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Recurrent marine heatwaves compromise the reproduction success and long-term viability of shallow populations of the Mediterranean gorgonian *Eunicella singularis*

Julia Sarda[a,b,c,*], Andrea Gori[a,b], Ruth Doñate-Ordóñez[a], Núria Viladrich[a,b], Federica Costantini[c,d], Joaquim Garrabou[e], Cristina Linares[a,b]

[a] *Department de Biologia Evolutiva, Ecologia i Ciències Ambientals, Universitat de Barcelona (UB), Avda. Diagonal 643, 08028, Barcelona, Spain*
[b] *Institut de Recerca de la Biodiversitat (IRBio), Universitat de Barcelona (UB), Avda. Diagonal 643, 08028, Barcelona, Spain*
[c] *Dipartimento di Scienze Biologiche, Geologiche ed Ambientali (BiGeA) & Centro Interdipartimentale di Ricerca per le Scienze Ambientali (CIRSA), University of Bologna, Via S. Alberto 163, I-48123, Ravenna, Italy*
[d] *Consorzio Nazionale Interuniversitario Per Le Scienze Del Mare (CoNISMa), Piazzale Flaminio 9, 00196, Roma, Italy*
[e] *Institut de Ciències del Mar (ICM-CSIC), Passeig Marítim de la Barceloneta 37-49, 08003, Barcelona, Spain*

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ABSTRACT

Mediterranean gorgonians are being threatened by the impact of recurrent extreme climatic events, such as marine heatwaves (MHWs). The white gorgonian *Eunicella singularis* was suggested to be the most resistant gorgonian species in the NW Mediterranean, mainly due to the presence of symbiotic algae. However, a substantial shift in the conservation condition of the species has been observed in the recent years. The aim of this study is to evaluate the lethal and sublethal effects of recent MHWs on the populations of *E. singularis*. Our results show that recurrent MHWs have impacted both the demography and reproduction of the species between 2002 and 2020, driving mortalities up to 36%, an increase in the percentages of non-reproducing adult colonies (11–58%), and a significant decrease in the recruitment rates. Although *E. singularis* is a highly dynamic species in comparison with other temperate gorgonians, the present study suggests that the persistence of this species may be severely compromised under recurrent MHWs, at least at shallowest depths.

1. Introduction

During the last decades, there has been an increase in the frequency and intensity of extreme climatic events driven by climate change, such as marine heatwaves (MHWs) (Smale et al., 2019). These events have been associated to mass mortality events (MMEs) of many habitat-forming species around the world, including reef-building corals, gorgonians, sponges, seagrasses, and kelp forests, causing profound changes at population and habitat levels (Hughes et al., 2017; Smale et al., 2019; Gómez-Gras et al., 2021a; Garrabou et al., 2022; Bell et al., 2023). As a climate change hotspot (Cramer et al., 2018; Lee et al., 2021), the Mediterranean Sea is experiencing an unprecedent increase in the occurrence of mass mortality events driven by MHWs (Garrabou et al., 2019, 2022), with seawater temperatures expected to rise at an average mean rate of 0.041 ± 0.006 °C/year, a rate 3.7 times faster than the global ocean estimates (0.011 °C/year) (Collins et al., 2019; Pisano et al., 2020).

Gorgonians are among the most affected by MMEs in the Mediterranean Sea (Garrabou et al., 2019). These species are considered habitat forming species in Mediterranean rocky bottom communities (Ballesteros, 2006; Gori et al., 2011a), displaying, in general, slow population dynamics, which limits their recovery capacity to face recurrent perturbations such as MHWs (Linares et al., 2007, 2008a; Garrabou et al., 2021; Gómez-Gras et al., 2021a). Consequently, MMEs could cause severe impacts for the populations persistence that cascade to dramatic changes at habitat level due to their ecological structural role (Pey et al., 2013; Garrabou et al., 2021; Gómez-Gras et al., 2021a), essential for the development and maintenance of highly diverse communities (Verdura et al., 2019; Gómez-Gras et al., 2021b).

Since the first event was reported in 1999 along the coasts of Italy

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* Corresponding author. Department de Biologia Evolutiva, Ecologia i Ciències Ambientals, Universitat de Barcelona (UB), Avda. Diagonal 643, 08028, Barcelona, Spain.

E-mail address: jsarda@ub.edu (J. Sarda).

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and France (Cerrano et al., 2000; Perez et al., 2000), mass mortalities driven by MHWs have been affecting many different populations of the gorgonian *Eunicella singularis* in the Mediterranean Sea (Garrabou et al., 2009, 2019, 2022). Even so, the impact on *E. singularis* has been extremely variable, with up to 80% mortality in some areas (Coma et al., 2006; Garrabou et al., 2009), contrasting with a mean mortality around 2% in others (Linares et al., 2008a; Pey et al., 2013). The potential advantage of some *E. singularis* populations facing these disturbances could be related to the presence of symbiotic microalgae from the family Symbiodiniaceae in their tissue (Carpine and Grasshoff, 1975), that can contribute to the recovery from perturbations by increasing the organic matter, energy input, and oxygen available to maintain the energetic requirements of the gorgonians (Fava et al., 2009).

While our understanding of the vulnerability of marine invertebrates to MHWs is improving, their recovery capacity is far less understood (Gómez-Gras et al., 2021b). In many species, the recovery of the populations depends on the reproductive success of the species (Giangrande et al., 1994; Chen et al., 2020). In tropical corals, recruitment may significantly decrease after mass mortality events (Hughes et al., 2019), as well as in Mediterranean gorgonians (Coma et al., 2006), jeopardizing the possibility of compensating the lethal effects of MHWs (Linares et al., 2005). However, despite its importance for the maintenance of populations, possible sublethal effects of MHWs on the species reproduction are still poorly assessed (Linares et al., 2008b). In the Mediterranean gorgonian *Paramuricea clavata*, thermal stress may affect reproduction by altering the energy available for the development of reproductive output (Arizmendi-Mejía et al., 2015) and its effects can persist during years (Linares et al., 2005). At larval stage, thermal stress also significantly decreases larval survival, promoting abnormal embryonic development and malformations in *P. clavata* (Kipson et al., 2012). In contrast, it has been suggested that reproductive output of *E. singularis* is less affected by thermal stress due to their trophic plasticity, being the only mixotrophic gorgonian in the Mediterranean Sea (Viladrich et al., 2017). Still, larval dispersal capacity and, consequently, genetic connectivity among populations can be drastically reduced (Viladrich et al., 2022).

Considering the abundance and ecological importance of the gorgonians in the Mediterranean Sea providing many ecosystem functions and services (Coppari et al., 2019; Gómez-Gras et al., 2021b), and their vulnerability to the increased MHWs (Garrabou et al., 2009, 2022), this study aims to explore the lethal and sublethal impacts of recurrent MHWs on populations of the gorgonian *Eunicella singularis* by exploring the sex ratio, the reproductive output, the symbiosis with dinoflagellate algae, and their relationship with demographic parameters in populations impacted by recent MMEs along an environmental gradient in the north of the Catalan coast in the NW Mediterranean Sea.

2. Material and methods

2.1. *Study species*

The temperate gorgonian *Eunicella singularis* (Esper, 1791) is a common and abundant octocoral in Mediterranean coralligenous and precoralligenous assemblages from 10 to 70 m depth, mainly found in rocky bottom areas exposed to near-bottom currents (Linares et al., 2008a; Gori et al., 2011a). As most Mediterranean gorgonians, it is a long-lived species, with slow growth, low adult mortality, and low reproductive output, but it differentiates from other species because *E. singularis* presents high recruitment rates (Linares et al., 2008a; Ghanem et al., 2021).

Regarding the reproduction of the species, *E. singularis* is an iteroparous and gonochoric species (Ribes et al., 2007), with an annual reproductive cycle (Gori et al., 2007; Ribes et al., 2007). Spermatogenesis takes 4–6 months (Weinberg and Weinberg, 1979; Ribes et al., 2007), whereas oogenesis takes up to 13–17 months, therefore females present two overlapping cohorts of oocytes (Ribes et al., 2007).

After male sexual products are released to the environment, the fecundation takes place inside the female polyps, where the ciliate planula larvae with endosymbionts will develop following an holoblastic segmentation of the zygote (Weinberg and Weinberg, 1979; Ribes et al., 2007). Breeding period seems to be related with increasing temperatures and the stabilization of the thermocline occurring in June–July (Forcioli et al., 2011; Gori et al., 2012), and larvae present a positive phototaxis (Weinberg, 1979), which is a response for identifying suitable habitats for the settlement of new individuals (Mundy and Babcock, 1998; Linares et al., 2008b). Settlement of the larvae takes place a few days after the release and ends with the development of a primary polyp (Weinberg and Weinberg, 1979; Zelli et al., 2020).

2.2. *Study locations and sampling*

This study is focused on ten populations of *E. singularis* in two different marine protected areas (MPAs) located at the north of the Catalan coast in the NW Mediterranean Sea (Fig. 1a, b, c). Specifically, the first six populations located at the *Parc Natural del Cap de Creus,* whereas the other four located at the *Parc Natural del Montgrí, Illes Medes i Baix Ter*. The north of the Catalan coast is characterized by a thermal latitudinal gradient, with lower temperatures in the northern area in comparison with the southern one (Fig. 1d). Moreover, the north of the Cap de Creus is highly exposed to strong currents (Northern Current) coming from the Gulf of Lions, which provide food and hydrodynamics to the area, essential for the development of coralligenous assemblages and the associated gorgonian populations (Ballesteros, 2006; Linares et al., 2008a) (Fig. 1e).

The studied populations were sampled by scuba divers between 10 and 20 m depth, at the upper limit distribution of the species.

Demographic sampling. The demographic data were obtained in July 2020 for seven of the ten studied populations, in Cap de Creus (three populations: Forcats, La Reparada and El Gat) and Medes Islands (four populations: Medallot, Pedra de Déu, La Reina and Tascons). The other three populations from Cap de Creus (Portaló, Culleró and Es Caials) were sampled for reproduction but no demographic data were available. Temperature data and information on MHW events were obtained from the T-MedNET database, an initiative that has been monitoring temperatures from the surface to a depth of 40 m over the past two decades, analyzing temperature trends and MHWs in nearshore waters (Bensoussan et al., 2019; Garrabou et al., 2019, 2022). Contrasting to other NW Mediterranean localities, these populations were not affected by MMEs in 2003 (Garrabou et al., 2009). In fact, the Catalan coast was one of the latest mediterranean areas to suffer large MMEs, which were first observed in 2017. And from then, the frequency and severity have been annually increasing (Zentner et al., 2023; Rovira et al., 2024). This coincides with the pattern observed over the entire Mediterranean Sea, showing that the 2015–2019 period has been the warmest since the beginning of satellite records (Garrabou et al., 2022). At each location, between 30 and 60 50 × 50 cm squares (surface = 0.25 m^2) were sampled. From each square we obtained information of the number of colonies, their maximum height, and the percentage of colony surface covered by necrosis (recent mortality) and epibiosis (old mortality).

Reproductive condition sampling. To assess the reproductive condition of the studied populations, we sampled approximately 100 fragments of about 5 cm of length of primary branches of *E. singularis* colonies at each of the ten studied populations, and preserved them in 96% ethanol. Only those colonies higher than 30 cm were sampled to ensure that they were reproductive colonies (Ribes et al., 2007). The sampling was performed on June 10 and 14 of 2021 in Cap de Creus and Medes Islands, respectively, before male sexual products were released to ensure that colonies contained sexual products. Overall, a total of 1094 colony fragments were recollected and analyzed.

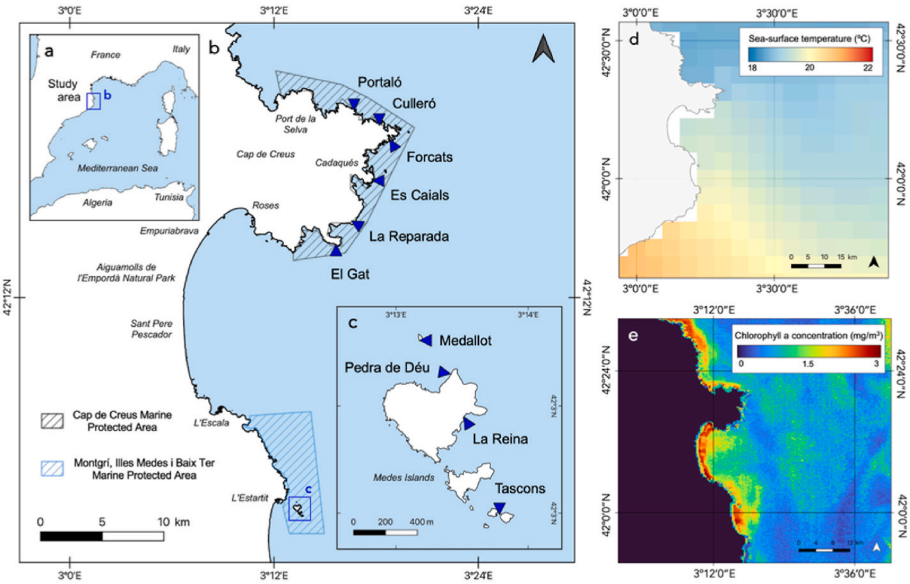

Fig. 1. Location of the studied *Eunicella singularis* populations along the north of the Catalan coast (NW Mediterranean Sea), in Cap de Creus and Medes Islands. Demographic data and samples for the study of reproduction were collected for each population except for Portaló, Culleró and Es Caials, for which no demographic data was collected. a) Western Mediterranean, b) North of the Catalan coast, c) Medes Islands. Source: qGIS and the Hydrographic Institute of the Navy (IHM). d) Mean sea-surface temperature from May to August of 2021 in the Northwestern Mediterranean. Data from the Group for High Resolution Sea Surface Temperature. Source: NOAA and qGIS. e) Mean chlorophyll concentration (mg/m^3) in the sea surface of the study area between April and June of 2021 (mean error percentage of 18%). Source: Sentinel 3A OLCI (Ocean and Land Color Instrument), SNAP and qGIS.

2.3. Demography

To study the size structure of the populations, we used two different descriptors, skewness and kurtosis. The skewness is an indicator of the symmetry of a distribution, and a positive value indicates a higher frequency of small-sized colonies compared to large ones. The kurtosis is a descriptor of the distribution of a variable, and the larger the value, the higher concentration of values around the mean. The skewness and kurtosis are only significant if the division between the standard error of the skewness (SES) and the standard error of the kurtosis (SEK) with the distribution indicator value is higher than 2 (Linares et al., 2008a; Sokal and Rohlf, 1995). For each population we calculated the mean density (colonies/m^2), the mean height of the colonies (cm), the percentage of affected colonies, the percentage of affected tissue per colony (necrosis and epibiosis), the percentage of colonies with less than 10% of affected tissue (considered healthy colonies), the percentage of colonies with 10–99% of affected tissue (considered impacted colonies), and the percentage of dead colonies (100% affected). The results were compared with the demographical parameters obtained in 2002 for the same populations, when no mass mortality events had still been observed in the study area (Garrabou et al., 2009), to determine how these populations are evolving with time and study the impacts of MMEs (Linares et al., 2008a).

2.4. Reproduction

2.4.1. Population sex ratio

A sex identification of all the fragments sampled for each population was performed in the laboratory using a stereo microscope to determine male, female, and indetermined colonies (colonies not reproducing). As the gorgonian *E. singularis* does not present secondary sexual characters, differentiation between male and female colonies is based on observing the color, texture, morphology, and appearance of their sexual products (Gori et al., 2007; Fig. S1). Female colonies present sexual products throughout the year, so, if the colonies are reproductively active, it is always possible to determine their sex. Conversely, male colonies can only be identified during the reproductive period from February to the summer (Ribes et al., 2007).

2.4.2. Reproductive effort

To assess the reproductive effort of each population, 10 female and 10 male fragments were analyzed. For each fragment, 10 different polyps were dissected under a stereo microscope and all sexual products inside photographed. The image analysis was performed with the image-processing software Image J (*Image J 1.52p 2019, National Institute of Health, US*), which allowed to determine the area (A) and circularity index of each sexual product. Circularity index is calculated as the ratio between the perimeter of a circle with the same area of a sexual product, and its measured perimeter. Since circularity (proximity of the shape of the sexual products to a perfect circle) was always high, the diameter (d) of each sexual product was obtained from the measured area with the following equation: $d = 2(A/\pi)^{-2}$, whereas the volume of the sexual products (V) was inferred through the diameter with the following equation: $\mathrm{V} = 4/3{\cdot}\pi(\mathrm{d}/2)^3$. From this data, we could determine for each population the fertility (expressed as the percentage of fertile polyps), the mean diameter (μm) of female and male sexual products, the mean fecundity (number of sexual products per polyp), and the mean total volume of sexual products per polyp (mm^3/polyp). A total of 1,600 polyps were dissected, and 17,465 sexual products were measured individually. Finally, the relationship between the population's mean diameter of sexual products and mean fecundity was assessed for each sex using regression analysis, after verifying that the model assumptions were met.

2.5. Symbiotic algae

To study the sublethal effects of the mass mortality events in the relationship of *Eunicella singularis* with their photosymbionts, a first identification of the Symbiodiniaceae species inhabiting *E. singularis* was conducted in order to assess potential genetic differences between populations using the ribosomal internal transcribed spacer 2 (ITS2) and the non-coding region of the psbA gene markers.

The DNA extraction was performed at the laboratory using the cetyltrimethylammonium bromide (CTAB) protocol (Winnepenninckx et al., 1994). Two different specific genes for Symbiodiniaceae (ITS2 and psbA^{ncr}) were amplified using polymerase chain reaction (PCR). The ribosomal internal transcribed spacer 2 (ITS2) is one of the most common used marker gene for the Symbiodiniaceae analyses and was amplified with the primer itsD (5′-GTGAATTGCAGAACTCCGTG-3'; Pochon et al., 2001) and its2rev2 (5′-CCTCCGCTTACTTATATGCTT-3'; Stat et al., 2009) for 26 individuals. PCR conditions were the following: initial denaturing at 95 °C for 2 min, 35 cycles each at 95 °C for 45s, 56 °C for 45s, and 72 °C for 45s, followed by a final extension step at 72 °C for 5 min. The non-coding region of the psbA gene (psbA^{ncr}) from the photosystem II of the chloroplast was amplified later to differentiate between species with the primer psbAFor_1 (5′-GCAGCTCATGGT-TATTTTGGTAGAC-3'; LaJeunesse and Thornhill, 2011) and psbARev_1 (5′-AATTCCCATTCTCTACCCATCC-3'; LaJeunesse and Thornhill, 2011) for 12 individuals, from which only 4 amplified successfully. PCR conditions were the following: initial denaturing at 94 °C for 2 min, 40 cycles each at 94 °C for 10s, 55 °C for 30s, and 72 °C for 2 min; followed by a final extension step at 72 °C for 10 min. The amplifications of both genes were sent to Macrogen Europe for Sanger Sequencing (Sanger et al., 1977), chromatograms were checked, and the obtained sequences were edited and aligned using Clustal W Multiple Alignment (BioEdit Sequence Alignment Editor) and MEGA11 (Molecular Evolutionary Genetic Analysis). Phylogenetic analyses using Maximum Likelihood were conducted with the software MEGA11 and RStudio (packages: *ape*, *adegenet* and *phangorn*) on aligned sequences including also sequences of other Symbiodiniaceae (GenBank PopSet accession number: 2292923562) and using the species *Cladocopium latusorum* as the outgroup (GenBank accession numbers: OP279755.1, OP279756.1, OP279774.1), and was tested with bootstrap support of branching nodes based on 100 replicates. The sequences found in our samples have been uploaded to GenBank (GenBank accession numbers: ITS2 (PP382812-PP382837) and psbA (PP382511-PP382514)).

2.6. Statistical analyses

All statistical analyses were performed with the software RStudio version 3.5.3 (2022.12.0 + 353). Differences in the demographic results were tested with analysis of variance statistics (ANOVA) and permutational analysis of variance (PERMANOVA) for those cases in which the model assumptions were not met. The model assumptions were tested with the Shapiro-Wilk test and Fligner-Killeen test. All the demographic analyses were performed considering three different factors, the year (fixed factor, 2 levels), area (fixed factor, 2 levels) and population (random factor nested to area, 7 levels). Deviation from 1:1 in population sex ratio was tested by means of a chi-square test and a generalized linear model (binomial family), as sex ratio proportions are expected to conform to a logistic distribution (Wilson and Hardy, 2002). Reproduction results were also tested with ANOVA and PERMANOVA analyses, using instead of the factor year, the factor sex (fixed factor, 2 levels). For all the reproduction analyses we excluded the few larvae found inside the polyps (11 in total), and the non-mature oocytes (smaller than 450 μm in diameter, Ribes et al., 2007). The correlations were studied using linear regressions, after verifying that the model assumptions were met using the Shapiro-Wilk test and the Breusch-Pagan test.

3. Results

3.1. Demography

The seven studied populations exhibited a unimodal size structure distribution based on the data from 2020, whereas in 2002, the size structure was more indicative of a bimodal distribution. The percentage of colonies of the first size-class (0−10 cm) has decreased significantly when compared with results obtained in 2002 (*two-way ANOVA, F = 5.53, p-value = 0.041*) (Fig. 2a).

A significant decrease in density was observed between years (*three-way PERMANOVA, F = 51.88, p-value = 0.001*). The mean density in 2002 was 19.5 ± 13.4 colonies/m^2 (mean ± SD), while, in 2020 the density had decreased considerably to 12.2 ± 4.8 colonies/m^2 (mean ± SD) (Fig. 2b). The most important decreases in densities were suffered in Tascons, Pedra de Déu and Medallot (all Medes Islands populations), while only two populations increased their densities (both in Cap de Creus) (Fig. 2b).

Significant differences in height were observed between years and areas (*three-way PERMANOVA, F = 32.51, p-value < 0.001*), with mean values of 16.1 ± 5.5 cm (mean ± SD) in 2020 and 19.0 ± 3.8 cm (mean ± SD) in 2002. Populations from Medes Islands exhibited lower values compared to those from Cap de Creus (Table 1). Additionally, differences in the minimum height of each population were also found between 2002 and 2020, with values of 2.7 ± 1.1 cm (mean ± SD) and 1.5 ± 0.9 cm (mean ± SD), respectively (*three-way ANOVA, F = 18.21, p-value = 0.008*).

The mean percentage of affected tissue significantly increased from 1.9 ± 2.3% (mean ± SD) in 2002, to 32.1 ± 13.7% (mean ± SD) in 2020 (*three-way PERMANOVA, F = 780.6, p-value < 0.001*). Significant differences were also observed between populations from Cap de Creus and Medes Islands (*three-way PERMANOVA, F = 19.1, p-value < 0.001*). In 2002, 92.7 ± 8.8% (mean ± SD) of the colonies were unaffected, decreasing to 44.8 ± 20.7% (mean ± SD) in 2020. Similarly, the percentage of total dead colonies increased from 0.1 ± 0.3% (mean ± SD) in 2002 to 16.5 ± 11.2% (mean ± SD) in 2020 (*three-way PERMANOVA, F = 21.7, p-value < 0.001*) (Fig. 3). Forcats and La Reparada (north of Cap de Creus) had the lowest percentages of affected tissue, while La Reina (Medes Islands) exhibited the highest. A similar trend was noted in the percentage of total mortality, with a value of 36% of dead colonies in La Reina and 5.3% in Forcats (Table 2).

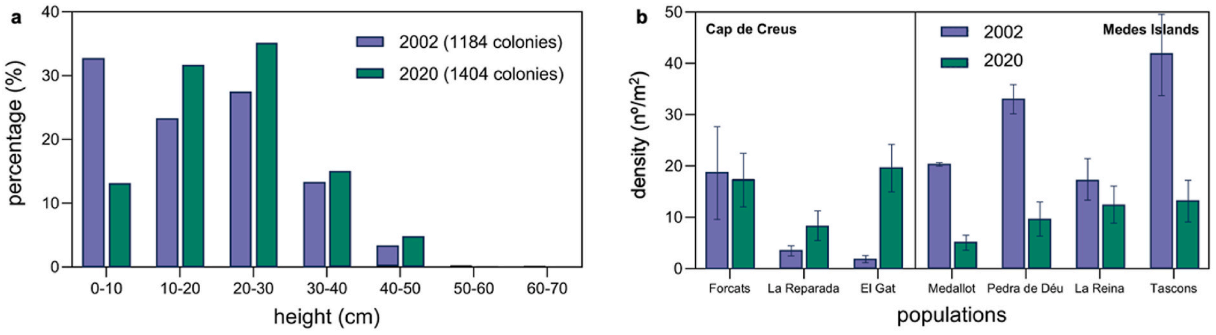

Fig. 2. a) Size distribution of the colonies studied in 2002 (in purple) and 2020 (in green) for all seven populations, b) Comparison of the density values (nº colonies/m^2) observed in each studied population.

Table 1
Demographic characteristics of the studied *Eunicella singularis* populations, compared with the results obtained in a previous study in 2002 (Linares et al., 2008a); depth, density (colonies/m^2), mean height (mean H), minimum and maximum height (Min, Max), skewness (g_1) and kurtosis (g_2). If the absolute value of the coefficient/standard error (SE) is greater than 2, the skewness and kurtosis are considered significant.

Year	Area	Population	Depth	Density	Mean H	SD	Min	Max	g_1	SE g_1	Sig. (>2)	g_2	SE g_2	Sig. (>2)
2002	Cap de Creus	Forcats	13	18.80	12.58	9.10	2	47	1.69	0.35	4.86	3.63	0.68	5.33
		La Reparada	15–20	3.62	24.53	11.89	2	61	0.50	0.18	2.75	0.34	0.36	0.94
		El Gat	15–20	1.80	14.70	8.89	3	50	1.13	0.25	4.46	2.21	0.50	4.39
	Medes Islands	Medallot	13	20.31	20.99	10.05	1.9	42	−0.46	0.30	−1.54	−0.55	0.59	−0.93
		Pedra de Déu	13	33.13	7.49	6.22	0.6	31	1.61	0.24	6.86	2.32	0.47	5.00
		La Reina	18	17.20	15.87	12.82	1	51	0.70	0.26	2.68	−0.43	0.51	−0.83
		Tascons	18	41.91	16.36	13.61	0.1	57	0.44	0.16	2.81	−0.76	0.31	−2.43
2020	Cap de Creus	Forcats	17–19	17.47	23.74	10.31	3.5	62	0.60	0.21	2.79	0.98	0.43	2.28
		La Reparada	19	8.21	19.33	12.74	2	49	0.39	0.27	1.42	−0.67	0.55	−1.23
		El Gat	14–16	19.72	18.95	7.44	4	50	0.39	0.17	2.30	0.57	0.34	1.70
	Medes Islands	Medallot	11	5.08	12.70	5.98	4	24	0.14	0.36	0.40	−1.16	0.72	−1.63
		Pedra de Déu	14	9.62	16.27	6.88	1	37	0.32	0.22	1.46	−0.02	0.44	−0.05
		La Reina	13	12.41	18.76	6.04	3	44	0.39	0.18	2.12	1.03	0.37	2.81
		Tascons	18	13.26	22.98	12.37	2	60	0.30	0.23	1.31	0.08	0.46	0.17

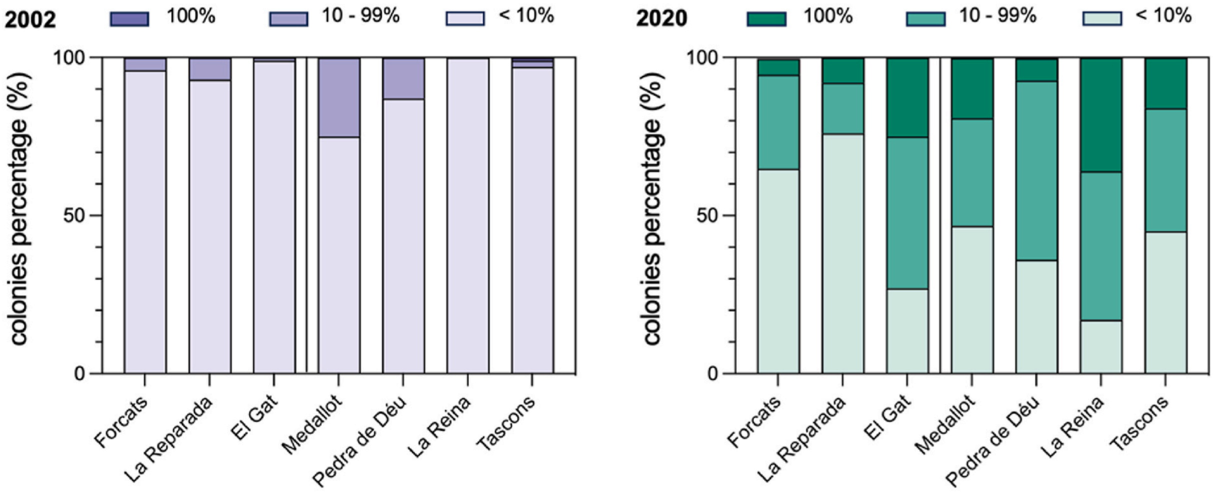

Fig. 3. Percentage of colonies with different levels of affected tissue in 2002 (purple) and in 2020 (green). Colonies with less than 10% of affected tissue (in light color), colonies with 10–99% of affected tissue (in medium color), and dead colonies (in dark color). The vertical line represents the separation between populations from Cap de Creus (on the left) and from Medes Islands (on the right).

Table 2
Number of studied colonies (N), mean percentages of affected tissue (%), and the proportion of healthy colonies (with <10% of injured tissue), affected colonies (between 10 and 99% of injured tissue) and dead colonies (with 100% injured tissue) in the studied populations of *Eunicella singularis*.

Year	Area	Population	N	% affected tissue	SD	% colonies with <10%	% colonies with 10–99%	% colonies with 100%
2002	Cap de Creus	Forcats	47	0.43	2.04	95.74	4.26	0.00
		La Reparada	181	1.71	8.42	94.48	5.52	0.00
		El Gat	90	0.56	5.27	98.89	1.11	0.00
	Medes Islands	Medallot	65	6.85	12.86	75.38	24.62	0.00
		Pedra de Déu	106	2.74	8.40	86.79	13.21	0.00
		La Reina	86	0.00	0.00	100	0.00	0.00
		Tascons	241	1.33	10.04	97.51	1.66	0.83
2020	Cap de Creus	Forcats	131	18.99	31.55	64.89	29.77	5.34
		La Reparada	80	16.46	32.45	76.25	16.25	7.50
		El Gat	212	43.71	39.75	27.36	48.11	24.53
	Medes Islands	Medallot	47	37.85	42.11	46.81	34.04	19.15
		Pedra de Déu	125	22.91	28.74	36.00	56.80	7.20
		La Reina	180	53.48	40.86	16.67	47.22	36.11
		Tascons	116	31.55	39.00	45.69	38.79	15.52

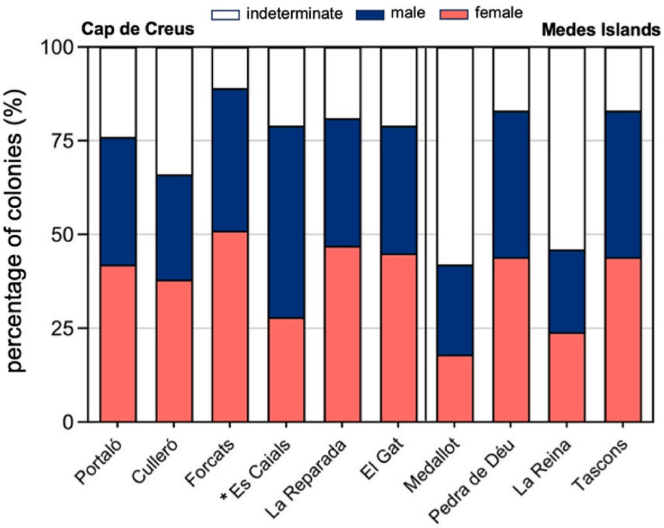

Fig. 4. Sex ratio of the studied populations of *Eunicella singularis*; female colonies are indicated in pink, male colonies in blue, and indeterminate colonies in white. All populations showed a sex ratio not significantly different than 1:1, except for the population of Es Caials (indicated with the asterisk, p-value = 0.011).

3.2. Reproduction

3.2.1. Sex ratio

Sex ratio was not significantly different from 1:1 ($\chi^2 = 2.414$, *p-value = 0.129*) when studying all the colonies sampled in 2021 together (423 female and 379 male colonies), and the only population with a significant deviation was Es Caials ($\chi^2 = 6.531$, *p-value = 0.011*). In contrast, the percentage of indetermined colonies was very high for all populations (11−58%), with a mean value of non-reproducing colonies in the study area of 28 ± 16% (mean ± SD). The maximum percentage of indetermined colonies was found in Medallot (58%), followed by La Reina (54%) (Fig. 4).

3.2.2. Fertility

Differences between sexes were observed regarding the fertility in all the populations (*three-way PERMANOVA, F = 11.4, p-value < 0.001*). While the fertility of male colonies was very high, with an average value of 99.0 ± 1.6% (mean ± SD) and more than half of the populations showing a 100% fertility rate (i.e. all the analyzed polyps presented sexual products), female colonies showed a significantly lower fertility with a mean of 75.2 ± 7.3% (mean ± SD). La Reina was the population with the lowest female fertility, with 65% of reproductive polyps. Higher female fertility was associated to less indetermined colonies in the population ($R^2 = 42.1\%$, *p-value = 0.025*). Consequently, the populations that presented a higher percentage of non-reproducing colonies, also presented lower female fertility values.

3.2.3. Reproductive effort

Significant differences in the size of sexual products were found between sexes and among populations (Fig. 5), with female colonies exhibiting two overlapping cohorts in all studied populations. Both female and male sexual products presented significant differences in size, with populations from northern Cap de Creus (Portaló, Culleró and Forcats) and Medes Islands presenting higher values than the other Cap de Creus populations (*three-way PERMANOVA, F = 42.49, p-value = 0.010; three-way PERMANOVA, F = 56.54, p-value = 0.010*).

In contrast, the opposite pattern was observed regarding the fecundity (i.e. number of sexual products), with no significant differences among Cap de Creus and Medes Islands (*three-way PERMANOVA, F = 0.41, p-value = 0.747*), but important differences between sexes (*three-way PERMANOVA, F = 135.36, p-value < 0.001*). These results indicate a significant inverse relationship between the diameter and the number of sexual products per polyp both for female colonies ($R^2 = 57.2\%$, *r = - 0.79, p-value = 0.006*) and male colonies ($R^2 = 35.5\%$, *r = - 0.65, p-value = 0.041*). The populations that presented larger female sexual products and lower fecundity, also presented the same pattern in male colonies (Fig. 6).

Differences in the volume of sexual products per polyp were observed among sexes and areas (*three-way PERMANOVA, F = 30.30, p-value < 0.001*). Male polyps exhibited higher gonadal volumes than females, with a mean value of 0.303 ± 0.052 mm^3 (mean ± SD) in males and 0.240 ± 0.047 mm^3 (mean ± SD) in females. Finally, La Reina population showed the lowest gonadal volume both in female and male colonies, with mean values of 0.142 ± 0.037 mm^3/polyp (mean ± SD) and 0.231 ± 0.011 mm^3/polyp (mean ± SD), respectively.

3.3. Demography vs reproduction

A significant negative correlation was observed between the fertility of female colonies and the mean percentage of affected tissue in each population ($R^2 = 55.95\%$, *p-value = 0.021*). Populations with a higher percentage of affected tissue exhibited lower fertilities in female colonies compared to those with lower percentages of affected tissue (Fig. 7). Conversely, no significant relationship was found between the fertility of male colonies and the mean percentage of affected tissue ($R^2 = 8.85\%$, *p-value = 0.579*).

3.4. Symbiotic algae

The phylogenetic analyses performed based on the psbA^{ncr} genes showed that the photosymbionts present in the individuals studied of *E. singularis* at four populations of the north of the Catalan coast are more closely related with the species *Philozoon paranemonium* than with the other species of the genus (Fig. 8). All the individuals analyzed clustered together and are differentiated from *Philozoon paranemonium* with a high bootstrap value. No differences were observed in the sequences of the ITS2 marker among the studied populations, neither in a latitudinal gradient nor between more affected and less affected populations.

4. Discussion

The current conservation status of *E. singularis* populations in the study area has been severely compromised during the last two decades, compared to studies from 2002 (Linares et al., 2008a). A significant change in the demographic structure has been observed between 2002 and 2020, explained by a reduction in the recruitment of the species. This is evidenced by the decline in small young colonies (less than 10 cm height), resulting in a significant increase in the minimum heights observed within the populations. This recruitment limitation could be related to sublethal reproductive effects. Given that *E. singularis* is commonly characterized by a high recruitment rate (Linares et al., 2008a), these findings suggest an important change in population dynamics with potential crucial consequences for their resilience to the increasing perturbations. A previous study has already reported decreases in the recruitment rates of *E. singularis* after mass mortality events (Coma et al., 2006), as occurs for other coral species (Hughes et al., 2019). Therefore, sublethal effects can be as important, if not more so, than lethal effects for the future populations' dynamics (Coma et al., 2006; Linares et al., 2005).

Another notable difference from 2002 results is the increased percentage of affected tissue in all studied populations in 2020. Our results show that nearly 60% of the studied colonies were affected in 2020, with 22% being completely dead. A previous experimental study demonstrated that shallow colonies are less tolerant to thermal stress than deeper ones (Pey et al., 2011), since living closer to their maximum thermal limit makes them more susceptible to any increase of

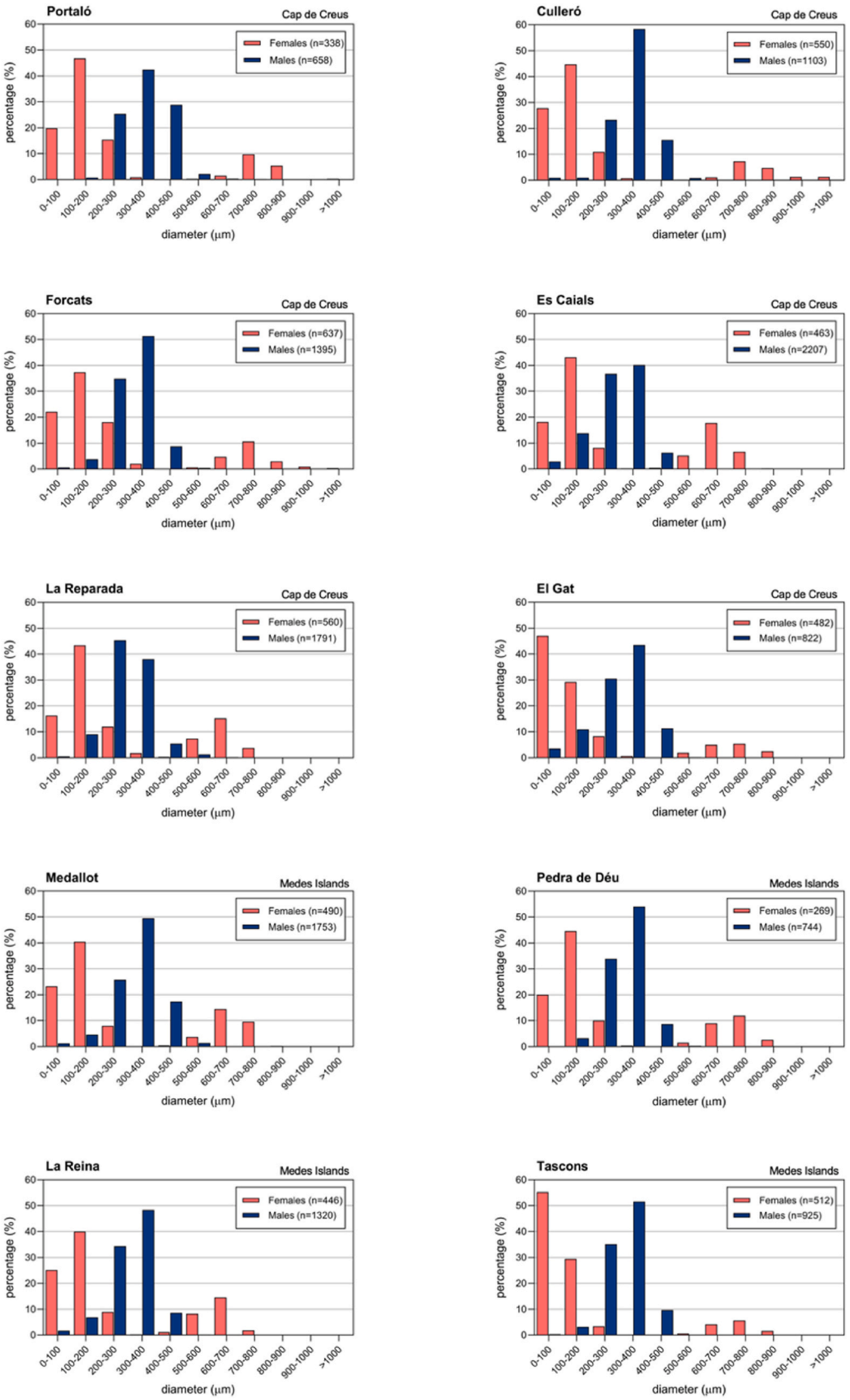

Fig. 5. Size-frequency distribution of the sexual products found in the studied populations. The female sexual products are represented in pink and the male sexual products in blue.

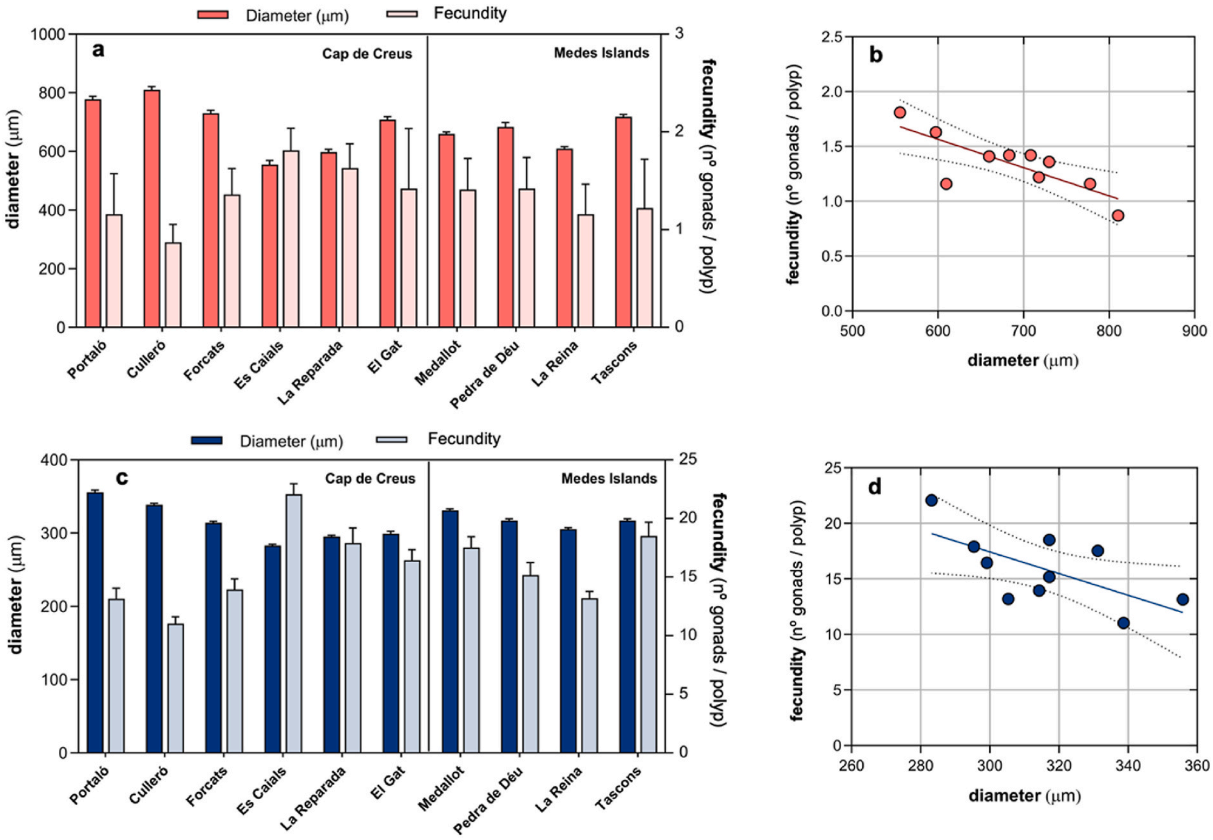

Fig. 6. Reproductive output of the studied populations. a) Diameter (in dark pink) and fecundity (in light pink) for the studied female colonies (mean ± SE). b) Linear regression between fecundity and diameter of female sexual products ($R^2 = 57.2\%$, *p-value* $= 0.006$). c) Diameter (in dark blue) and fecundity (in light blue) for the studied male colonies (mean ± SE). d) Linear regression between fecundity and diameter of male sexual products ($R^2 = 35.5\%$, *p-value* $= 0.041$).

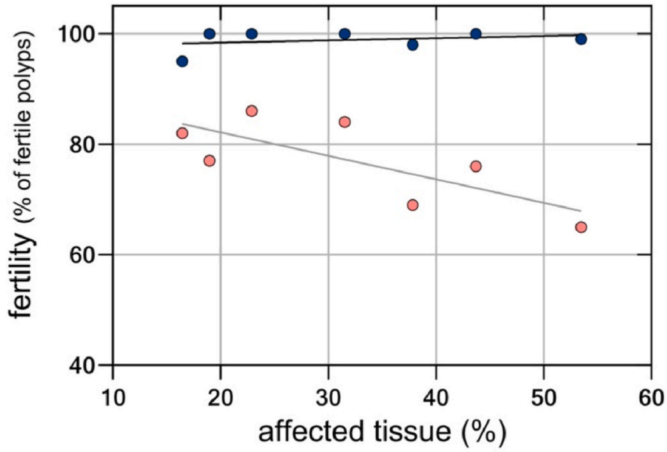

Fig. 7. Linear regression between the fertility of colonies (% of fertile polyps) and the percentage of affected tissue. Female colonies presented a significant relationship ($R^2 = 55.95\%$, *p-value* $= 0.021$) and each population is represented in pink, whereas male colonies (represented in blue) did not show any significant relationship ($R^2 = 8.85\%$, *p-value* $= 0.579$).

temperature. Temperatures above 24 °C contribute to the collapse of the photosynthetic capacity of the symbionts, negatively impacting the energetic resources of the gorgonians and potentially explaining recent mortality events (Ezzat et al., 2013). In contrast, deeper populations are thought to have less stress-induced protein degradation and a higher physiologic plasticity to withstand thermal stress (Pey et al., 2011). Additionally, larvae from Mediterranean gorgonians are highly susceptible to temperature changes, significantly affecting species recruitment (Kipson et al., 2012; Viladrich et al., 2022). Our study focused exclusively on the shallowest range of the species distribution given that the more severe impacts from marine heatwaves have been observed above 25 m depth (Garrabou et al., 2022). Nevertheless, future research should explore deeper depths to fully understand the current condition of *E. singularis* populations along its entire depth distribution (Gori et al., 2011a, 2011b).

Over time, all populations seem to be worsening, with a latitudinal gradient showing higher densities and lower mortalities in northern populations compared to southern ones. This could be attributed to the seawater temperature gradient in the area, as northern populations experience colder conditions than the southern ones (*T-MedNET* database; Bensoussan et al., 2019). Populations from Cap de Creus exhibit a relatively lower percentage of affected tissue and mortality, as well as higher densities, compared to those from Medes Islands. Indeed, Medes Islands populations have been more exposed to MHWs in the last years than those from Cap de Creus, as temperatures in the southern part of the study area are closer to the species' thermal limit (Ezzat et al., 2013), with more days of seawater temperature above 24 °C in the depth range of studied populations (*T-MedNET* database).

Our results indicate a balanced sex ratio (1:1), consistent with previous studies in the same area (Gori et al., 2007; Ribes et al., 2007). However, the proportion of indetermined colonies observed in this study was much higher. In a study performed in 1999–2000 in Tascons (Medes Islands), the mean percentage of indetermined colonies was 4% (Ribes et al., 2007). In contrast, this value increased to 17% in the same population twenty-two years later. Indetermined colonies were important across all studied populations, with Medallot and La Reina exhibiting alarmingly high percentages of non-reproducing colonies (over 50%).

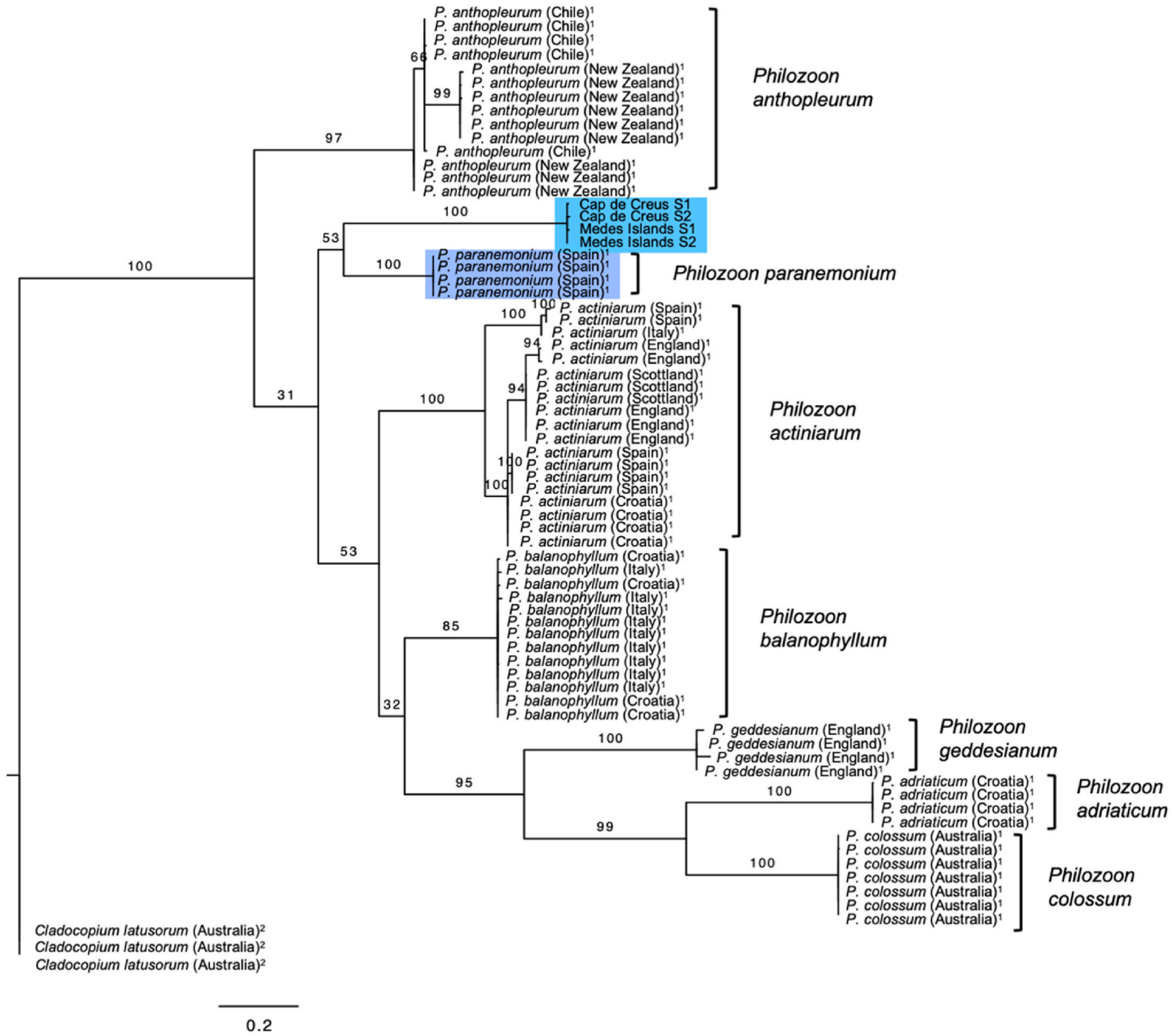

Fig. 8. Maximum likelihood phylogeny of the genus *Philozoon* inferred from psbA^{ncr} sequence alignments (ML: HKY + G as nucleotide substitution model) with four individuals obtained from our *Eunicella singularis* samples, $^{(1)}$ sequences from LaJeunesse et al. (2022), and $^{(2)}$ outgroup sequences from Brown et al. (2022). The Cap de Creus samples were extracted from individuals of Forcats, while the Medes Islands were one from Pedra de Déu and the other from Medallot. Bootstrap support values (100 replicates) given at nodes. Scale bar indicates the expected number of substitutions per site.

The significant correlation between female fertility and the proportion of non-reproducing colonies suggest that stress causes female colonies to first reduce fertility and eventually stop reproducing. While male fertility was consistently high, female colonies showed variable reproductive investment, indicating a greater impact of MHWs on female colonies.

Increased temperature prompt corals to relocate energy to maintain essential functions, reducing resources for reproduction (Gelais et al., 2016). Indeed previous studies have suggested that decreased fecundity in gorgonians could result from an energetic relocation towards producing fewer but larger oocytes (Gori et al., 2013; Linares et al., 2008c; Viladrich et al., 2016), as we observed in our study in the northern Cap de Creus and Medes Islands. The production of larger oocytes has been shown to enhance larval survival and settlement (Johnston et al., 2020). Our results agree with previous findings showing that thermal stress reduces reproductive output in several tropical and Mediterranean species, resulting in the production of fewer but larger sexual products to ensure viable larvae (Airi et al., 2014; Leinbach et al., 2021; Levitan, 2006). Additionally, coral polyps may reabsorb some oocytes to increase energy availability for stress response (Airi et al., 2014; Arizmendi-Mejía et al., 2015; Johnston et al., 2020). Therefore, oocytes resorption could be a possible mechanism explaining the reduced sexual product output in populations affected by MHWs. Conversely, the production of numerous small oocytes in southern Cap de Creus populations may indicate additional stress in this area.

Food input may also significantly affect the reproductive output of Mediterranean gorgonians (Gori et al., 2013). While the mixotrophic strategy of *E. singularis* provides an energetic surplus from symbiotic algae (Cocito et al., 2013; Ezzat et al., 2013), this contribution depends on nutrients availability and heterotrophic inputs (Bedgood et al., 2020; Denis et al., 2024; Tremblay et al., 2016). Reduced nutrient and food availability during summer, combined with increased temperatures and enhanced stratification (Coma et al., 2009), can significantly alter the metabolism and biological processes of benthic organisms (Coma et al., 2000). Previous studies have shown that reproductive output in gorgonians is related to the nutritional conditions of the colonies (Stimson, 1987; Ben-David-Zaslow & Benayahu, 1999; Gori et al., 2012; Viladrich et al., 2017), and food availability is crucial for reducing mortality from thermal stress (Coma et al., 2009; Pey et al., 2013). In our study area, we observed contrasting patterns of primary productivity, with high chlorophyll concentrations in northern Cap de Creus and Medes Islands, and low concentrations along the eastern and southern coast of Cap de Creus (Fig. 1e). Consequently, MHWs in lower-nutrient areas (as the east and south of Cap de Creus in 2021), may cause important sublethal impacts on *E. singularis*, resulting in smaller oocytes. Further studies are needed to better understand the primary productivity patterns between populations, as we only considered satellite data from the sampling months, potentially overlooking the influence of the Ter River on Medes Islands.

For the first time we observed that Symbiodiniaceae individuals inhabiting *E. singularis* are more closely related to the species *Philozoon paranemonium*. However, further analyses are needed to clarify this, as our samples show clear differentiation from *P. paranemonium* sequences. This could be explained by; a) a spatial genetic differentiation, since *P. paranemonium* sequences were collected over 500 km south (Múrcia) of our samples, or b) different species, as *P. paranemonium* is associated with symbionts from *Paranemonia cinerea*, while our host is *E. singularis*. Symbiont species diversity may be more related to the host than geographical distance, as all described *Philozoon* species are closely related to specific host genus (LaJeunesse et al., 2022). Therefore, further studies are required to determine if maybe a new *Philozoon* species is exclusively associated with *E. singularis*.

The genetic marker used to study the population structure of *Philozoon* sp. showed no significant differences between populations or between less and more affected areas. All samples had the same ITS2 marker sequence, closely matching those from previous studies in Italy and southern Spain (with only three base pair differences), corresponding to the previously named *Symbiodinium* temperate clade A (GenBank accession numbers: JN242191.1, Meron et al., 2012; KP761372.1, Grajales et al., 2016), now classified under the genus *Philozoon* (LaJeunesse et al., 2018). This low ITS2 sequence variability suggests some connectivity at the analyzed spatial scale. A similar lack of genetic structure was found for the host *E. singularis* over a spatial scale of 15 km (Costantini et al., 2016). However, more variable markers could help determine if population structure exists. Additionally, a quantitative study of symbiont abundance could reveal differences in concentration, as populations with higher impacts, such as La Reparada or Medallot, might have lower symbiont concentrations. This aligns with findings in other coral species, where temperature increases led to algae cell expulsion, known as coral bleaching (Hoegh-Guldberg, 1999; Roveta et al., 2023).

5. Conclusions

This study reveals a dramatic decline of the populations of *E. singularis* in the north of the Catalan coast, primarily due to the increase of the percentage of affected tissue and dead colonies, but also the increase of the percentage of non-reproducing colonies, the reduced reproductive output, and the decrease in the recruitment of the species. Therefore, our findings demonstrate the importance to investigate both lethal and sublethal effects of MHWs at different temporal and spatial scales. Additionally, our results highlight that this species is much more vulnerable to climate change than previously expected, threatening its long-term viability and conservation, at least at shallow depths.

CRediT authorship contribution statement

Julia Sarda: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation. **Andrea Gori:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Ruth Doñate-Ordóñez:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Núria Viladrich:** Writing – review & editing, Supervision, Methodology, Investigation, Data curation. **Federica Costantini:** Writing – review & editing, Supervision, Resources, Methodology. **Joaquim Garrabou:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Cristina Linares:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marenvres.2024.106822.

Data availability

Data will be made available on request.

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