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## Estimations of free fatty acids (FFA) as a reliable proxy for larval performance in Mediterranean octocoral species

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### Abstract

The survival, behavior, and competence period of lecithotrophic larvae depends not only on the energy allocation transferred by maternal colonies, but also on the amount of energy consumed to sustain embryonic, larval, and post-larval development. The objective of the present work is to understand the effect of energy consumption on the performance of lecithotrophic larvae. To this aim, we analysed free fatty acid (FFA) content and composition of the larvae of three Mediterranean octocorals (*Corallium rubrum*, *Eunicella singularis*, and *Paramuricea clavata*) as a proxy for energy consumption. Results showed that *C. rubrum* larvae consume more FFA than *P. clavata*, whereas the energy consumed by *E. singularis* larvae is high but highly variable. These results are in accordance with the larval behavior of these three species, since *C. rubrum* larvae are characterized by their high swimming activity frequency, *P. clavata* larvae are almost inactive, and the swimming activity frequency of *E. singularis* larvae is high, although variable. The differences in FFA composition of the larvae suggest contrasting energetic strategies that could explain the differences in survival and recruitment rates. In fact, high dispersal and recruitment capacities for *E. singularis* larvae can be inferred from the FFA composition, whereas the high spatial and temporal variability of recruitment observed in *C. rubrum* may be related to the non-selective transfer of fatty acid (FA) from maternal colonies. Finally, the high recovery rates after mass mortality events observed in *P. clavata* could be favored by the presence of a specific FA [22:6(n-3)] related to adaptation mechanisms under environmental stresses during the first developmental stages.

**Keywords:** Energy consumption; Gorgonians; Fatty acids; Octocorals.

### Introduction

Maternal energy investment in lecithotrophic larvae of marine invertebrates mainly consists in the allocation of lipids (Richmond, 1987; Arai *et al.*, 1993), being wax esters, triacylglycerols, sterols, and polar lipids the most abundant (Figueiredo *et al.*, 2012). Some symbiotic species also present photosynthetic dinoflagellates of the family Symbiodiniaceae (*sensu* LaJeunesse *et al.*, 2018) in their larvae, which may provide additional energy to the offspring during development (Kopp *et al.*, 2016; Mies *et al.*, 2017). Differences in the energetic alloca-

tion from maternal colonies to larvae, or in the capacity to obtain energy during the planktonic phase, may influence larvae survival rates. For example, it is commonly assumed that symbiotic larvae have higher survival rates than non-symbiotic ones (Yakovleva *et al.*, 2009; Harii *et al.*, 2010). However, survival, behavior, and the competence period of larvae also depends on the amount of energy consumed to sustain embryonic, larval, and post-larval development (Holland & Spencer, 1973; Gallager & Mann, 1986; Pechenik, 1990; Qian *et al.*, 1990). For instance, since the metabolic demands in lecithotrophic larvae are related with swimming behavior, the active-

ly swimming larvae are expected to have a high energy consumption (Okubo *et al.*, 2008). Moreover, the energy consumption could increase for substrate recognition, competition for space, and early growth on sessile organisms, which may have consequence on their recruitment success (Adjeroud *et al.*, 2017).

The study of free fatty acid (FFA) content in the larval stage may be used as a proxy for lipids used at a particular time, since FFAs are obtained from the oxidation of lipid reserves (Gurr *et al.*, 2002), which in turn are beta-oxidized to provide a source of highly efficient energy (high ATP/Fatty Acid (FA) molecule) (Sargent *et al.*, 1988). Therefore, the FFA content is directly related to the energy consumed at a given time. Moreover, studying the FFA composition may help to understand the nature of energetic requirements, such as the attainment and maintenance of optimal health and physiological functions. This approach has been thoroughly used in the fish culture industry (Bell & Sargent, 1996; Izquierdo, 1996; Copeman *et al.*, 2002; Bransden *et al.*, 2005) and has recently been applied to understand some important ecological processes on coral species (Viladrich *et al.*, 2016; Conlan *et al.*, 2017; Grinyó *et al.*, 2018). Studies that looked at total FA showed that a dietary deficiency in some FAs can reduce the nutritional condition and growth in adult colonies (Latyshev *et al.*, 1991; Imbs, 2013; Radice *et al.*, 2019), as well as lower swimming activity and the survival of the larvae (Figueiredo *et al.*, 2012), which can eventually lead to lower recruitment rates (Conlan *et al.*, 2017). Hence, the different types of FAs (Saturated Fatty Acids, SFA; MonoUnsaturated Fatty Acids, MUFA, and PolyUnsaturated Fatty Acids, PUFA) could be a good proxy for larval condition. In general, when FAs 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 certain PUFA is considered essential for larval development and health status (DeMott & Muller-Navarra, 1997; Wen *et al.*, 2002; Figueiredo *et al.*, 2012).

Among benthic sessile invertebrates, gorgonians play a paramount role as ecosystem engineers in many benthic communities around the world (Gili & Coma, 1998; Wild *et al.*, 2011; Velásquez & Sánchez, 2015), and are considered one of the main three-dimensional constituents of the “marine animal forests” (*sensu* Rossi, 2013). Gorgonians exhibit three different strategies for sexual reproduction: (1) broadcast spawning: sperm and oocytes are released in the water column and fertilization is external, (2) surface brooding: oocytes/zygotes are retained by mucous material on the surface of the female colonies during larval development, although it is unclear if fertilization is internal or external, and (3) internal brooding: the oocytes are internally fertilized and the female colonies retain the zygotes and embryos within their body during their development (Kahng *et al.*, 2011).

In the Mediterranean Sea, the non-symbiotic gorgonians *Corallium rubrum* (Linnaeus, 1758) and *Paramuricea clavata* (Risso, 1826), together with the symbiotic *Eunicella singularis* (Esper, 1791), are characteristic species of shallow benthic communities in coastal areas (Weinberg,

1979a). They are gonochoric, releasing lecithotrophic larvae once a year during the summer months (Coma *et al.*, 1995; Santangelo *et al.*, 2003; Ribes *et al.*, 2007). The gorgonians, *C. rubrum* and *E. singularis*, are internal brooders, 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), *E. singularis* larvae, such as the adult colonies, contain photosynthetic dinoflagellates that belong to the *Symbiodinium* genus (phylogenotypes that were previously assigned to “clade A”) (Forcioli *et al.*, 2011; Weinberg, 1979b). The larval competence (i.e., the period during which pelagic larvae are able to settle) is approximately 8 days for *E. singularis*, 11 days for *P. clavata* and 27 days for *C. rubrum* (Zelli *et al.*, 2020).

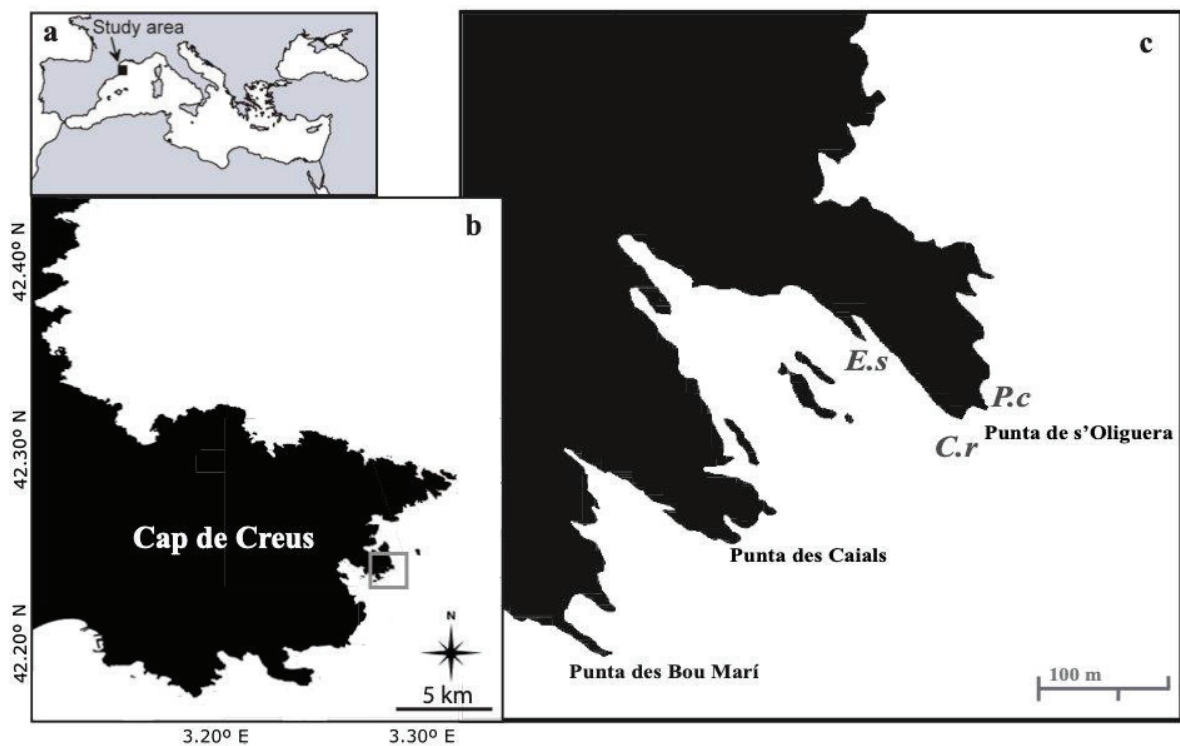
The aim of this study is to explore the link between energy consumption and larval performance in the three most characteristic and widely distributed shallow-water Mediterranean octocoral species. To achieve this objective, FFA were considered as a proxy for energy consumption, and their content and composition were analysed in the larvae of the three species just after release. The FFA content was used to quantify the energy consumed in the pelagic phase, whereas the FFA composition of larvae was compared between species to explore different energy requirements, in the light of data on larval performances (i.e., swimming activity rates and settlement rates) of the three species. These results can provide clues to understand how the energy used by larvae relates to their performance.

## Materials and Methods

### Sampling Procedure

Larvae of *P. clavata* (surface brooder) were collected from the surface of at least 5 different females using 50 ml syringes by SCUBA divers at 25-30 m depth at Punta de s’Oliguera in Cap de Creus on June 22<sup>nd</sup>, 2012 (Spain, NW Mediterranean, 42.284167 °N; 3.299722 °E) (Fig. 1). Larvae of the two internal brooder species (*C. rubrum* and *E. singularis*) are difficult to obtain *in situ*. For this reason, 5 female colonies of *C. rubrum* collected at 25–30 m depth and 5 female colonies of *E. singularis* collected at 15–20 m depth at Punta de s’Oliguera were maintained separately in 8 L tank at 20 ± 1.0 °C for 1–2 days until the larval release. A chiller (Tank chiller line TK 2000) was used to keep a constant seawater temperature, and the water was filtered using a biological filter (SERA fil bioactive 250+UV). Larvae of *E. singularis* were collected on July 19<sup>th</sup>, 2012, and larvae of *C. rubrum* on July 27<sup>th</sup>, 2012. Studied species and their larvae is shown in Figure 2.

For each species, three replicates of 30 different larvae were fixed on pre-combusted GF/F filters, cold shocked with liquid nitrogen and stored at -80 °C. Filters were then freeze-dried for 24 h at -110 °C and a pressure of 100 mbar. The freezer-dried material was stored at -20°C for further analyses.



**Fig. 1:** Location of the study area in the NW Mediterranean (a, b) and sampling sites on the eastern coast of Cap de Creus (c). *C.r.*, *E.s* and *P.c* indicate the position of the *Corallium rubrum* (25-30 m depth), *Eunicella singularis* (15-20 m depth), and *Paramuricea clavata* (25-30 m depth) populations, respectively.

### **Free Fatty Acid (FFA) Content and Composition in Larvae**

FFA content and composition were assessed for three replicates of each species according to the method described by Viladrich *et al.* (2016). Each filter with 30 larvae was dissolved in dichloromethane:methanol (3:1) spiked with an internal standard (2-octyldodecanoic acid and 5 $\beta$ -cholanic acid) to estimate recuperation. The extraction solvent was eluted through an aminopropyl glass column resulting in 3 fractions (neutral lipids, FFAs, and polar lipids). In this study, the FFA fraction was methylated using a solution of 20% boron trifluoride-methanol reagent heated at 90 °C for 1 h.

The methyl esters of FA (FAMES) were separated and analysed by gas chromatography with mass spectrometry detection (GC/MS, 7820A GC from Agilent Technologies) equipped with a DB-5ms Agilent column (60 m length, 0.25 mm internal diameter and 0.25  $\mu$ m phase thickness). Hydrogen was used as a carrier gas at 30 mL $\cdot$ min<sup>-1</sup>. The high compound numbers in the samples and the similarity of retention required a complex method of temperature ramps, using the oven temperature programmed to increase from 50 °C to 160 °C at 20 °C min<sup>-1</sup>, from 160 °C to 188 °C at 0.5 °C min<sup>-1</sup>, from 188 °C to 229 °C at 20 °C min<sup>-1</sup>, from 229 °C to 235 °C at 2 °C min<sup>-1</sup> and, finally, from 235 °C to 300 °C at 5 °C min<sup>-1</sup> (Viladrich *et al.*, 2016). The injector and detector temperatures were 300 °C and 320 °C, respectively. FAMES were identified by comparing their retention times with those of commercial standards of FA (Supelco 37 Component FAME Mix and Supelco® Mix C<sup>4</sup>-C<sup>24</sup>) and were quantified by inte-

grating areas under peaks in the chromatograms (Chromquest 4.1 software) using calibration curves derived from the Supelco 37 Component FAME Mix. The results are presented in  $\mu$ g FFA larvae<sup>-1</sup> and in percentage of saturated free fatty acids (free SFA), monounsaturated free fatty acids (free MUFA), and polyunsaturated free fatty acids (free PUFA), besides each FFA component percentage.

### **Statistical Analyses**

Differences in FFA content and percentage of free SFA, free MUFA, and free PUFA between larvae of the different species were tested using a one-way ANOVA. Before performing the ANOVAs, normality of data residuals and variance homogeneity were tested using the Shapiro-Wilk and Bartlett tests (functions “shapiro.test” and “bartlett.test” of the package *stats* in R environment). One-way ANOVA tests were performed with the function “aov” of the package *stats* (Chambers & Hastie, 1992).

A correspondence analysis (CA) was used to check for associations among FFA composition of larvae using the function “ca” of the *ca* package in R environment (Nenadic & Greenacre, 2007). Before performing the CA, FFA compounds that represented less than 2% of the total concentration were eliminated and percentages recalculated so that the sum was equal to 100%. After this transformation, the CA was performed on a cross table containing 13 FFA compounds and 9 samples of larvae (3 per species).

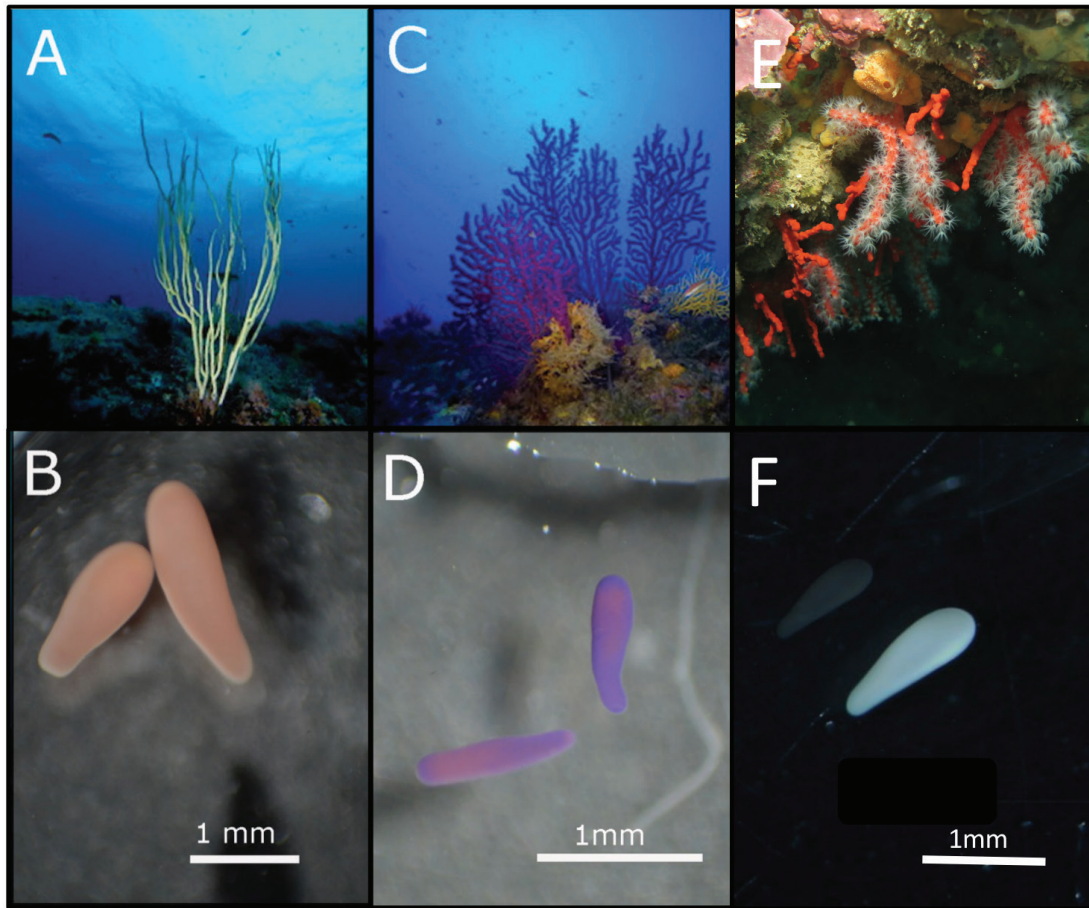


Fig. 2: Colony and larvae of *Eunicella singularis* (a, b), *Paramuricea clavata* (c, d), and *Corallium rubrum* (e, f).

## Results

### Free Fatty Acid (FFA) Content in Larvae

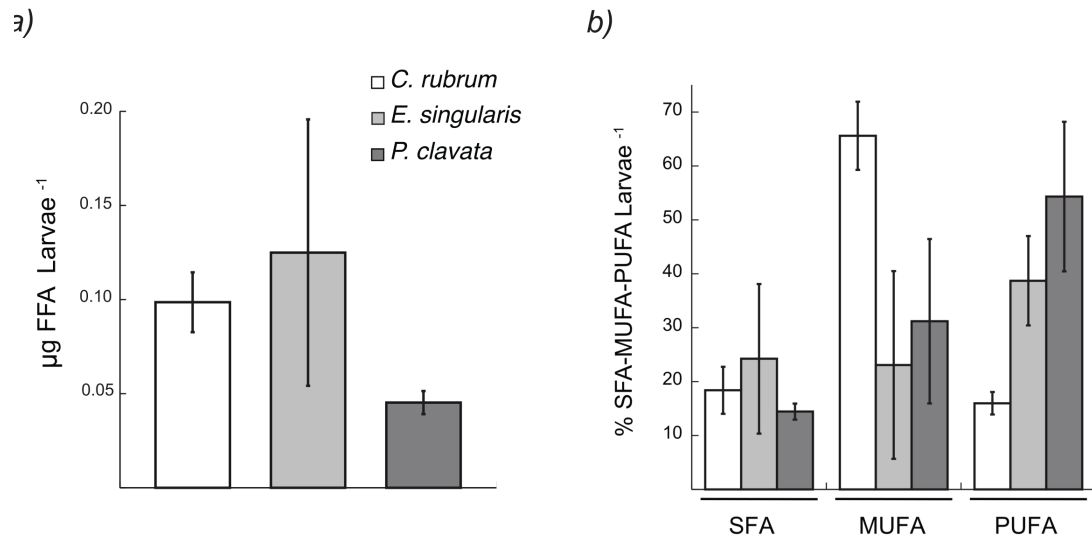
FFA content was  $0.098 \pm 0.015$   $\mu\text{g}$  per larvae (mean  $\pm$  SD) for *C. rubrum*,  $0.045 \pm 0.005$  for *P. clavata*, and  $0.125 \pm 0.07$  for *E. singularis* (Fig. 3a). ANOVA results showed significant differences between *C. rubrum* and *P. clavata* (one-way ANOVA,  $p < 0.005$ ), whereas no differences were observed between *E. singularis* and *C. rubrum* or *P. clavata* larvae, possibly due to the high variability of the *E. singularis* values (one-way ANOVA,  $p > 0.1$ ) (Fig. 3a). The ANOVA for free SFA, expressed as a percentage of total fatty acids, did not show significant differences between species (one-way ANOVA,  $p > 0.5$ ) (Fig. 3b), being  $18.39 \pm 4.37\%$  for *C. rubrum*,  $14.45 \pm 1.48\%$  for *P. clavata*, and  $26.98 \pm 11.60\%$  for *E. singularis* (mean  $\pm$  SD). The percentage of free MUFA was significantly higher in *C. rubrum* ( $65.61 \pm 6.32\%$ ) than in *P. clavata* and *E. singularis* larvae ( $31.21 \pm 15.26\%$  and  $26.77 \pm 17.40\%$ , respectively) (one-way ANOVA,  $p < 0.05$ ), while the free PUFA percentage was significantly higher in *P. clavata* and *E. singularis* ( $54.33 \pm 13.87\%$  and  $46.26 \pm 6.17\%$ , respectively) than in *C. rubrum* larvae ( $15.98 \pm 2.08\%$ ) (one-way ANOVA,  $p < 0.01$ ) (Fig. 3b).

### Free Fatty Acid (FFA) Composition in Larvae

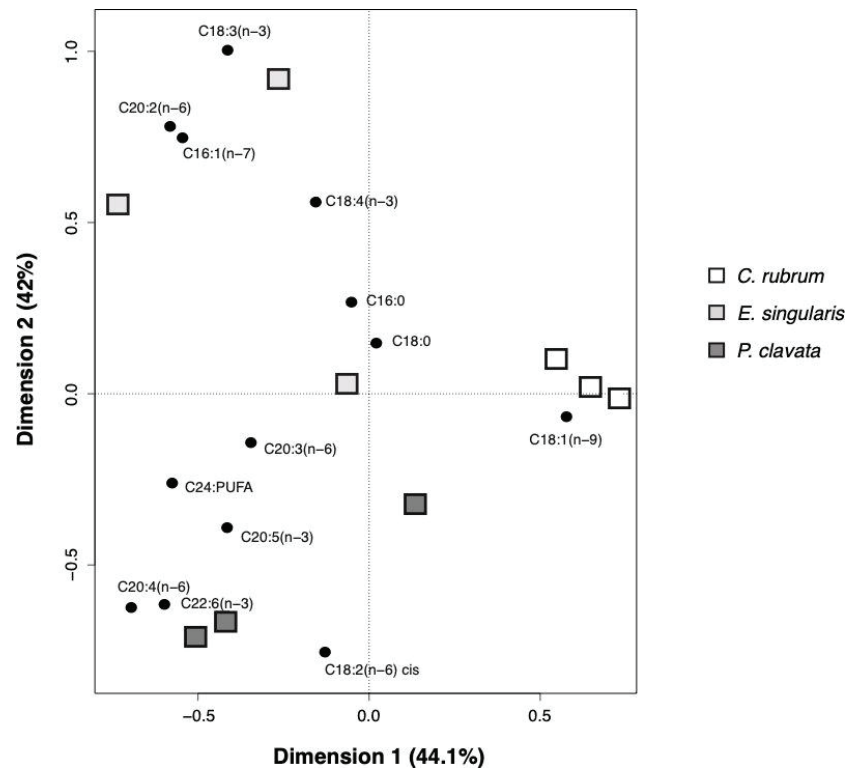
A total of 25 FFA markers for *C. rubrum*, 29 for *P. clavata*, and 34 for *E. singularis* were identified (ESM, Supplementary Material, Table S1). The first two dimensions explained 86% of the total variance of the CA. As it can be observed in the biplot, Dimension 1 (44% of total inertia) separates *C. rubrum* larvae from those of *P. clavata* and *E. singularis* based on the FFA descriptors (Fig. 4). Dimension 2 (42% of total inertia) shows that there are also differences between *P. clavata* and *E. singularis* larvae, indicating that larvae from the three species displayed distinct FFA compositions (Fig. 4). Samples of *C. rubrum* larvae, which all lay very close to one another, are characterized by the presence of 18:1(n-9). *Paramuricea clavata* samples, form a second and less compact group, with 20:4(n-6), 20:5(n-3), and 22:6(n-3) as the dominant FFA. Finally, the samples of *E. singularis* larvae are widely spread on the biplot and associated with different FFA, being 18:3(n-3), 18:4(n-3), 20:2(n-6), 16:1, and 16:0 as the principal markers.

## Discussion

This study shows, for the first time, that FFA content and composition are a reliable proxy to understand lecithotrophic larvae performance. Until now, FFA portion in coral larvae had been largely neglected, probably due to



**Fig. 3:** Free fatty acid content (mean  $\pm$  SD) in *Corallium rubrum* larvae (white), *Eunicella singularis* larvae (grey), and *Paramuricea clavata* larvae (dark grey) expressed as (a)  $\mu\text{g Larvae}^{-1}$  and (b) percentage of saturated fatty acids (SFA), monounsaturated Fatty acids, (MUFA), and polyunsaturated fatty acids (PUFA) (n = 3).



**Fig. 4:** Correspondence analysis (CA) biplot illustrating the ordering of the studied larvae in the first two dimensions regarding their free fatty acid composition (black points). *Corallium rubrum* in white, *Eunicella singularis* in grey, and *Paramuricea clavata* in dark grey.

their low concentration (Figueiredo *et al.*, 2012; Conlan *et al.*, 2017). Our results underscore the importance of FFAs during the larval period of *C. rubrum*, *P. clavata*, and *E. singularis*, despite their low concentrations ( $<0.12 \mu\text{g}$  per larva) (Fig. 3a).

According to our results, *C. rubrum* larvae had a 2-fold higher FFA content than *P. clavata*, whereas the FFA content in *E. singularis* was highly variable ( $\pm 57\%$ ). Since FFA are the main sources of energy for ATP production, these values represent a measure of larvae en-

ergetic consumption (Gurr *et al.*, 2002). Moreover, as seawater temperature at the time of release was the same for the three species (Viladrich *et al.*, 2016), the influence of temperature on the observed variability can be excluded, and larval energetic consumption can be related to the active movement of the larvae (Okubo *et al.*, 2008). Therefore, based on FFA content, we would expect *C. rubrum* larvae to be highly mobile, whereas *P. clavata* quite passive, and *E. singularis* larval motility high and highly variable. Measurements of larval swimming activity

frequency (i.e., percentage of time during which active swimming or crawling behavior is displayed by larvae) (*sensu* Guizien *et al.*, 2020) for the three species are in accordance with the values of FFA content measured, with 77% activity for *C. rubrum* (Martínez-Quintana *et al.*, 2015), 5% for *P. clavata* and 20-90% for *E. singularis* (Guizien *et al.*, 2020). Therefore, energy consumption may be a good proxy to estimate coral larvae swimming activity frequency.

As previously highlighted, the energy used depends on the specific metabolic requirements (Viladrich *et al.*, 2016, 2017; Grinyó *et al.*, 2018), and therefore, a detailed study of the FFA composition during the larval stage in different species may also help understanding the mechanisms underlying larval performance. In *E. singularis*, the high quantity of FFA 18:4(n-3) (Fig. 4) supports the hypothesis that the Symbiodiniaceae are transferred from the mother colonies to the larvae, as previously proposed by Weinberg and Weinberg (1979). This fatty acid, in fact, is a robust tracer of the photosynthetic activity of symbiotic dinoflagellates (Papina *et al.*, 2003; Treignier *et al.*, 2008; Pupier *et al.*, 2021), since it cannot be synthesized *de novo* by heterotrophs (Volkman *et al.*, 1989; Dalsgaard *et al.*, 2003). Therefore, the presence of 18:4(n-3) indicates a surplus of energy which can increase survival rates and the competency of larvae (Ben-David-Zaslow & Benayahu, 1998; Harii *et al.*, 2010; Figueiredo *et al.*, 2012). Interestingly, this is in line with the lack of genetic structuring at large spatial scales observed for this species (Costantini *et al.*, 2016). The high variability of this FFA (18:4(n-3)) observed in *E. singularis* larvae could be caused by the number of symbionts per larva, which varies according to the supply from mother colonies (Gaither & Rowan, 2010; Roth *et al.*, 2013). Nevertheless, not all the symbiotic larvae obtain an energy surplus from symbiosis, as showed by Kopp *et al.* (2016) and Mies *et al.* (2017). Future studies should focus on the role of Symbiodiniaceae in *E. singularis* larvae energetic budget. Finally, large amounts of 18:3(n-3) in *E. singularis* larvae could explain the higher recruitment rates of this species with respect to *C. rubrum* and *P. clavata* (Bramanti *et al.*, 2005; Linares *et al.*, 2008; Linares *et al.*, 2012) since this marker may have beneficial effects on larval settlement, following a trend already observed in bivalve and polychaete larvae (Pawlik & Faulkner, 1986; Jonsson *et al.*, 1999; da Costa *et al.*, 2011).

In the case of *C. rubrum*, our results suggest that the nutritional status of the adult colonies may directly affect the viability of the larvae due to a non-selective transfer of the most conspicuous FA, as previously suggested for different marine organisms (Qian & Chia, 1991; Harland *et al.*, 1993; Dalsgaard *et al.*, 2003; Figueiredo *et al.*, 2012). In fact, 18:1(n-9) is the main component for both *C. rubrum* larvae (62%) and maternal colonies (30%) (Fig. 4) (Viladrich *et al.*, 2016), and it has been associated with detritus (Schultz & Quinn, 1973; Fahl & Kettner, 1993), which is the main food source for this species (Tsounis *et al.*, 2006). This non-selective transfer of lipids from maternal colonies to larvae may result in a dependence of recruitment on the nutritional condition

of the mother colonies (Lasker, 1990; Yoshioka, 1996; Dunstan & Johnson, 1998), which in turn is affected by environmental conditions (Rossi & Tsounis, 2007). Indeed, the high spatial and temporal variability of recruitment rates observed in *C. rubrum* (Garrabou & Harmelin, 2002; Santangelo *et al.*, 2012) may be partially explained by the spatial and temporal variability of environmental conditions. Moreover, the high variability of recruitment rates could also be related to the low free PUFA content in *C. rubrum* larvae (Fig. 3b), since PUFA are mainly devoted to growth and resistance to stress conditions, thus enhancing larval metamorphosis and survival (Bell & Sargent, 1996; Pond *et al.*, 1996; Sargent *et al.*, 1997; Albessard *et al.*, 2001; Rossi *et al.*, 2006; Figueiredo *et al.*, 2012; Conlan *et al.*, 2017). Therefore, small changes in the energy storage reserves of mother colonies may have serious consequences on the following generations.

Interestingly, some FFAs may be the key to understanding the resilience of some species after thermal stress episodes. Previous studies showed higher recruitment rates of *P. clavata* after a mass mortality events caused by increased temperature, if compared to *C. rubrum* and *E. singularis* (Coma *et al.*, 2006; Cupido *et al.*, 2009; Santangelo *et al.*, 2015). High recruitment rates after disturbances are associated to high resilience, attributed either to the high reproductive output of local *P. clavata* survivors (Cupido *et al.*, 2009), or to migration from distant populations (Padrón *et al.*, 2018). However, it is possible that such high recruitment rates in *P. clavata* could also be concurrently favored by the presence of the FFAs 22:6(n-3), 20:4(n-6), and 20:5(n-3) (Fig. 4), which are key components of lipids for larval performance. Indeed, 22:6(n-3) is known to influence membrane fluidity and permeability, which can have a positive impact on enzyme activity, immune functions, and adaptation to heat stress, among many other cellular processes (Dratz & Holte, 1992; Hall *et al.*, 2000; Bergé & Barnathan, 2005; Kneeland *et al.*, 2013). Furthermore, the FFA 20:4(n-6) and 20:5(n-3) are essential fatty acids for larval development, which have been related to the production of biologically active eicosanoids under stress or unfavorable conditions (Sargent *et al.*, 1999), supporting the immune system functioning and osmoregulation (Chapelle, 1986; Mazorra *et al.*, 2003).

The present results are thus in accordance with the literature on larval performance of the studied species. The FFA content in larvae might then be used to predict the swimming activity frequency of different sessile invertebrate species, and their composition might also give a more detailed understanding of the mechanisms underlying larval performances. This new approach paves the path for future studies of early life-history stages of lecithotrophic larvae.

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## Supplementary Data

The following supplementary information is available online for the article:

**Table S1.** Mean values and standard deviation (SD) of free fatty acid (FFA) markers and saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA) for *Corallium rubrum*, *Eunicella singularis* and *Paramuricea clavata* larvae.