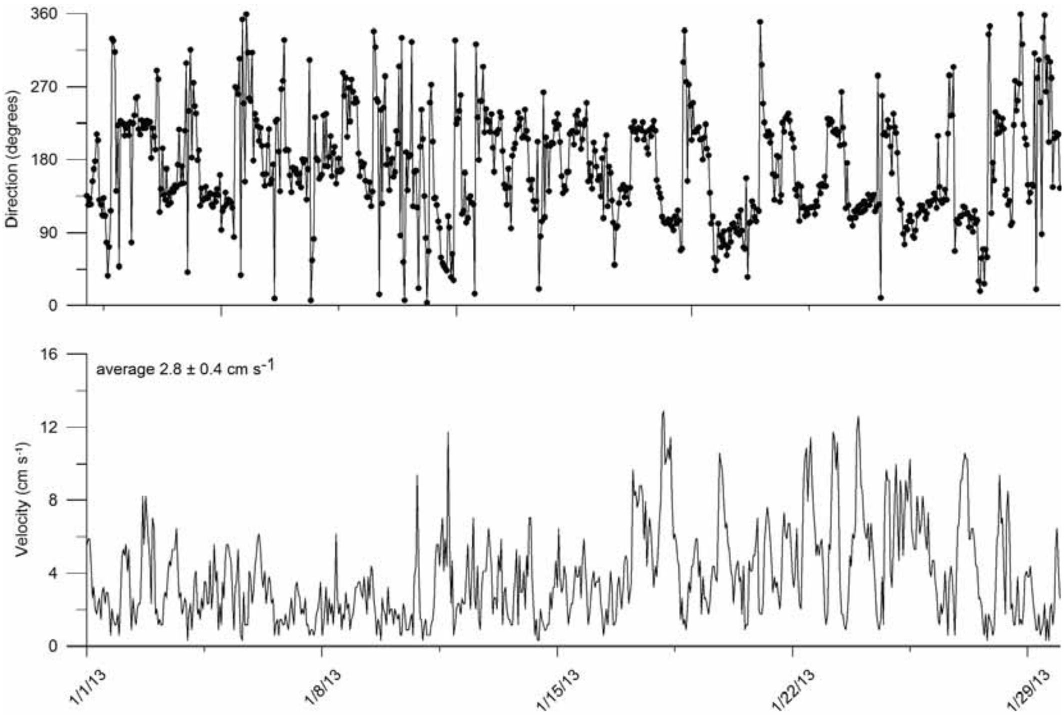
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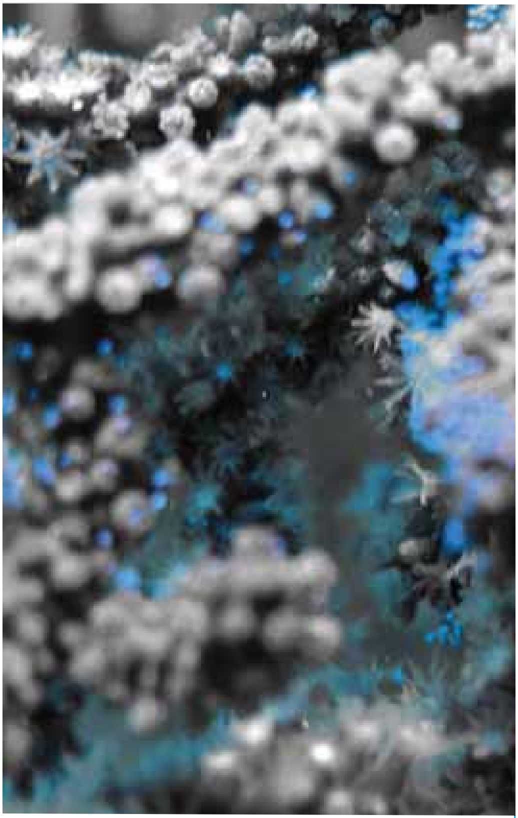
Annex 2

Tables Chapter 1



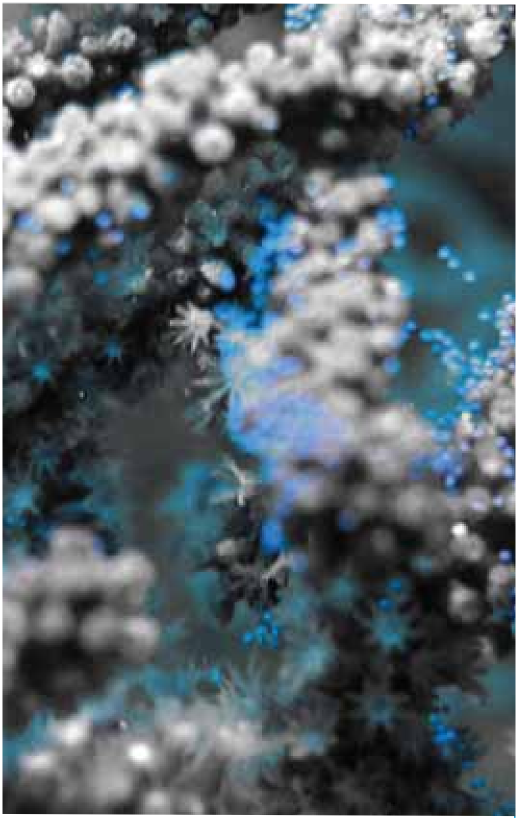
VILADRICH N (2015) Doctoral Thesis pp 103-105

Corallium rubrum	2010								2011							
	Before				After				Before				After			
	Mascles		Femelles		Mascles		Femelles		Mascles		Femelles		Mascles		Femelles	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
C12:0	0,38	0,14	0,46	0,07	0,26	0,04	0,20	0,02	0,07	0,11	0,16	0,14	0,24	0,22	0,31	0,00
C13:0	0,00	0,00	0,00	0,00	0,00	0,00	0,11	0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C14:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C14:0	3,78	0,37	3,94	0,51	2,35	0,32	2,54	0,20	3,58	0,34	3,31	0,61	2,00	0,18	1,51	0,14
C15:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C15:0	1,67	0,21	1,59	0,33	0,85	0,16	0,98	0,08	1,18	0,12	1,17	0,13	0,66	0,15	0,60	0,07
C16:1(n-7)	2,79	0,48	2,95	1,22	3,23	0,36	2,82	0,01	5,09	1,15	5,66	0,93	2,41	0,43	1,71	0,20
C16:0	31,27	1,16	31,22	2,14	17,96	2,39	19,84	3,30	29,19	1,98	26,17	1,68	18,92	0,82	15,92	2,62
C17:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	2,34	0,41	2,28	0,26	1,39	0,26	1,60	0,28	1,77	0,15	1,66	0,15	0,82	0,71	1,11	0,21
C18:3(n-6)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:4(n-3)	0,72	1,02	1,97	1,08	0,00	0,00	0,00	0,00	0,87	0,77	1,24	0,39	0,00	0,00	0,00	0,00
C18:2(n-6) cis	2,44	0,53	2,32	0,98	2,52	0,34	1,50	0,99	2,68	0,25	2,62	0,04	2,83	1,53	2,82	0,26
C18:3(n-3)	4,31	6,09	0,00	0,00	0,00	0,00	0,47	0,67	0,00	0,00	0,33	0,29	0,39	0,68	0,00	0,00
C18:1(n-9)	24,82	0,82	25,30	2,46	21,89	4,05	22,06	5,10	27,50	3,64	32,45	1,66	20,31	1,16	18,72	2,24
C18:2(n-6) trans	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	9,54	1,18	10,44	0,89	7,11	1,13	9,76	4,72	9,91	1,32	8,26	0,98	8,10	0,66	7,01	1,28
C20:4(n-6)	3,40	1,52	4,43	1,23	26,59	5,31	22,95	11,75	4,34	1,12	3,66	0,48	24,72	2,15	32,76	6,97
C20:5(n-3)	0,73	1,03	0,82	1,43	2,29	0,75	2,23	0,67	2,44	0,46	2,27	0,38	2,28	0,45	2,18	0,22
C20:3(n-6)	2,18	0,59	2,31	0,09	2,64	0,32	2,36	0,21	2,05	0,43	1,95	0,46	3,89	0,60	3,20	0,40
C20:4(n-3)	0,00	0,00	0,00	0,00	0,15	0,26	0,22	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C20:2(n-6)	0,95	0,44	1,05	0,09	1,00	0,03	0,86	0,03	0,91	0,21	0,79	0,23	1,35	0,25	1,20	0,13
C20:1(n-9)	1,66	0,38	1,64	0,21	1,32	0,12	1,05	0,01	1,72	0,35	2,20	0,55	1,64	0,24	1,47	0,10
C20:0	0,23	0,00	0,25	0,04	0,22	0,03	0,19	0,03	0,24	0,01	0,15	0,14	0,22	0,02	0,23	0,05
C21:0	0,53	0,20	0,52	0,04	0,14	0,25	0,12	0,17	0,42	0,73	0,35	0,60	0,11	0,19	0,41	0,07
C22:5	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C22:6(n-3)	0,00	0,00	0,00	0,00	2,16	1,87	2,62	0,58	0,09	0,15	0,10	0,18	3,02	0,40	2,67	0,41
C22:4	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C22:5(n-x)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C22:2(n-6)	1,82	0,63	1,62	0,93	1,07	0,96	0,98	0,78	1,27	0,28	0,97	0,59	0,00	0,00	0,20	0,35
C22:1(n-9)	0,00	0,00	0,51	0,88	0,36	0,63	0,43	0,60	0,00	0,00	0,29	0,51	1,22	0,36	0,92	0,29
C22:0	0,56	0,13	0,62	0,06	0,35	0,04	0,72	0,03	0,46	0,10	0,35	0,09	0,49	0,16	0,55	0,20
C23:0	0,58	0,23	0,56	0,06	0,61	0,42	0,28	0,09	0,40	0,09	0,17	0,15	0,54	0,18	0,48	0,13
C24:PUFA	0,05	0,08	0,04	0,07	1,14	0,77	1,28	0,96	0,24	0,03	0,22	0,19	0,99	0,38	1,55	0,51
C24:1(n-9)	2,02	0,00	1,86	0,19	1,58	0,36	1,14	0,04	2,58	0,54	2,69	0,59	1,68	0,14	1,48	0,11
C24:0	1,23	0,49	1,30	0,15	0,79	0,11	0,68	0,24	1,01	0,25	0,78	0,23	1,17	0,43	0,98	0,29
SFA	52,11	3,37	53,18	3,51	32,04	4,59	37,02	8,91	48,22	3,62	42,54	1,58	33,27	1,64	29,11	4,91
MUFA	33,10	0,29	33,38	3,06	29,09	4,80	28,06	5,84	38,17	5,14	43,98	1,87	26,04	0,95	23,60	2,40
PUFA	14,79	3,09	13,44	1,91	38,87	8,76	34,92	14,75	13,61	1,81	13,48	0,36	40,69	2,57	47,29	7,03

Paramuricea clavata	2010								2011							
	Before				After				Before				After			
	Mascles		Femelles		Mascles		Femelles		Mascles		Femelles		Mascles		Femelles	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
C12:0	0,07	0,01	0,04	0,05	0,05	0,04	0,05	0,08	0,05	0,07	0,11	0,02	0,12	0,03	0,11	0,05
C13:0	0,03	0,04	0,00	0,00	0,00	0,00	0,00	0,00	0,02	0,03	0,01	0,02	0,02	0,04	0,04	0,01
C14:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,12	0,21	0,07	0,04
C14:0	1,80	0,15	1,05	0,15	1,28	0,27	1,32	0,44	1,39	1,96	1,78	0,17	1,92	0,42	2,01	0,06
C15:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C15:0	0,56	0,03	0,29	0,03	0,59	0,10	0,58	0,14	0,34	0,48	0,43	0,05	0,79	0,19	0,82	0,14
C16:1(n-7)	2,79	0,16	1,68	0,20	2,10	0,37	2,31	0,25	2,27	2,40	3,19	0,69	2,08	0,36	2,06	0,50
C16:0	25,92	0,79	19,90	3,52	17,72	1,55	17,41	0,20	15,68	1,91	11,83	1,39	14,65	1,75	14,36	2,53
C17:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	1,31	0,34	0,67	0,05	1,47	0,10	1,65	0,14	1,02	0,13	0,82	0,11	1,30	0,19	1,33	0,22
C18:3(n-6)	0,10	0,15	0,00	0,00	0,00	0,00	0,00	0,00	1,50	1,76	0,31	0,03	0,00	0,00	0,00	0,00
C18:4(n-3)	0,45	0,26	0,47	0,10	0,08	0,13	0,08	0,07	2,18	0,31	0,77	0,26	0,20	0,06	0,24	0,10
C18:2(n-6) cis	1,84	0,07	1,80	0,12	1,83	0,15	1,33	1,16	0,78	0,10	2,48	0,39	2,78	1,10	3,15	0,40
C18:3(n-3)	0,49	0,17	0,55	0,05	0,27	0,07	0,47	0,36	0,96	0,07	0,72	0,13	0,44	0,38	0,66	0,08
C18:1(n-9)	11,05	1,69	8,50	0,55	11,29	1,04	11,77	0,65	6,72	0,98	7,32	0,87	4,26	3,60	2,05	0,23
C18:2(n-6) trans	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	4,41	5,31	0,91	0,15	0,00	0,00	0,00	0,00
C18:0	14,75	5,19	11,51	2,04	10,02	1,78	11,68	1,91	6,09	0,59	5,60	1,13	7,22	0,67	6,94	0,76
C20:4(n-6)	19,97	4,00	31,10	4,61	36,62	3,30	31,34	2,00	20,98	0,04	26,62	3,29	34,90	9,13	36,52	13,69
C20:5(n-3)	8,59	3,96	9,13	1,24	3,32	0,60	3,20	0,55	12,96	1,76	10,30	0,61	7,52	3,60	7,15	4,52
C20:3(n-6)	0,73	0,12	1,20	0,16	0,80	0,11	1,15	0,40	0,98	0,26	1,71	0,52	0,86	0,12	0,75	0,06
C20:4(n-3)	0,40	0,02	0,80	0,14	0,00	0,00	0,00	0,00	0,55	0,09	1,18	0,62	0,00	0,00	0,00	0,00
C20:2(n-6)	0,38	0,12	0,43	0,02	0,43	0,05	0,66	0,22	0,19	0,03	0,39	0,06	0,40	0,03	0,38	0,00
C20:1(n-9)	0,42	0,09	0,56	0,07	0,52	0,12	0,75	0,33	0,77	0,15	1,34	0,37	0,44	0,03	0,39	0,02
C20:0	0,15	0,02	0,15	0,03	0,20	0,02	0,24	0,03	0,07	0,01	0,08	0,01	0,17	0,03	0,17	0,02
C21:0	0,47	0,56	1,16	0,09	0,26	0,02	0,36	0,07	0,09	0,03	0,27	0,14	0,29	0,08	0,26	0,01
C22:5	0,84	1,19	0,00	0,00	0,12	0,12	0,18	0,04	0,39	0,05	0,48	0,15	0,52	0,13	0,54	0,11
C22:6(n-3)	1,39	0,86	2,51	0,18	3,60	1,14	3,23	0,57	9,96	1,19	8,80	2,26	7,62	2,40	8,93	2,47
C22:4	0,10	0,02	0,12	0,03	0,00	0,00	0,21	0,04	0,15	0,01	0,24	0,07	0,32	0,02	0,34	0,00
C22:5(n-x)	0,00	0,00	0,09	0,11	0,00	0,00	0,05	0,04	0,18	0,00	0,24	0,21	0,12	0,10	0,13	0,18
C22:2(n-6)	0,39	0,03	0,60	0,10	0,44	0,38	0,55	0,43	0,17	0,13	0,24	0,19	0,33	0,13	0,32	0,24
C22:1(n-9)	0,23	0,07	0,28	0,04	0,42	0,01	0,58	0,15	0,23	0,16	0,51	0,09	0,48	0,15	0,39	0,02
C22:0	0,15	0,00	0,19	0,07	0,28	0,02	0,36	0,10	0,08	0,00	0,13	0,03	0,25	0,03	0,23	0,00
C23:0	0,16	0,02	0,22	0,06	0,24	0,03	0,34	0,11	0,07	0,01	0,13	0,02	0,23	0,02	0,19	0,00
C24:PUFA	3,17	0,65	3,69	0,41	4,22	3,76	5,83	0,74	7,29	0,20	9,37	1,64	8,04	1,64	7,98	1,47
C24:1(n-9)	0,99	0,25	0,92	0,14	1,32	0,10	1,61	0,14	1,27	0,16	1,38	0,38	1,10	0,23	1,09	0,21
C24:0	0,31	0,07	0,41	0,07	0,53	0,06	0,73	0,29	0,17	0,01	0,30	0,06	0,52	0,05	0,40	0,04
SFA	45,68	5,56	35,59	5,95	32,61	1,01	34,70	2,14	25,07	5,23	21,50	2,81	27,49	2,94	26,87	3,64
MUFA	15,63	2,23	12,26	0,75	15,67	1,38	16,99	0,97	15,62	1,76	14,38	0,88	8,32	3,06	5,97	0,68
PUFA	38,69	7,78	52,16	5,72	51,72	1,37	48,31	2,88	59,30	3,47	64,13	2,16	64,19	0,32	67,16	4,32

Annex 3

Tables Chapter 2



VILADRICH N (2015) Doctoral Thesis pp 107-109

Corallium	2010								2011							
	Before				After				Before				After			
	Mascles		Femelles		Mascles		Femelles		Mascles		Femelles		Mascles		Femelles	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
C12:0	0,38	0,14	0,46	0,07	0,26	0,04	0,20	0,02	0,07	0,11	0,16	0,14	0,24	0,22	0,31	0,00
C13:0	0,00	0,00	0,00	0,00	0,00	0,00	0,11	0,15	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C14:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C14:0	3,78	0,37	3,94	0,51	2,35	0,32	2,54	0,20	3,58	0,34	3,31	0,61	2,00	0,18	1,51	0,14
C15:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C15:0	1,67	0,21	1,59	0,33	0,85	0,16	0,98	0,08	1,18	0,12	1,17	0,13	0,66	0,15	0,60	0,07
C16:1(n-7)	2,79	0,48	2,95	1,22	3,23	0,36	2,82	0,01	5,09	1,15	5,66	0,93	2,41	0,43	1,71	0,20
C16:0	31,27	1,16	31,22	2,14	17,96	2,39	19,84	3,30	29,19	1,98	26,17	1,68	18,92	0,82	15,92	2,62
C17:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	2,34	0,41	2,28	0,26	1,39	0,26	1,60	0,28	1,77	0,15	1,66	0,15	0,82	0,71	1,11	0,21
C18:3(n-6)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:4(n-3)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:2(n-6) cis	2,44	0,53	2,32	0,98	2,52	0,34	1,50	0,99	2,68	0,25	2,62	0,04	2,83	1,53	2,82	0,26
C18:3(n-3)	4,31	6,09	0,00	0,00	0,00	0,00	0,47	0,67	0,00	0,00	0,33	0,29	0,39	0,68	0,00	0,00
C18:1(n-9)	24,82	0,82	25,30	2,46	21,89	4,05	22,06	5,10	27,50	3,64	32,45	1,66	20,31	1,16	18,72	2,24
C18:2(n-6) trans	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	9,54	1,18	10,44	0,89	7,11	1,13	9,76	4,72	9,91	1,32	8,26	0,98	8,10	0,66	7,01	1,28
C20:4(n-6)	3,40	1,52	4,43	1,23	26,59	5,31	22,95	11,75	4,34	1,12	3,66	0,48	24,72	2,15	32,76	6,97
C20:5(n-3)	0,73	1,03	0,82	1,43	2,29	0,75	2,23	0,67	2,44	0,46	2,27	0,38	2,28	0,45	2,18	0,22
C20:3(n-6)	2,18	0,59	2,31	0,09	2,64	0,32	2,36	0,21	2,05	0,43	1,95	0,46	3,89	0,60	3,20	0,40
C20:4(n-3)	0,00	0,00	0,00	0,00	0,15	0,26	0,22	0,31	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C20:2(n-6)	0,95	0,44	1,05	0,09	1,00	0,03	0,86	0,03	0,91	0,21	0,79	0,23	1,35	0,25	1,20	0,13
C20:1(n-9)	1,66	0,38	1,64	0,21	1,32	0,12	1,05	0,01	1,72	0,35	2,20	0,55	1,64	0,24	1,47	0,10
C20:0	0,23	0,00	0,25	0,04	0,22	0,03	0,19	0,03	0,24	0,01	0,15	0,14	0,22	0,02	0,23	0,05
C21:0	0,53	0,20	0,52	0,04	0,14	0,25	0,12	0,17	0,42	0,73	0,35	0,60	0,11	0,19	0,41	0,07
C22:5	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C22:6(n-3)	0,00	0,00	0,00	0,00	2,16	1,87	2,62	0,58	0,09	0,15	0,10	0,18	3,02	0,40	2,67	0,41
C22:4	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C22:5(n-x)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C22:2(n-6)	1,82	0,63	1,62	0,93	1,07	0,96	0,98	0,78	1,27	0,28	0,97	0,59	0,00	0,00	0,20	0,35
C22:1(n-9)	0,00	0,00	0,51	0,88	0,36	0,63	0,43	0,60	0,00	0,00	0,29	0,51	1,22	0,36	0,92	0,29
C22:0	0,56	0,13	0,62	0,06	0,35	0,04	0,72	0,03	0,46	0,10	0,35	0,09	0,49	0,16	0,55	0,20
C23:0	0,58	0,23	0,56	0,06	0,61	0,42	0,28	0,09	0,40	0,09	0,17	0,15	0,54	0,18	0,48	0,13
C24:PUFA	0,05	0,08	0,04	0,07	1,14	0,77	1,28	0,96	0,24	0,03	0,22	0,19	0,99	0,38	1,55	0,51
C24:1(n-9)	2,02	0,00	1,86	0,19	1,58	0,36	1,14	0,04	2,58	0,54	2,69	0,59	1,68	0,14	1,48	0,11
C24:0	1,23	0,49	1,30	0,15	0,79	0,11	0,68	0,24	1,01	0,25	0,78	0,23	1,17	0,43	0,98	0,29
SFA	52,11	3,37	53,18	3,51	32,04	4,59	37,02	8,91	48,22	3,62	42,54	1,58	33,27	1,64	29,11	4,91
MUFA	33,10	0,29	33,38	3,06	29,09	4,80	28,06	5,84	38,17	5,14	43,98	1,87	26,04	0,95	23,60	2,40
PUFA	14,79	3,09	13,44	1,91	38,87	8,76	34,92	14,75	13,61	1,81	13,48	0,36	40,69	2,57	47,29	7,03

Eunicella	2010								2011							
	Before				After				Before				After			
	Mascles		Femelles		Mascles		Femelles		Mascles		Femelles		Mascles		Femelles	
	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd	Mean	Sd
C12:0	0,69	0,06	0,59	0,10	0,23	0,18	0,14	0,02	0,42	0,02	0,45	0,12	0,31	0,29	0,26	0,20
C13:0	0,01	0,02	0,00	0,00	0,01	0,01	0,01	0,01	0,01	0,02	0,07	0,13	0,00	0,00	0,02	0,04
C14:1(n-5)	0,03	0,04	0,00	0,00	0,17	0,15	0,03	0,03	0,03	0,04	0,02	0,03	0,00	0,00	0,00	0,00
C14:0	1,37	0,19	1,20	0,12	1,67	0,55	0,61	0,06	1,59	0,78	1,86	0,61	1,67	0,75	1,93	0,55
C15:1(n-5)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,10	0,14	0,04	0,07
C15:0	0,25	0,08	0,21	0,01	0,43	0,19	0,21	0,04	0,43	0,26	0,50	0,20	0,49	0,20	0,63	0,25
C16:1(n-7)	9,18	0,70	8,84	0,99	3,58	2,88	7,38	0,53	3,22	3,53	3,12	3,28	1,58	0,23	1,04	0,91
C16:0	24,72	3,73	19,25	0,99	38,22	20,86	23,15	2,03	38,02	19,22	43,40	12,86	37,31	38,12	31,89	36,42
C17:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	0,32	0,10	0,28	0,05	1,00	0,47	0,26	0,03	0,70	0,42	0,73	0,36	0,91	0,07	0,99	0,25
C18:3(n-6)	0,78	0,21	0,79	0,02	0,55	0,47	1,54	0,21	0,83	1,18	0,37	0,34	0,00	0,00	0,32	0,56
C18:4(n-3)	5,53	0,06	5,93	0,78	4,74	3,60	4,11	0,22	4,65	4,38	1,84	0,36	5,68	2,74	6,01	5,28
C18:2(n-6) cis	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00
C18:3(n-3)	21,23	1,70	23,35	0,03	9,27	6,24	20,16	1,18	0,98	1,39	2,42	4,19	0,65	0,92	0,58	0,64
C18:1(n-9)	0,70	0,17	0,77	0,08	1,07	1,60	0,50	0,06	2,77	3,91	2,09	3,36	1,03	1,16	0,71	0,84
C18:2(n-6) trans	0,00	0,00	0,00	0,00	0,16	0,32	0,00	0,00	0,86	1,22	0,79	1,37	1,65	2,33	0,62	1,08
C18:1(n-7)	4,00	1,44	6,06	1,55	5,07	3,47	4,83	3,45	10,08	5,69	11,80	3,87	5,35	7,56	3,84	6,65
C18:0	4,10	0,69	3,60	0,10	17,18	9,51	4,65	0,59	10,83	5,66	10,62	4,69	9,32	3,99	8,27	5,16
C20:4(n-6)	7,18	0,79	9,42	2,06	7,44	5,40	16,48	4,05	13,67	19,33	10,68	9,56	22,60	27,88	26,54	22,17
C20:5(n-3)	1,77	0,08	1,64	0,07	0,87	0,39	1,19	0,13	0,98	1,39	1,90	2,30	1,97	2,79	4,14	1,97
C20:3(n-6)	0,52	0,11	0,60	0,01	0,38	0,27	0,60	0,11	0,83	0,20	0,40	0,69	0,87	0,62	0,77	0,19
C20:4(n-3)	0,08	0,04	0,19	0,01	0,03	0,02	0,14	0,02	0,10	0,14	0,07	0,12	0,05	0,07	0,12	0,10
C20:2(n-6)	0,52	0,09	1,13	0,17	0,44	0,11	1,12	0,23	0,62	0,18	0,53	0,30	0,31	0,01	0,20	0,18
C20:1(n-9)	0,24	0,08	0,39	0,11	0,12	0,09	0,27	0,02	0,14	0,19	0,10	0,18	0,30	0,01	0,22	0,20
C20:0	0,11	0,02	0,09	0,00	0,26	0,08	0,13	0,01	0,30	0,06	0,28	0,05	0,26	0,25	0,20	0,13
C21:0	2,97	5,85	0,04	0,05	0,11	0,12	0,06	0,00	0,26	0,17	0,19	0,03	0,20	0,09	0,13	0,11
C22:5	0,06	0,07	0,13	0,00	0,06	0,05	0,13	0,01	0,10	0,14	0,09	0,10	0,08	0,12	0,22	0,13
C22:6(n-3)	8,86	6,73	10,57	1,11	3,50	2,55	6,17	1,48	3,26	4,61	2,56	1,58	2,40	1,85	5,18	4,60
C22:4	0,03	0,04	0,13	0,01	0,03	0,03	0,14	0,07	0,12	0,18	0,07	0,08	0,14	0,20	0,11	0,13
C22:5(n-x)	0,11	0,13	0,49	0,42	0,01	0,02	0,08	0,02	0,14	0,20	0,06	0,10	0,10	0,00	0,13	0,13
C22:2(n-6)	0,52	0,61	0,21	0,09	0,22	0,18	0,20	0,08	0,07	0,09	0,24	0,22	0,00	0,00	0,08	0,13
C22:1(n-9)	0,33	0,05	0,29	0,07	0,20	0,16	0,34	0,17	0,38	0,17	0,22	0,08	0,27	0,00	0,25	0,21
C22:0	1,57	0,27	1,43	0,27	0,94	0,58	1,52	0,58	0,53	0,28	0,49	0,07	0,46	0,42	0,38	0,26
C23:0	0,32	0,50	0,07	0,00	0,14	0,15	0,06	0,01	0,22	0,18	0,17	0,03	0,15	0,02	0,07	0,12
C24:PUFA	0,49	0,10	0,43	0,05	0,70	0,54	1,79	0,38	1,10	1,55	0,80	0,66	2,00	2,71	1,84	1,77
C24:1(n-9)	0,25	0,10	0,21	0,01	0,22	0,16	0,56	0,17	0,42	0,59	0,07	0,12	1,01	1,41	1,13	1,02
C24:0	0,71	0,36	1,43	0,21	0,59	0,31	0,69	0,31	0,99	0,57	0,86	0,53	0,80	0,38	1,01	0,56
SFA	36,71	7,48	27,26	0,81	60,48	21,76	31,00	3,23	53,77	27,30	59,15	18,81	51,41	44,32	45,23	42,42
MUFA	15,09	0,89	17,19	1,03	10,51	4,25	13,69	3,93	16,82	1,80	17,86	3,23	8,80	6,58	6,48	7,45
PUFA	48,20	7,83	55,55	0,22	29,00	17,52	51,20	7,33	29,41	25,50	22,99	17,37	39,79	37,74	48,29	36,68

Annex 4

Table Chapter 3

VILADRICH N (2015) Doctoral Thesis pp 111-112

	Corallium rubrum		***Eunicella singularis***		***Paramuricea clavata***	
	Mean	Sd	Mean	Sd	Mean	Sd
C12:0	0,04	0,07	0,10	0,03	0,45	0,05
C13:0	0,00	0,00	0,02	0,03	0,00	0,00
C14:1(n-5)	0,00	0,00	0,04	0,05	0,00	0,00
C14:0	0,50	0,13	0,38	0,07	2,52	0,31
C15:1(n-5)	0,00	0,00	0,04	0,06	0,00	0,00
C15:0	0,23	0,07	0,19	0,04	1,37	0,13
C16:1(n-7)	0,66	0,22	5,12	0,58	1,95	0,36
C16:0	10,12	2,82	10,76	2,33	24,67	1,25
C17:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00
C17:0	0,51	0,10	0,30	0,04	0,74	0,64
C18:3(n-6)	0,00	0,00	1,22	0,88	0,00	0,00
C18:4(n-3)	2,37	0,80	0,17	0,15	0,00	0,00
C18:2(n-6) cis	4,30	0,94	11,29	13,67	10,19	4,35
C18:3(n-3)	5,39	1,22	1,84	2,09	5,51	0,56
C18:1(n-9)	62,44	6,70	24,01	15,08	0,00	0,00
C18:2(n-6) trans	0,00	0,00	0,00	0,00	11,24	1,83
C18:1(n-7)	0,00	0,00	0,00	0,00	0,00	0,00
C18:0	3,92	0,84	2,90	0,18	10,76	1,29
C20:4(n-6)	1,69	0,09	5,82	6,91	9,84	3,35
C20:5(n-3)	0,49	0,43	1,35	1,30	1,43	1,29
C20:3(n-6)	0,59	0,52	1,93	0,96	1,66	1,44
C20:4(n-3)	0,00	0,00	0,16	0,14	0,16	0,27
C20:2(n-6)	0,49	0,09	5,17	2,75	1,46	0,08
C20:1(n-9)	0,75	0,04	1,02	0,51	1,40	0,15
C20:0	0,17	0,02	0,11	0,01	0,24	0,05
C21:0	0,35	0,05	0,40	0,06	0,00	0,00
C22:5	0,00	0,00	0,11	0,06	0,00	0,00
C22:6(n-3)	0,00	0,00	2,55	2,57	1,75	1,66
C22:4	0,30	0,09	0,27	0,38	0,00	0,00
C22:5(n-x)	0,00	0,00	0,08	0,11	0,00	0,00
C22:2(n-6)	1,08	0,10	0,20	0,06	0,00	0,00
C22:1(n-9)	0,00	0,00	0,51	0,11	1,43	0,11
C22:0	0,67	0,05	0,33	0,07	0,63	0,09
C23:0	0,94	0,33	0,46	0,01	0,61	0,09
C24:PUFA	0,36	0,18	2,40	1,48	1,14	0,56
C24:1(n-9)	0,69	0,08	0,31	0,05	2,02	0,45
C24:0	0,94	0,12	0,37	0,00	1,56	0,18
SFA	18,39	4,37	16,31	2,61	43,53	2,74
MUFA	65,62	6,32	30,73	16,07	5,37	0,78
PUFA	15,99	2,09	52,97	18,68	51,10	3,06