Reproduction capacity of the white gorgonian *Eunicella singularis* after restoration of colonies sourced from bycatch

Capacidad reproductiva de la gorgonia blanca *Eunicella singularis* después de la restauración de colonias recuperadas de la pesca accidental

Gal·la Edery Jimenez

Master's Degree in Marine Biology: Biodiversity and Conservation

Universidad de La Laguna

September 2021

Núria Viladrich Canudas, Investigadora postdoctoral Marie Sklodowska-Curie, Universitat de Barcelona, y **Marta Sansón Acedo**, Profesora Titular del Departamento de Botánica, Ecología y Fisiología Vegetal de la ULL,

HACEN CONSTAR:

Que la memoria presentada por la Graduada en Biología por la Universitat de Girona **Gal·la Edery Jiménez**, titulada *Reproduction capacity of the white gorgonian Eunicella singularis after restoration of colonies sourced from bycatch* / *Capacidad reproductiva de la gorgonia blanca Eunicella singularis después de la restauración de colonias recuperadas de la pesca accidental*, ha sido realizada bajo nuestra dirección, alcanzando todas las competencias, condiciones de calidad y rigor científico que se requieren para optar a su presentación y defensa como Trabajo de Fin de Máster, en el Máster Universitario en Biología Marina: Biodiversidad y Conservación de la Universidad de La Laguna, curso 2020-2021.

Y para que conste a los efectos oportunos, firmamos el presente, a 06 de septiembre de 2020.

Fdo. Dra. Núria Viladrich Canudas   Fdo. Dra. Marta Sansón Acedo

ACKNOWLEDGMENTS

I feel like this journey has only just begun…

ABSTRACT

In the WE Mediterranean sublitoral communities, gorgonian species play an important role as ecosystem engineers, which are protected by the European Union. However, artisanal fisheries are causing damage to gorgonian populations as they get entangled on nets. Ecological restoration is a trending technic nowadays and its one of the goals included on the Sustainable Development Goals. Several studies have reported survival and growth success in corals restoration, but few is known about the effects in reproduction, needed for a long-term viability of the population. In this study, the reproduction capacity of *Eunicella singularis* has been tested, one, two, and three years after being sourced from the Cap de Creus artisanal fisheries bycatch and transplanted. Three different colony sizes (small, medium, and large) were also tested, as has been seen as an important factor in reproduction. Diameter of sexual products, number and volume per polyp had been measured to estimate the reproductive capacity. Results suggest that transplantation cause a reduction in the reproduction capacity on *E. singularis* colonies, with higher effect on males, contrary to what has been seen previously. Despite the loss of colonies produced, smaller females show better reproductive capacity than males, which has been observed in large colonies. Moreover, colonies appear not to recover during the study period, what suggest that transplantation could lead to the complete mortality of transplants over time. An unexpected algal bloom occurs during the experiment and has been suggested to also cause a sublethal effect, changing oogenesis timing and reducing the reproduction capacity of both sexes, creating a synergy with transplantation.

KEY WORDS: *Eunicella singularis* · Gorgonian · Restoration · Reproduction · Mediterranean Sea

RESUMEN

En las comunidades sublitorales del Mediterráneo Occidental, las especies de gorgonias juegan un papel importante como ingenieras de ecosistemas, y cuyos hábitats están protegidos por la Unión Europea. Sin embargo, la pesca artesanal está causando daños a las poblaciones de gorgonias al enredarse en las redes. La restauración ecológica es una técnica en tendencia actualmente y es uno de los objetivos incluidos en los Objetivos de Desarrollo Sostenible. Varios estudios han reportado buenos resultados en la supervivencia y crecimiento de la restauración corales, pero pocos han evaluado los efectos en la reproducción, necesaria para la viabilidad a largo plazo de la población. En este estudio se examina la capacidad de reproducción de *Eunicella singularis* tras uno, dos y tres años después de haber sido recuperada de la captura incidental de la pesca artesanal en el Cap de Creus y trasplantada de nuevo a su hábitat. Se realizaron tres tamaños diferentes de colonias (pequeñas, medianas y grandes), ya que se ha visto como un factor importante en la reproducción. Se midió el diámetro de los productos sexuales y el número y el volumen de estos pólipo para estimar la capacidad reproductiva. Los resultados sugieren que el trasplante provoca una reducción de la capacidad reproductiva en las colonias de *E. singularis*, con mayor efecto en los machos, al contrario de lo visto anteriormente. A pesar de la pérdida de colonias producida, las hembras más pequeñas muestran mejor capacidad de reproducción a diferencia de los machos, que se ha observado en las colonias grandes. Además, las colonias no parecen recuperarse durante el período de estudio, lo que sugiere que el trasplante podría conducir a la mortalidad completa de los trasplantes con el tiempo. Se produjo un bloom algal inesperado durante el experimento y se ha sugerido que también causa un efecto subletal, cambiando el tiempo de ovogénesis y reduciendo la capacidad de reproducción de ambos sexos, creando una sinergia con el trasplante.

PALABRAS CLAVE: *Eunicella singularis* · Gorgonia · Restauración · Reproducción · Mar Mediterráneo

CONTENTS

INTRODUCTION

Gorgonians belong to the Subcalss Octocorallia (Cnidaria: Antozoa) and comprise more than 3,500 species (Daly et al., 2007; Williams & Cairns, 2019). Gorgonians are sessile invertebrates of benthic communities, with a colonial polypoid structure supported by an axial organic skeleton made of proteinaceous material called gorgonine (Ehrlich, 2010). Each polyp is composed by (1) the coelenteron, which has several functions such as digestion, circulation and reproduction, (2) the oral disk and (3) the eight pinnately branched tentacles (Fautin & Mariscal, 1991). They are long lived organisms with generally slow-growing and low population dynamics (Coma et al., 1998; Garrabou & Harmelin, 2002; Linares et al., 2007; Linares et al., 2010).

In the Mediterranean sublittoral communities, gorgonians are the main structural species of the benthic sessile invertebrates and they play an important role as ecosystem engineers (Jones et al., 1994). Their three-dimensional structure can change the hydrodynamic regime by flow retention and cause sediment accumulation (Eckman, 1985; Bruno & Bertness, 2001). They also creates a gradient of environmental conditions that provide habitat for other species, increasing the biomass and the diversity of the community (Wendt & O'Rourke, 1985; Gili & Coma, 1998; Cerrano et al., 2009; Ponti et al., 2018), which is comparable with the tropical coral reefs (Ballesteros, 2006; Boudouresque et al., 2016). Moreover, by capturing plankton and dissolved organic matter (DOM) from the water column, they play a key role in benthic-pelagic coupling processes and biogeochemical cycles (Gili & Coma, 1998).

As previously mentioned, gorgonians are sessile organisms with slow-growing and slow populations dynamics, making them especially vulnerable to the ongoing global changes. Several studies have reported mass mortality events in the Mediterranean Sea due to storms (Bavestrello et al., 1994; Betti et al., 2021) or extreme temperatures (Cerrano et al., 2005; Coma et al., 2006; Garrabou et al., 2009). These events do not only cause mortality, but also changes on distribution patters, lower growth rates, higher risk of diseases and reduction of reproductive capacity to gorgonians populations (Coma et al., 2004; Linares et al., 2007; Arizmendi-Mejía et al., 2015), that can result in a drastic loss of their populations but also at both the community and the ecosystem level. However, the gorgonians population also declines due to other direct effects caused by

anthropogenic actions, such as anchoring (Bavestrello et al., 1997; Francour et al., 1999), diving (Coma et al., 2004; Silva, 2012) and fishing activities (Bavestrello et al., 1997; Witherell & Coon, 2000).

Particularly, as some commercial fish species are often associated to gorgonian communities (Krieger & Wing, 2002; Henry & Roberts, 2007), they are largely exploited by artisanal and recreational fisheries using trammel nets, longlines and fishing rods. Gorgonians, due to their erect and branching morphology, can get entangled in the nets causing branching fragmentation, total detachment of the colonies or coenenchyma abrasion, letting denuded the axial skeleton and getting them vulnerable to colonisation by epibionts (Bavestrello et al., 1997; Witherell & Coon, 2000; Glover & Smith, 2003; Dias et al., 2020). Moreover, the damage is not only produced during the fishing activity, but also when nets are lost, causing ghost fishing (Chiappone et al., 2005; Macfadyen et al., 2009).

As the natural recovery of corals and gorgonians could take long time due to their biological characteristics, ecological restoration is becoming a trending technic now a days. Ecological restoration was defined by SER (2004) as «the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed» and that «the restored ecosystem has to be self-sustainable and have the potential to persist indefinitely». It has been implemented in many parts of the world, from shallow tropical to deep water coral species (see Montero-Serra et al., 2018 and Boström-Einarsson et al., 2020).

The studies already done on coral restoration reported more than 50% average annual survival and a positive growth rate during the study periods (Yoshioka & Yoshioka, 1991; Fava et al., 2010; Montero-Serra et al., 2018), suggesting that transplantation could be a good technic to enhance its conservation and mitigate some impacts (Guzmán, 1991; Linares et al., 2007; Montseny et al., 2019, 2020; Ferreira, 2020).

Although restoration is practised trough local actions, it has regional and global benefits for nature and people (SER, 2021). In the Sustainable Development Goals (SDGs) recently published by the United Nations Development Programme (UNDP), the Goal 14 for Life Below Water includes a specific objective for «sustainably manage and protect marine and coastal ecosystems to avoid significant adverse impacts, including by strengthening their resilience, and take action for their restoration in order to achieve

healthy and productive oceans» (UNDP, 2021). Moreover, European Union has the Directive 2008/56/EC "Strategy for the marine environment", the first binding law for the conservation, protection, and restoration of marine ecosystems, and the Council Directive 92/43/CEE "on the conservation of natural habitats and of wild fauna and flora" which include de Mediterranean coralligenous habitat.

One of the gorgonians restoration projects that are currently being implemented in the Western Mediterranean Sea is RESCAP. They recovered *Eunicella cavolini* colonies from the fisheries bycatch at 40-60 m depth and reintroduced them using a technic they called 'badminton method' (Montseny et al., 2020). They were monitored to assess the success and found that, in good bottom conditions, 85% of the colonies where detected after a year (Montseny et al., 2021).

Despite this global concern, most restoration studies focus on survival and growth rates of corals after transplantation, neglecting the possible sublethal effects driven by adaptative mechanisms of corals. Adaptative mechanisms have an additional energetic cost, which could cause transgenerational effects on the viability of future offspring (Szmant & Gassman, 1990; Ayre & Hughes, 2004).

Accordingly, sexual reproduction is an important factor to consider. Although, asexual reproduction is a good strategy to increase the population (MacFadden, 1991), the dispersal capacity is limited and could result in less genetic variability. Contrary, sexual reproduction enhances genetic variability which confers greater resistance capacity to individuals to face adversities (Zayasu & Suzuki, 2019). Moreover, as larvae are mobile, the dispersal capacity is greater and, consequently, leads to an increase in gene flow between populations (Palumbi, 1994; Heller & Zavaleta, 2009; Hart & Marko, 2010). Thus, if those sublethal effects affect the sexual reproduction, it may severely undermine the long-term viability of coral populations.

There are few studies that assess the effect of transplantation on the sexual reproductive capacity of the colonies. Colony size fragmentation has been seen to have consequences in reproduction (Guest et al., 2007; Okubo et al., 2007; Okubo et al., 2009). Kai and Sakai (2008) have also tested the age of the transplants in the gamete production, suggesting that the age of the donor colony could determine the reproduction in the fragments. This possible effect of colony size on reproduction has also been observed in natural

populations. Indeed, Coma et al. (1995) showed that reproductive output differs between colony sizes with a highly contribution from large colonies. Additionally, no studies were found assessing the response of male colonies to transplantation, as they were assumed to be more resilient than females (Cerrano et al., 2005), or were directly discarded because they are more difficult to study due to the shorter spermatogenesis time period (Guest et al., 2007).

The aim of this study was to evaluate the reproductive capacity of *E. singularis* colonies after being rescued from fisheries bycatch and transplanted back to their habitat.

The white gorgonian *Eunicella singularis* (Esper, 1794) (Figure 1) is one of the most representative species of octocorals of the Western Mediterranean coralligenous and precoralligenous communities from 5 to 70 m depth (Carpine & Grassshoff, 1975; Gili & Ros, 1985; Gori et al., 2011; Harmelin & Garrabou, 2005; Weinberg, 1979). It is the only Mediterranean octocoral living in symbiosis with photosynthetic dinoflagellates of the genus *Symbiodinium* sp. (Weinberg, 1976; Forcioli et al., 2011), having mixotrophic strategy (i.e. autotrophic and heterotrophy feeding) (Ezzat et al., 2013). *E. singularis* is a long-lived specie and the fastest growing Mediterranean gorgonian (Viladrich et al., 2018).
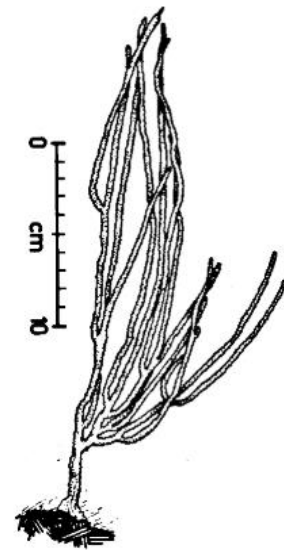
Figure 1. Draw of *Eunicella singularis* from Weinberg (1976).

E. singularis is a gonochoric species with annual sexual reproduction between May and June or July, depending on the location and the environmental factors (Gori et al., 2012). It presents different timing of sexual production, as spermatogenesis takes 4-6 months and oogenesis takes 13-17 months (Ribes et al., 2007). Females show two overlapping oocyte size cohorts: the first cohort comprises an oocytes diameter range between >0 - 300 µm and the second cohort comprises oocytes with diameters bigger than 300 µm. Female polyps have a low oocyte production compared to other Mediterranean gorgonians (Gori et al., 2007). Spermatic sacs in males comprises diameters between > 0 – 700 µm and a higher production of spermatic sacs per polyp (Ribes et al., 2007; Gori et al., 2007, 2012). Fertilization and embryogenesis take place within female polyps and larvae are released after a few days (Weinberg & Weinberg, 1979), as a strategy to maximize larval survival (Ribes et al., 2007).

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To achieve the objective, colonies of gorgonian *E. singularis* were collected and transplanted for three consecutive years (2016, 2017 and 2018). The first year, colonies were transplanted directly to the field, but many were lost, probably due to their large size and that the currents may have uprooted the colonies. To avoid this and considering the colony size as an important factor for reproduction, the following years colonies were fragmented into three different sizes (small, medium and large). The transplanted colonies were monitored annually, and a little fragment was collected in the year 2019. A fragment from control population was also sampled in the years 2017 and 2019. In the laboratory, reproductive capacity was estimated by measuring the diameter of sexual products, quantifying the number per polyp and their volume (Hall & Hughes, 1996).

The hypotheses are that (1) transplantation has negative effects on reproduction (2) with a greater effect in females (3) and in the smaller colonies, (4) and that the effect decreases over time.

MATERIAL AND METHODS

GORGONIAN COLLECTION AND MAINTENANCE

Colonies of gorgonian *E. singularis* were collected from artisanal fishing bycatch (trammel nets) in 2016, 2017 and 2018 in Cap de Creus (NW Mediterranean Sea, 42°19'12''N; 003°19'34''E) (Figure 2), in a depth range from 15 to 60 m. Fishermen collected gorgonians entangled in trammel nets and kept them in containers filled with surface seawater (~20–23°C). Once back on land (1–2 hr after collection), gorgonians were maintained for few days up to one week maximum, in a 100 L tank filled with seawater maintained at 18 ± 1.0 °C. A submersible pump provided continuous water movement in the tank with a flow rate of 320 L h^{-1}. A chiller (TECO TK2000) was used to maintain seawater temperature, and the water was filtered using a biological filter (SERA 250+UV). The size of the collected colonies ranged from 21 to 34 cm in height.
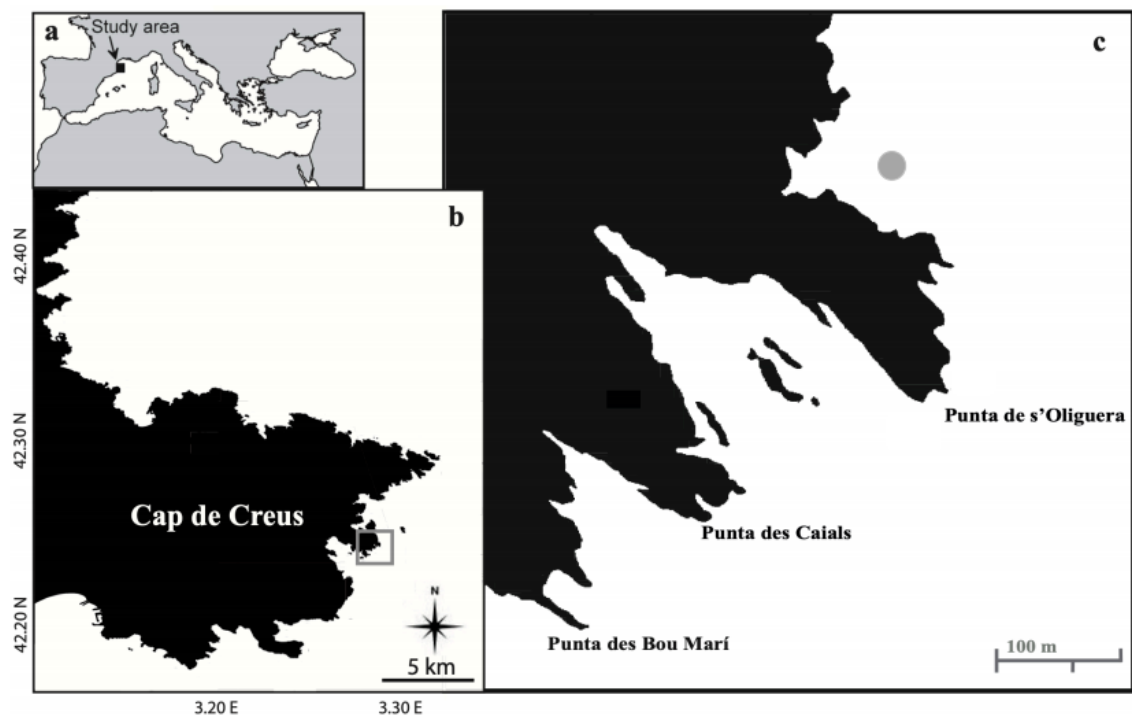

Figure 2. Map of the study area. The grey dot on Figure 2c shows the study area where colonies were transplanted.

GORGONIAN TRANSPLANTS PREPARATION

In 2016, entire gorgonian colonies were transplanted as they were collected from fishermen. Colonies collected in 2017 and 2018 were fragmented into three different

sizes: (A) small, (B) medium, and (C) large (Figure 3). Small colonies consisted in one-two single branches, whereas medium colonies had 2-4 ramifications, and large ones had more than 5 ramifications. All colonies used as transplants did not show any signal of necrotic tissue before being returned to the sea.

| Small | Medium | Large |
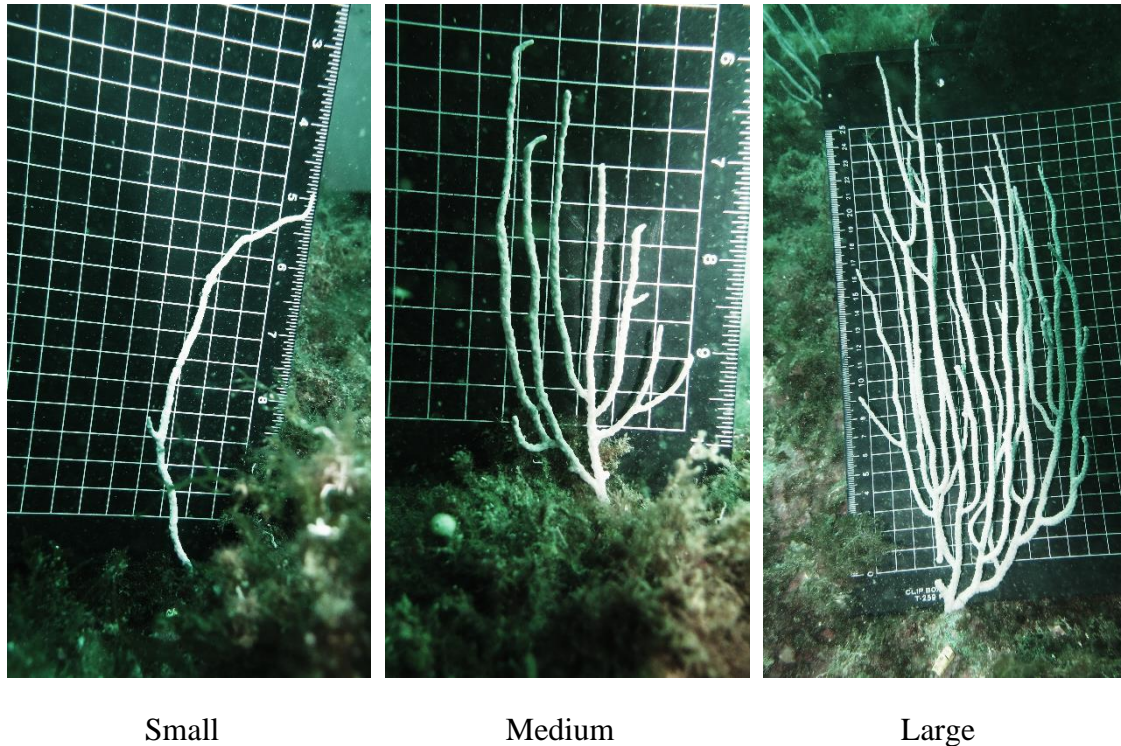
Figure 3. Pictures of transplant colonies of *E. singularis* taken on the field, where different colony sizes could be seen. Scale is in cm.

GORGONIAN TRANSPLANTATION AND MONITORING

Gorgonians were transplanted by scuba diving to 15-20 m depth in Es Bofill (Cap de Creus, 42°17'11''N; 003°17'59''E) using epoxy putty (GROTECH® Corafix SuperFast). The transplant area was characterised by two sub-vertical rocky walls. One wall was facing north and the other facing south. Both rocky walls presented natural *E. singularis* colonies. Colonies collected in 2016 and 2017 were all transplanted on the south-facing rocky wall. However, a bloom of algal turf occurred in spring and early summer of 2017, causing high colony mortality on the south-facing rocky wall (Figure 4). In order to avoid impacts from future algal blooms, all colonies collected in 2018 were transplanted on the north-facing rocky wall. In brief, 25 colonies were transplanted in 2016, 31 colonies in 2017, and 48 colonies in 2018. All colonies were transplanted at the end of spring and monitored every year (presence or absence) until 2019 (Table 1). In beginning of June

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2019, one fragment (~3 cm) of a primary branch was sampled by scuba diving from each living transplanted colony. The fragments were fixed in 10% formalin and analysed to quantify the reproductive effort of the transplanted colonies in terms of oocytes and spermatic sacs production (Ribes et al. 2007). Moreover, 30 additional natural colonies located on the north-facing rocky wall were monitored from year 2016 for survival (presence or absence), and one fragment was sampled from each of them in the beginning of June 2017 and 2019 to quantify the reproductive effort of the natural population (control).

Table 1. Presence and absence of the transplanted colonies and control population during the study period.

	2016	2017	2018	2019
Control	30	30	30	30
1 year			48	22
2 years		31	19	7
3 years	25	12	9	6
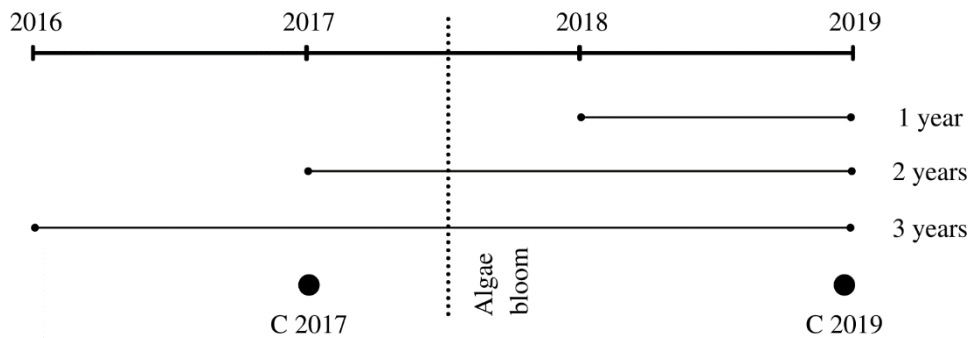
Figure 4. Chronogram of the experimental design. First dot of black bars indicates the year the colonies were transplanted, and the final dot indicates the year the samples were taken. Big black dots indicate the year of control population when samples were taken. Dotted line indicates when the algal bloom took place.

REPRODUCTIVE EFFORT

Sex identification was performed under an optical microscope and according to the colour and appearance of sexual products (Gori et al., 2007; Ribes et al., 2007). Spermatic sacs are pale, while oocytes present darker tonalities, harder consistency and are covered by a spotted membrane (Figure 5 and 6). Whenever possible, five female and five male colonies were examined for the natural population and the transplanted colonies.

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However, due to the observed mortality of transplanted colonies, this was not always possible (Table 2 and 3).

Figure 5. Oocytes from a female colony of *E. singularis*.
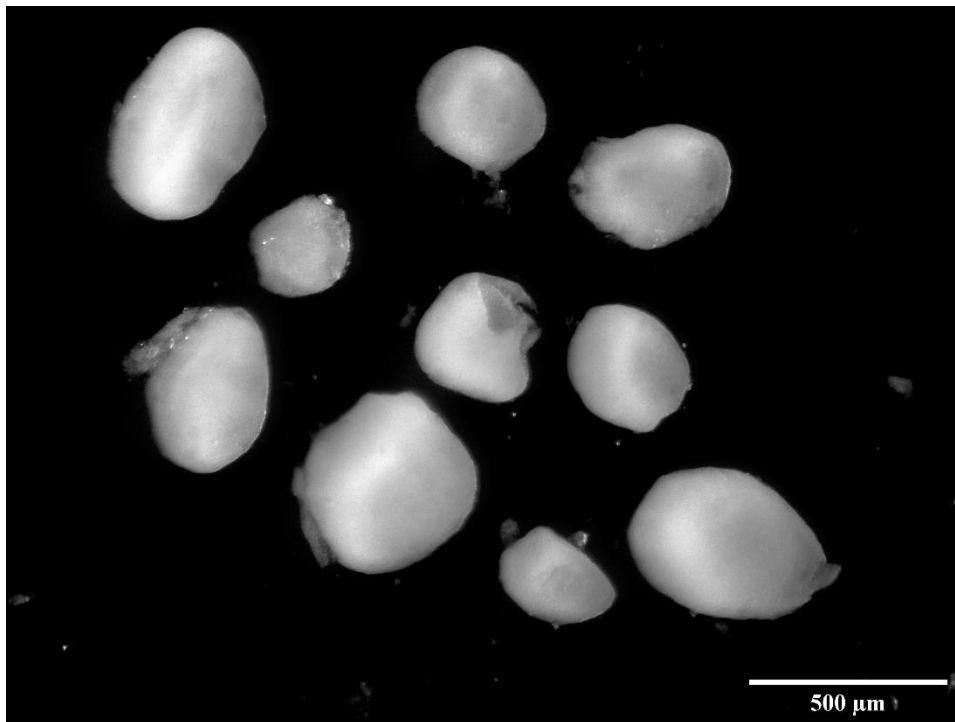
Figure 6. Spermatic sacs from a male colony of *E. singularis*.

Table 2. Colonies examined for each seax and treatment.
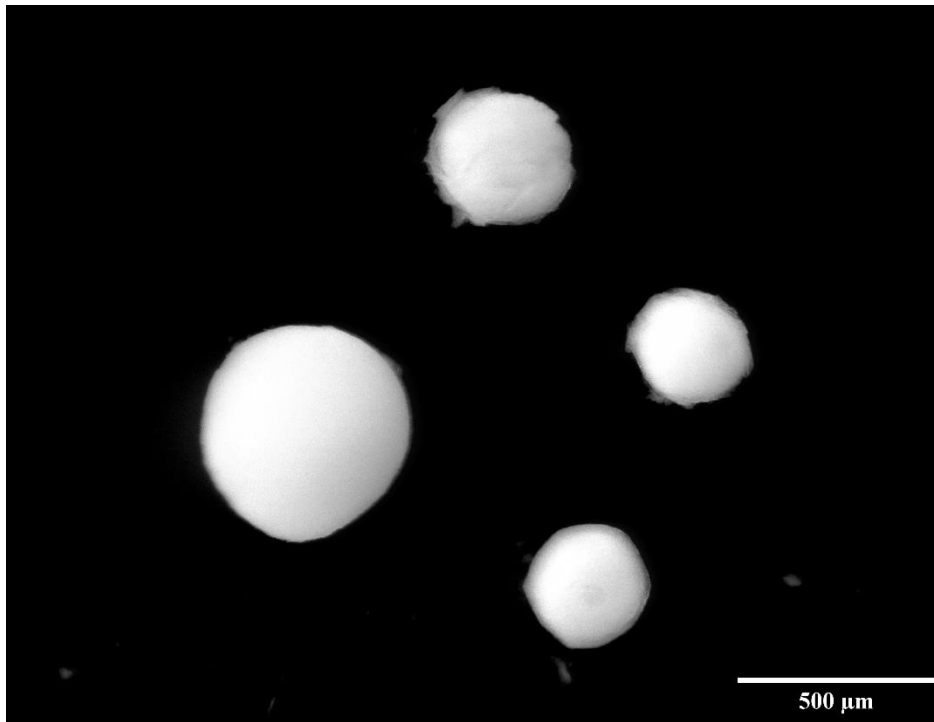
	C 2017	C 2019	1 year	2 years	3 years
F	5	5	15	5	4
M	5	5	6	1	2

Table 3. Colonies examined for each sex, colony size and treatment.

		1 year	2 years
F	Small	6	0
	Medium	5	2
	Large	4	3
M	Small	4	0
	Medium	1	0
	Large	1	1

For each colony, six polyps on the central portion of the branch were haphazardly selected and dissected under a binocular stereomicroscope (Olympus SZX7) (Figure 7). All sexual products from each polyp were photographed with a CMEX-12 camera, and pictures were analysed with the image-processing software ImageJ (Version 1.53e). The software automatically counts the number of sexual products and measures the area and circularity (the proximity of the shape of an object to a circle) of each. Since circularity was always higher than 0.8, all sexual products were considered as spherical, and their measured areas (A) were converted to diameter (d) and volume (V) with the equation:

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

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

A total of 53 polyps were dissected, and 1104 sexual products measured.
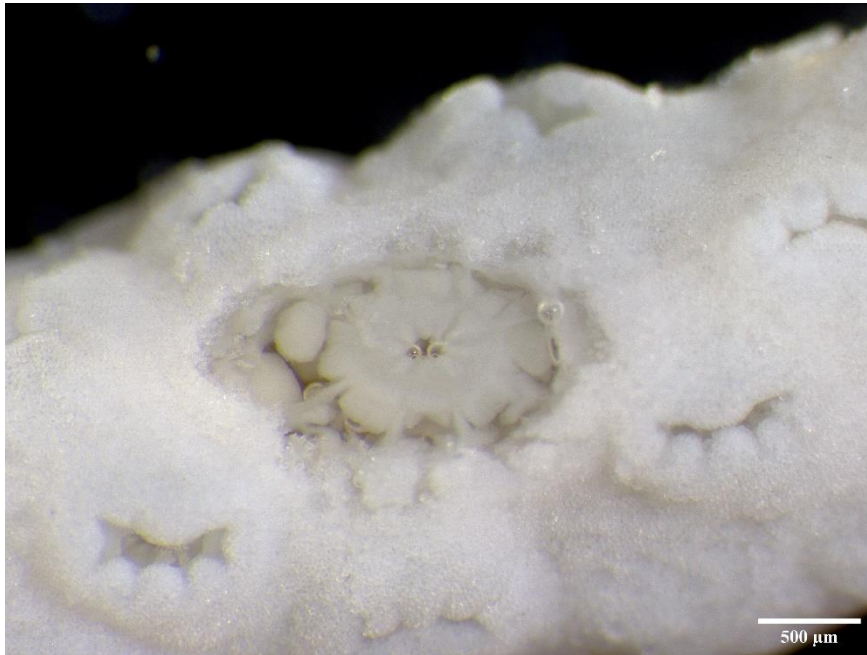
Figure 7. Detail of one opened polyp. It could be seen the coelenteron and oral disk and, on the left side, some sexual products.

DATA ANALYSIS

Diameter of sexual products, number of sexual products per polyp and volume per polyp were the variables used to determine the effect of the transplantation on the reproductive capacity of the colonies. The variables were examined separately by sexes and compared between treatments and colony size for the transplantations in 2018 and 2019. For female colonies, the two cohort where also separated and compared by the same factors.

As Shapiro Wilk test (R software, function 'saphiro.test') showed that data was not normally distributed, the non-parametric Kruskal-Wallis test (R software, function 'kruskal.test') followed by the pairwise Wilcoxon-Mann-Whitney test with Bonferroni adjust method (R software, function 'wilcox.test') were used to examine differences in diameter of sexual products, number of sexual products per polyp and volume per polyp between treatments and colony sizes for each sex. The significance level p-value > 0.05 was used.

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RESULTS

REPRODUCTIVE OUTPUT OF FEMALES

In the frequency distribution of diameters, female colonies showed two overlapping oocyte size cohorts for all treatments. First cohort (> 0 - 300 µm) always presented more oocytes than the second (> 300 - 700 µm) (Figure 8).

The diameter distribution of control population showed changes between 2017 and 2019. In 2017, no oocytes between 300 - 500 µm were observed, whereas in 2019, oocytes were present for all frequencies. Control population also showed less oocytes and higher maximum diameter in 2017 (97 oocytes; 700 µm) than in 2019 (132 oocytes; 650 µm).

Transplanted female colonies one year before, showed a similar diameter frequency distribution to control 2017, as no oocytes were observed between 350 - 450 µm, while the diameter distribution of transplanted colonies two years before was comparable to that of control 2019. After three years,
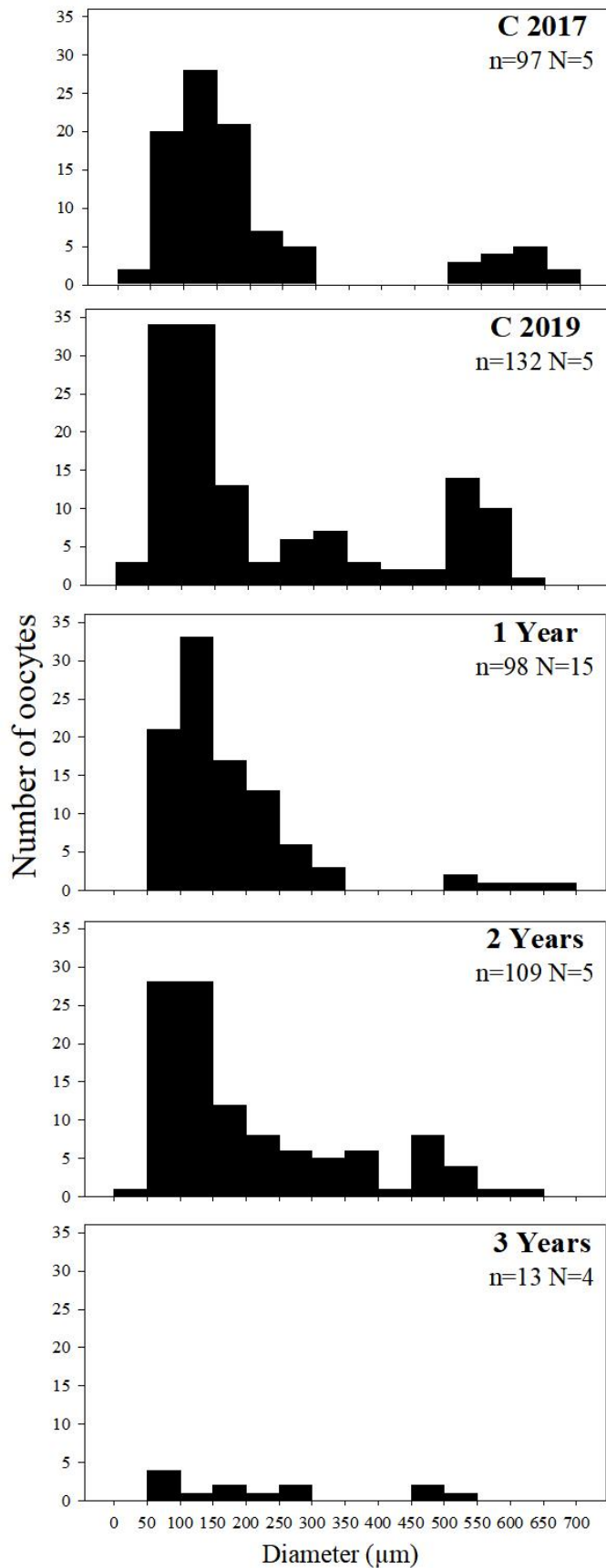

Figure 8. Distribution of oocytes diameter frequency per treatment; n=total number of oocytes, N=number of colonies.

the transplanted female colonies presented the most different diameter frequency distribution, showing very few sexual products (13 oocytes).

In transplanted colonies, the maximum diameter decreased over time, being 700 µm after the first year, 650 µm after the second year and 550 µm the last year.

Although the differences observed on maximum diameter, diameter of oocytes per polyp did not show significant differences between controls and treatments (Kruskal-Wallis test, $p > 0.05$), with a total average of 209.37 ± 7.49 µm (mean ± SE) (Figure 9).
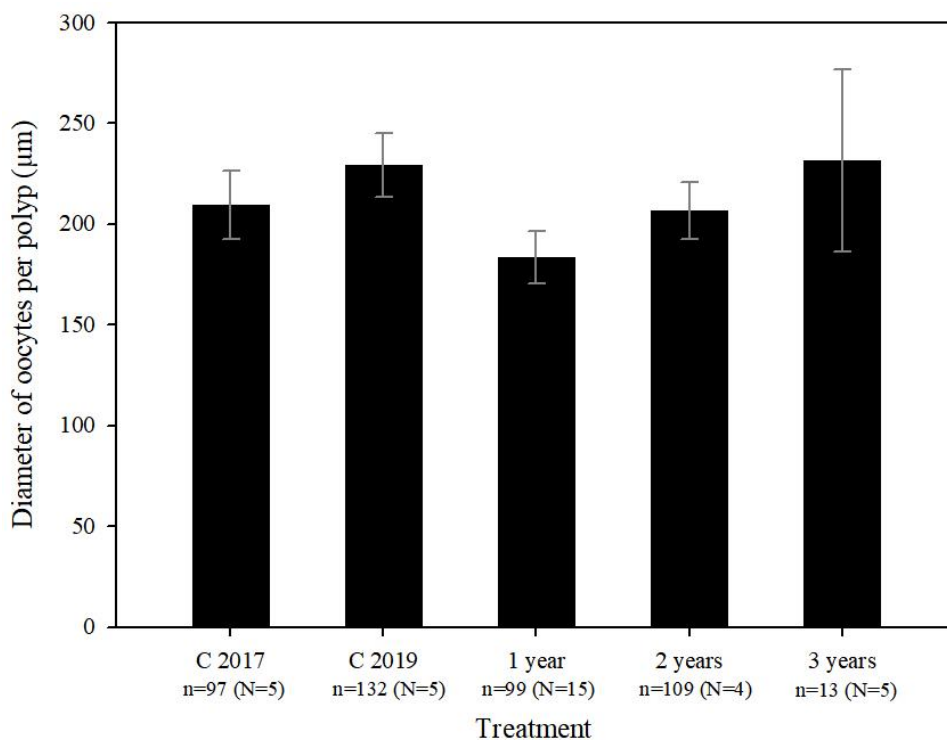
Figure 9. Diameter of oocytes per polyp per treatment (mean ± SE); n=total number of oocytes, N=number of colonies.

When the diameter of oocytes per polyp is analysed by first and second cohort separately, both cohorts of control population showed a significantly higher average in 2017 (144.24 ± 6.09 µm and 595.09 ± 13.09 µm, mean ± SE, respectively) than in 2019 (123.14 ± 5.86 µm and 482.23 ± 15.40 µm, mean ± SE, respectively) (Kruskal-Wallis test, $p < 0.05$) (Figure 10). The first one-year treatment cohort was the only one that showed a wider oocyte diameter average than control 2019 (149.81 ± 6.43 µm, mean ± SE) (Kruskal-Wallis test, $p < 0.05$). For the second cohort, no differences were observed between treatments and control 2019 (Kruskal-Wallis test, $p > 0.05$).
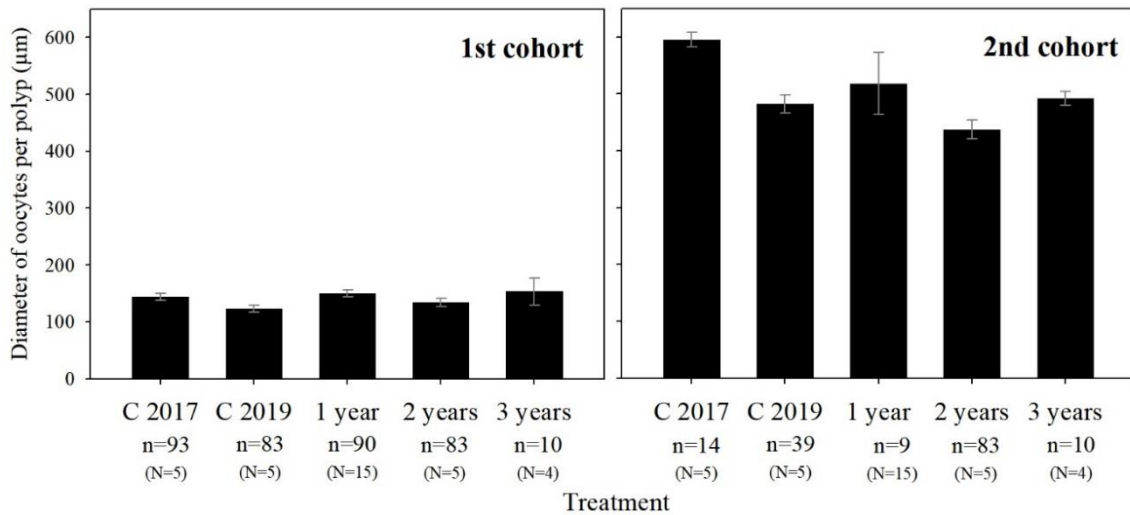
Figure 10. Diameter of oocytes per polyp per treatment (mean ± SE) for 1st and 2nd cohort; n=total number of oocytes, N=number of colonies.

Number of oocytes per polyp did not present statistically differences between controls (Kruskal-Wallis test, $p > 0.05$) (Figure 11). Two years after colonies transplantation, number of oocytes was similar to control 2019 (Kruskal-Wallis test, $p > 0.05$), showing a total average number of 4.02 ± 0.46 oocytes per polyp (mean ± SE). Conversely, transplanted colonies one and three years before presented a highly decline in the number of oocytes (0.98 ± 0.14 μm; total mean ± SE) respect control 2019 (Kruskal-Wallis test, $p < 0.001$).

Figure 11. Number of oocytes per polyp per treatment (mean ± SE); N=number of colonies.

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These differences between controls and treatments were exactly the same as the number of oocytes per polyp separated by cohorts (Figure 12). The couple cohorts did not show differences between controls and transplanted colonies after two years (2.88 ± 0.25 and 0.88 ± 0.15, mean $\pm$ SE, first and second cohort respectively) (Kruskal-Wallis test, $p > 0.05$). Conversely, number of oocytes per polyp was drastically reduced in transplanted colonies after one- and three-years treatments in first and second cohort (0.90 ± 0.14 and 0.11 ± 0.07, mean $\pm$ SE, respectively) respect control 2019 (Kruskal-Wallis test, $p < 0.001$). In all cases, except in the three-years treatment, first cohort showed higher number of oocytes per polyp than second (Kruskal-Wallis test, $p < 0.001$).
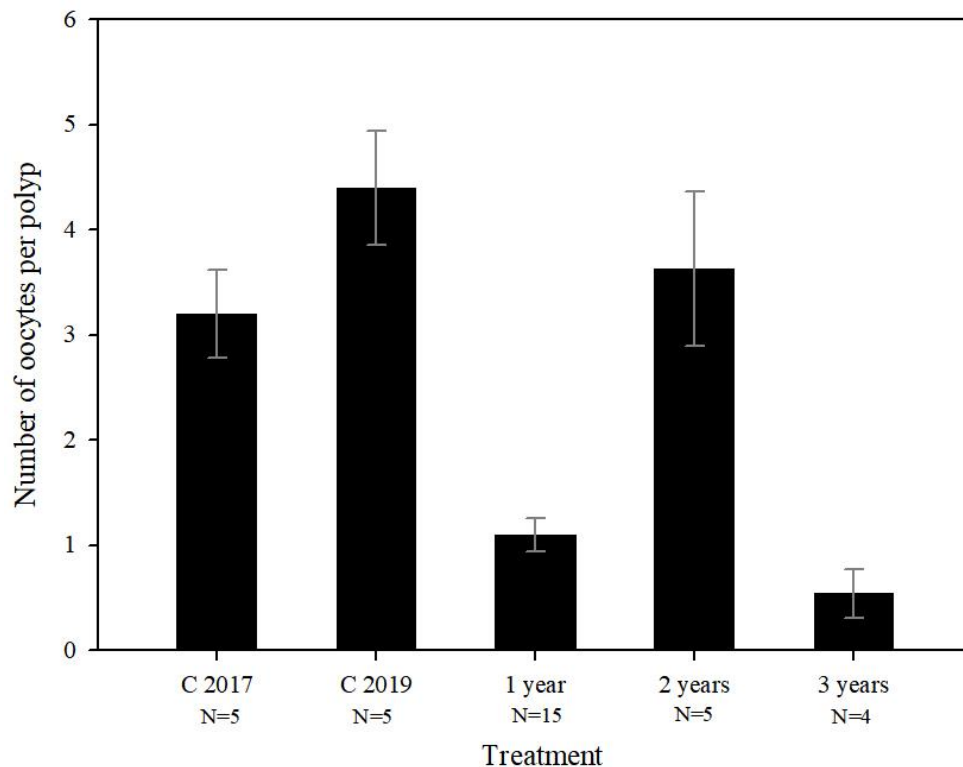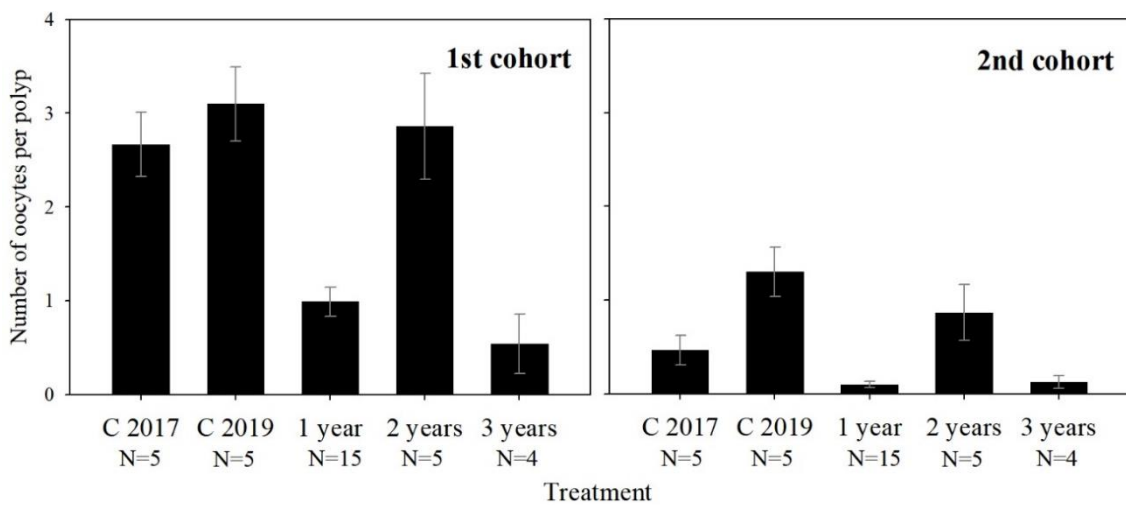

Figure 12. Number of oocytes per polyp per treatment (mean $\pm$ SE) for 1st and 2nd cohort; N=number of colonies.

Volume of oocytes per polyp did not show differences between controls (Kruskal-Wallis test, $p > 0.05$) (Figure 13). Transplanted colonies after two years did not show a different volume of oocytes than control 2019 (Kruskal-Wallis test, $p > 0.05$), showing an average total volume of $69.17 \times 10^6 \pm 13.02 \times 10^6$ μm^3 (mean $\pm$ SE). Contrary, the volume per polyp of the one- and three-years treatments was drastically reduced ($11.28 \times 10^6 \pm 30.55 \times 10^5$ μm^3, total mean $\pm$ SE) respect control 2019 (Kruskal-Wallis test, $p < 0.001$).
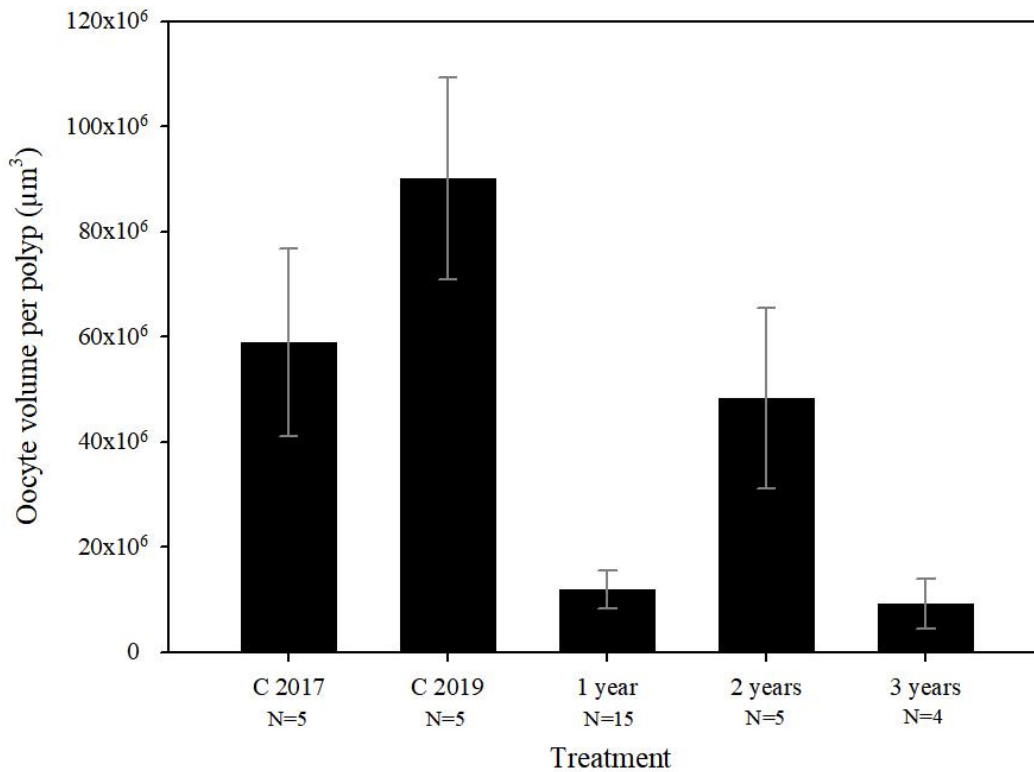
Figure 13. Oocyte volume per polyp per treatment (mean ± SE); N=number of colonies.

As for number of oocytes, the differences observed between volume per polyp and volume per polyp separated by cohorts were very similar (Figure 14). Controls and transplanted colonies after two years did not show differences in both cohorts ($58.61 \times 10^5 \pm 72.62 \times 10^4$ µm^3 and $48.92 \times 10^6 \pm 89.32 \times 10^6$ µm^3, mean ± SE, first and second cohort, respectively) (Kruskal-Wallis test, $p > 0.05$). Conversely, volume per polyp was drastically reduced in transplanted colonies from one- and three-years treatments in the first and the second cohort ($24.73 \times 10^5 \pm 48.33 \times 10^4$ µm^3 and $61.96 \times 10^5 \pm 27.88 \times 10^6$ µm^3, mean ± SE, respectively) respect control 2019 (Kruskal-Wallis test, $p < 0.005$). First cohort of control 2017 and one-year treatment showed lower volume of oocytes per polyp than second cohort (Kruskal-Wallis test, $p < 0.01$). However, control 2019, two- and three-years treatments did not show significant differences in oocyte volume between cohorts (Kruskal-Wallis test, $p > 0.05$).

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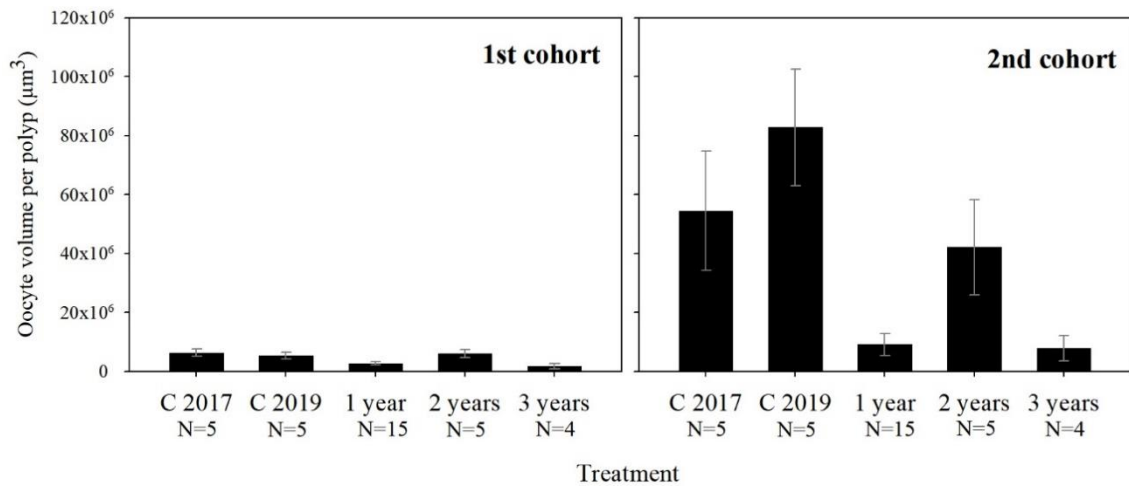
Figure 14. Oocyte volume per polyp per treatment (mean ± SE) for 1st and 2nd cohort; N=number of colonies.

REPRODUCTIVE OUTPUT OF FEMALES ACCORDING TO COLONY SIZE

For transplanted colonies after one year, all sizes had been taken up, however, replication after two-years was very low (2 colonies for medium size and 1 colony for large size) or non-existent for the small size (Figure 15). Therefore, all the presented results in this section on transplanted colonies after two years can only be interpreted as possible tendencies, as no statistical analyses could be performed.

After one year of transplantation, the frequency histogram for each colony size did not show oocytes for all diameter ranges between 0 – 700 µm, and most of them were from the first cohort. Small colonies showed the highest total number of oocytes per colony with 62 oocytes, whereas medium and large colonies only had 18 oocytes. The maximum diameter found for each colony size was 650 µm, 550 µm and 700 µm (small, medium and large, respectively). After two years of transplantation, medium colonies appeared to show a wider range of oocytes diameter with higher maximum diameter and number of oocytes (650 µm; 60) than the large colonies (400 µm; 49).
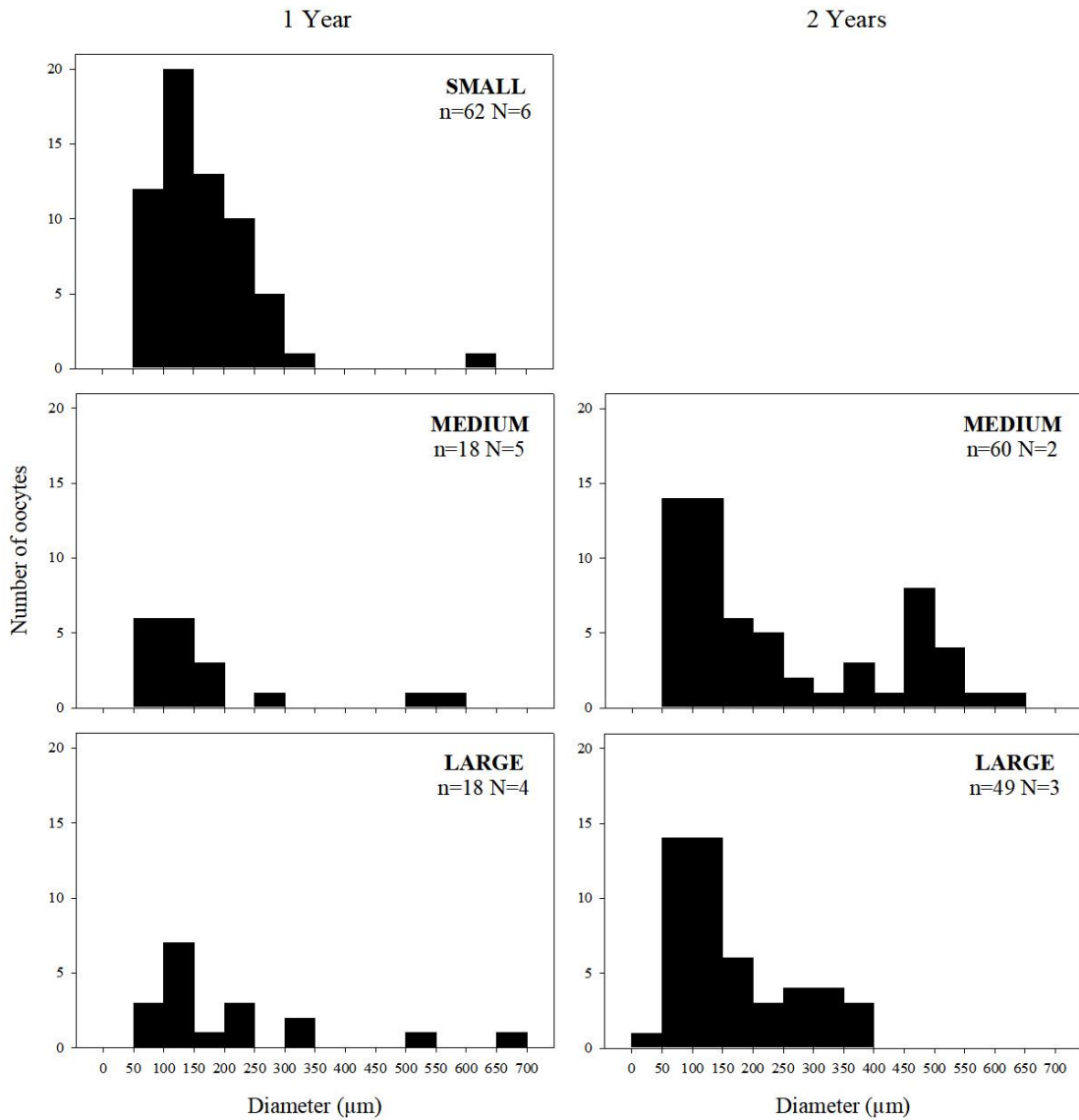
Figure 15. Distribution of oocytes diameter frequency per treatment and colony size; n=total number of oocytes, N=number of colonies.

Diameter of oocytes in transplanted colonies after one year did not show any significant differences between size, (183.92 ± 13.17, total mean ± SE) (Kruskal-Wallis test, $p >$ 0.05) (Figure 16). However, the average diameter tended to increase according to colony size (167.71 ± 11.45 µm, 180.34 ± 37.43 µm and 240.00 ± 45.11 µm, mean ± SE; small, medium, and large colony size, respectively). Respect to transplanted colonies after two years, they also did not show significant differences between size (206.45 ± 14.10 µm, total mean ± SE) (Kruskal-Wallis test, $p >$ 0.05), and the results showed a possible contradictory tendency respect to those of one-year treatment, as the average diameter of medium colonies was higher (240.67 ± 22.17 µm, mean ± SE) than large size (164.53 ± 13.72 µm, mean ± SE).

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Figure 16. Diameter of oocytes per polyp per colony size (mean ± SE) for 1 year and 2 years treatments; n=total number of oocytes, N=number of colonies.

Number of oocytes per polyp of transplanted colonies after one year was higher in small colonies (1.72 ± 0.30; mean ± SE) than medium size (0.6 ± 0.20; mean ± SE) (Kruskal-Wallis test, $p < 0.01$) (Figure 17). Large colonies presented an average of 0.79 ± 0.22 oocytes per polyp (mean ± SE) and did not show differences with the others colony sizes (Kruskal-Wallis test, $p > 0.05$). Conversely, however no significant differences were observed (Kruskal-Wallis test, $p > 0.05$) for two-years treatment, medium colonies presented a possible increase in number of oocytes per polyp (5.00 ± 1.60, mean ± SE) than large size (2.72 ± 0.56, mean ± SE). Moreover, colonies from two-years treatment appeared to have a higher number of oocytes per polyp than one-year treatment for medium and large size colonies.
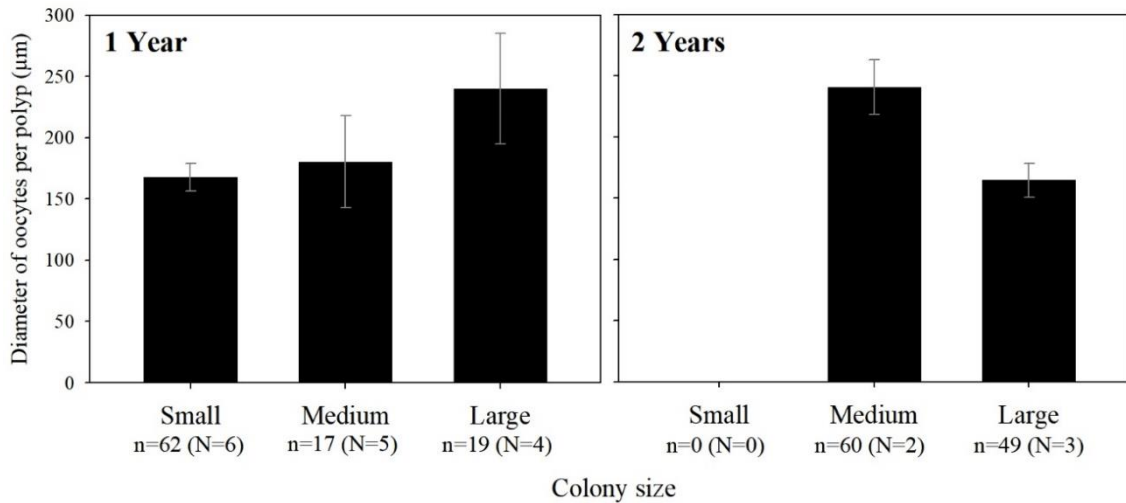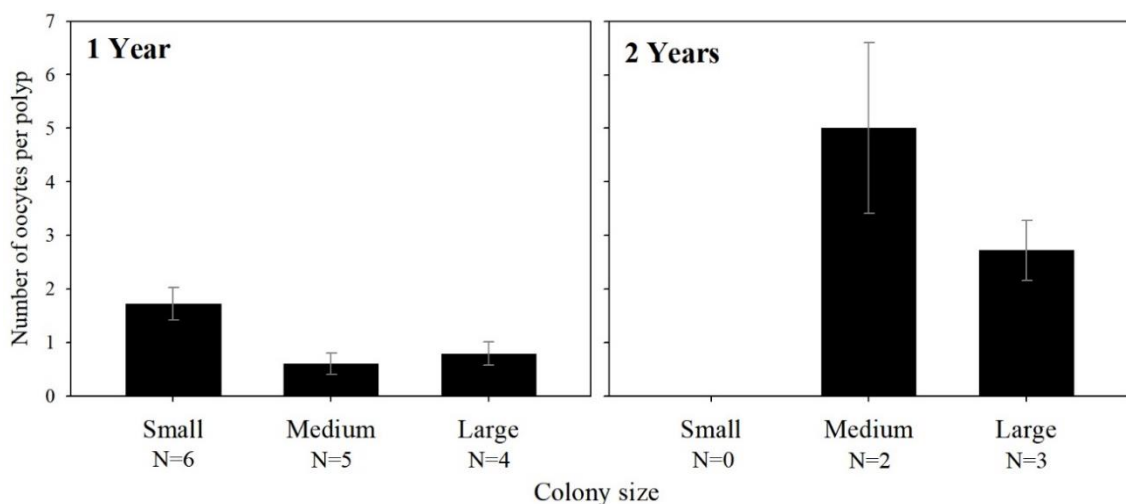

Figure 17. Number of oocytes per polyp per colony size (mean ± SE) for 1 year and 2 years treatments; N=number of colonies.

Volume of oocytes per polyp in one-year treatment presented the highest average volume of oocytes in large colonies ($20.88 \times 10^6 \pm 10.96 \times 10^6\,\mu m^3$, mean $\pm$ SE), however, it did not show significant differences with other sizes (Kruskal-Wallis test, $p > 0.05$) (Figure 18). Small colonies showed a significantly higher volume ($95.23 \times 10^5 \pm 40.20 \times 10^5\,\mu m^3$, mean $\pm$ SE) than the medium colonies ($73.89 \times 10^5 \pm 46.09 \times 10^5\,\mu m^3$, mean $\pm$ SE) (Kruskal-Wallis test, $p < 0.05$). Conversely, and although the few replicates, two-years treatment showed a possible higher volume of oocytes in medium colonies ($10.01 \times 10^7 \pm 38.21 \times 10^6\,\mu m^3$, mean $\pm$ SE) than in large colonies ($13.75 \times 10^6 \pm 51.06 \times 10^5\,\mu m^3$, mean $\pm$ SE). Volume of oocytes of medium size colonies appeared to show highest in two-years treatment, while large colonies appeared to show highest volume in one-year treatment.
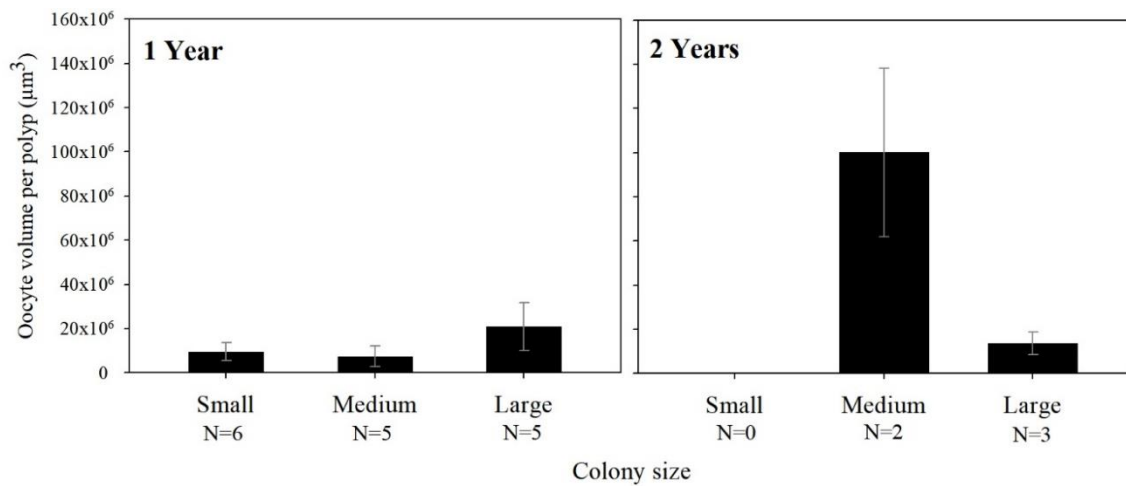
Figure 18. Oocyte volume per polyp per colony size (mean $\pm$ SE) for 1 year and 2 years treatments; N=number of colonies.

REPRODUCTIVE OUTPUT OF MALES

Male colonies for the control population show good replication with 5 colonies taken up for each year (Figure 19). However, the replicates of the treatments were decreasing over time, since for colonies transplanted after one year, 6 colonies were taken up, after two years 1 colony and after three years 2 colonies. Therefore, the results presented for two- and three-years treatments can only be interpreted as possible tendencies, as no statistical analyses could be performed.

Diameter distribution of spermatic sacs ranged between 0-500 µm. Control population exhibited different frequency distribution between years, since in 2017, maximum diameter as well as total number of spermatic sacs, was higher than in 2019 (500 µm, 335; 300 µm, 84, respectively).

The diameter range distribution from one-year treatment was similar to control 2017 (50-500 µm), even so, total number of
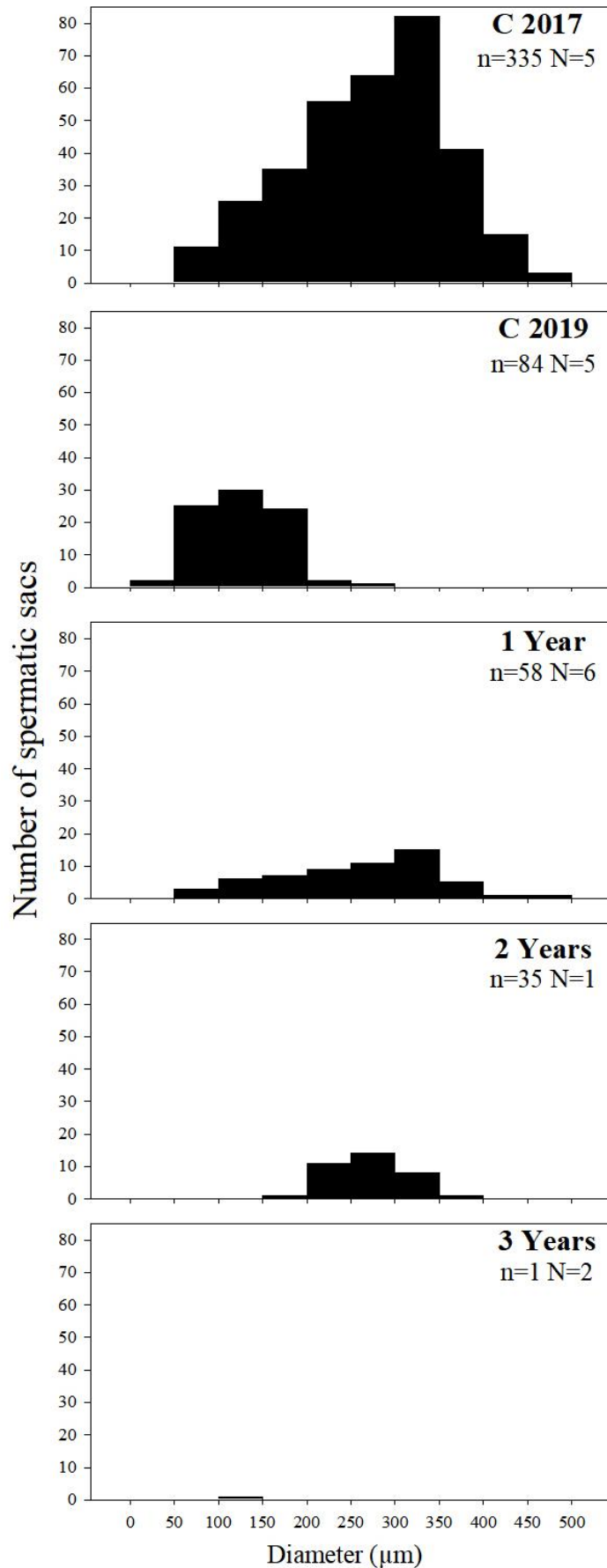
Figure 19. Distribution of spermatic sacs diameter frequency per treatment; n= total number of spermatic sacs, N=number of colonies.

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spermatic sacs was very lower (58 vs 335), and two-years treatment showed a lower range distribution (150 - 400 µm) than both controls. However, both treatments showed a higher maximum diameter than 2019 (300 µm). In three-years treatment, it is important to note that in two male colonies (12 polyps analysed), only one spermatic sac was found.

Spermatic sacs diameter in control population was higher in 2017 (271.39 ± 4.73 µm, mean ± SE) than in 2019 (125.62 ± 4.89 µm, mean ± SE) (Kruskal-Wallis test, $p < 0.001$) (Figure 20). The diameter average of control 2019 was also lower than one- and two-years treatments (261.17 ± 8.01 µm, total mean ± SE) (Kruskal-Wallis test, $p < 0.001$). However, it is important to note that only one colony was sampled in two-years treatment. Three-years treatment showed a single spermatic sac, so it was impossible to compare statistically.
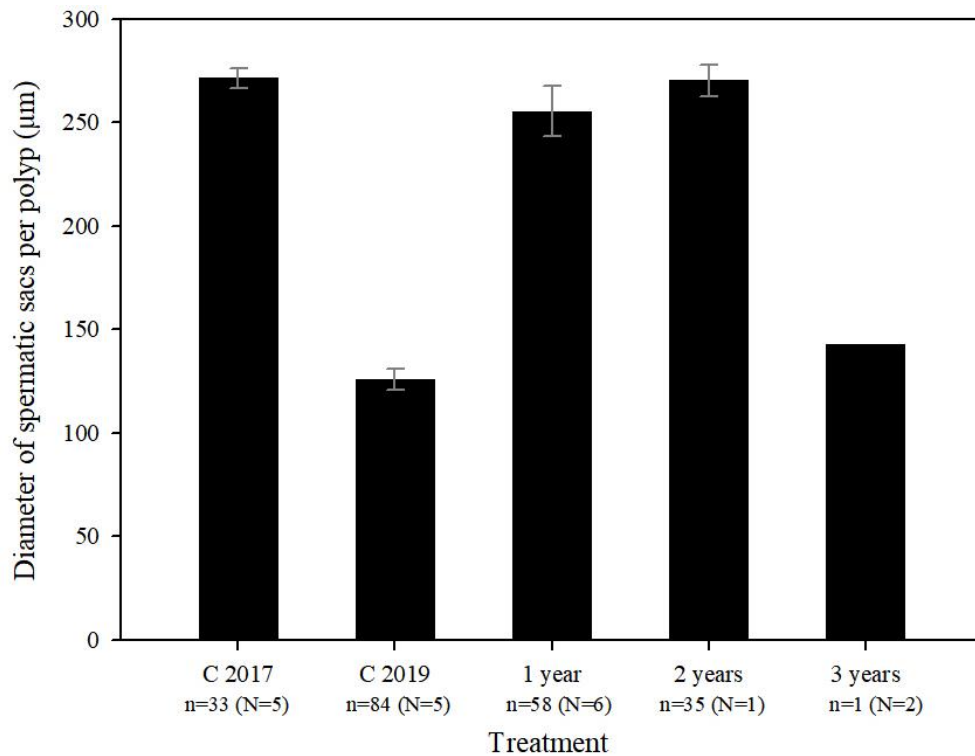
Figure 20. Diameter of spermatic sacs per polyp per treatment (mean ± SE); n=total number of spermatic sacs, N=number of colonies.

Number of spermatic sacs per polyp was drastically reduced in control population from 2017 to 2019 (10.80 ± 1.36 and 2.80 ± 0.89, mean ± SE, respectively) (Kruskal-Wallis test, $p < 0.001$) (Figure 21). One-year treatment did not show significant differences with control 2019 (Kruskal-Wallis test, $p > 0.05$). Even the low replicates, two-years treatment showed a higher average of spermatic sacs (5.83 ± 0.60, mean ± SE) than one-year

treatment, and three-years treatment, as only one spermatic sac was found, the number of spermatic sacs per polyp was the lowest (0.08 ± 0.08, mean ± SE).
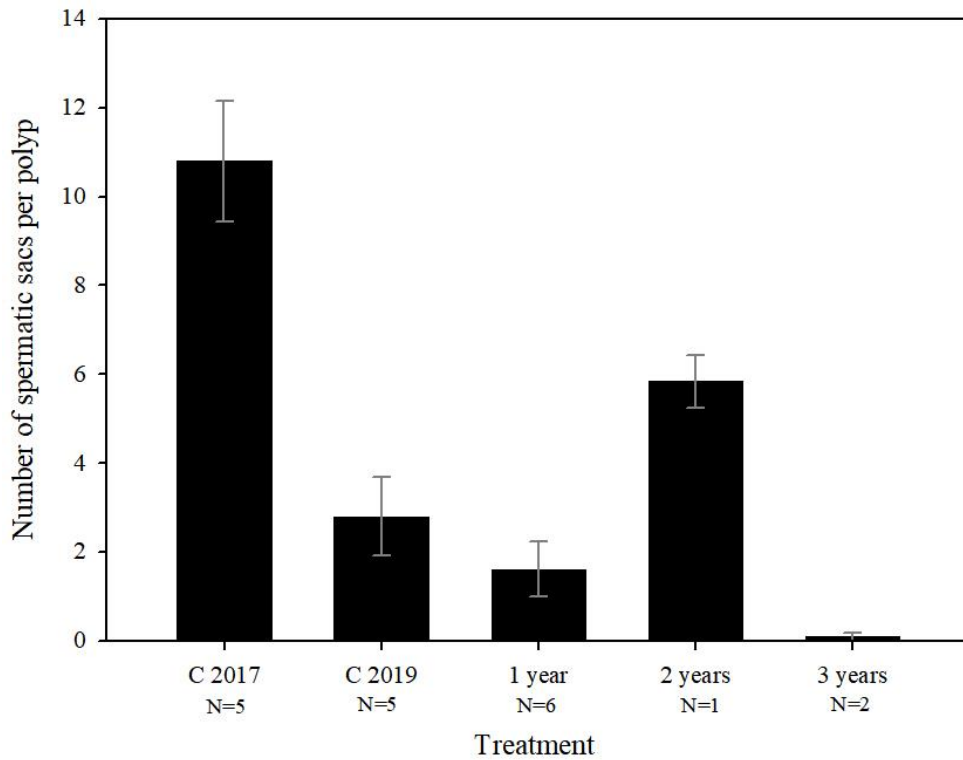
Figure 21. Number of spermatic sacs per polyp per treatment (mean ± SE); N=number of colonies.

As number of spermatic sacs, volume per polyp of control 2017 (149.75x10^6 ± 178.09x10^5 μm^3, mean ± SE) was higher than control 2019 (40.72x10^5 ± 13.17x10^5 μm^3, mean ± SE) (Kruskal-Wallis test, $p < 0.001$) (Figure 22). One year-treatment did not show differences with control 2019 (Kruskal-Wallis test, $p > 0.05$). Also as before, even the few replicates, two-years treatment showed the higher spermatic sacs volume per polyp (65.42x10^6 ± 91.22x10^4 μm^3, mean ± SE) and three-years treatment showed the lowest (12.77x10^4 ± 12.77x10^4 μm^3, mean ± SE).
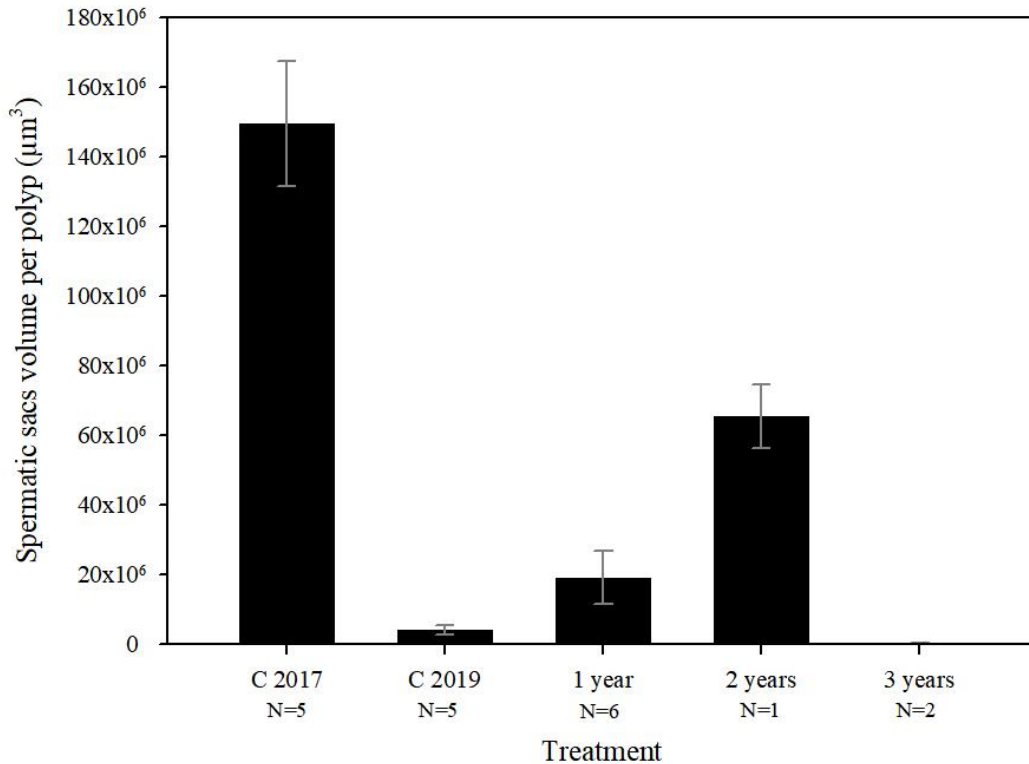
Figure 22. Spermatic sacs volume per polyp per treatment (mean ± SE); N=number of colonies.

REPRODUCTIVE OUTPUT OF MALES ACCORDING TO COLONY SIZE

To colony sizes, replicates were very limited, being four small colonies and one colony of medium and large size for one-year treatment, and only one large colony for two-years treatment (Figure 23). Therefore, all the results presented in this section can only be interpreted as possible tendencies, as no statistical analyses could be done.

The frequency distribution of diameters only presented spermatic sacs in large colonies. Indeed, it is important to note that in four small colonies any spermatic sacs were found. Although only one large colony was analysed for both years, one-year treatment showed wider range of diameters from 50 µm to a maximum diameter of 500 µm and higher number of spermatic sacs (58). Two-years treatment showed a narrower range, from 150 µm to a maximum diameter of 400 µm, and a smaller number of spermatic sacs (35).
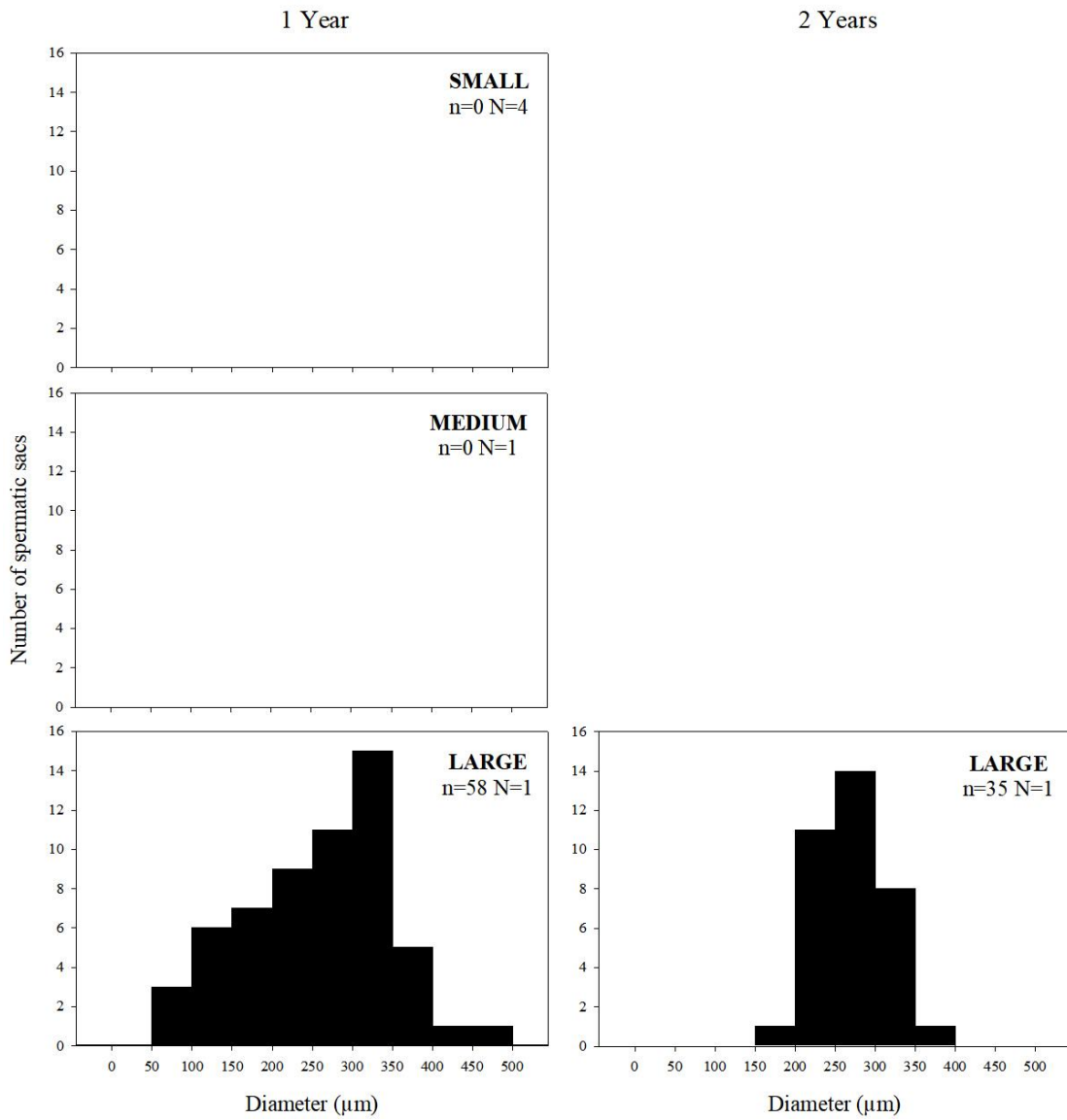
Figure 23. Distribution of spermatic sacs diameter frequency per treatment and colony size; n= total number of spermatic sacs, N=number of colonies.

However, average diameter did not seem to show differences between years (255.62 ± 11.94 μm and 270.35 ± 7.68 μm, mean ± SE, respectively) (Figure 24).
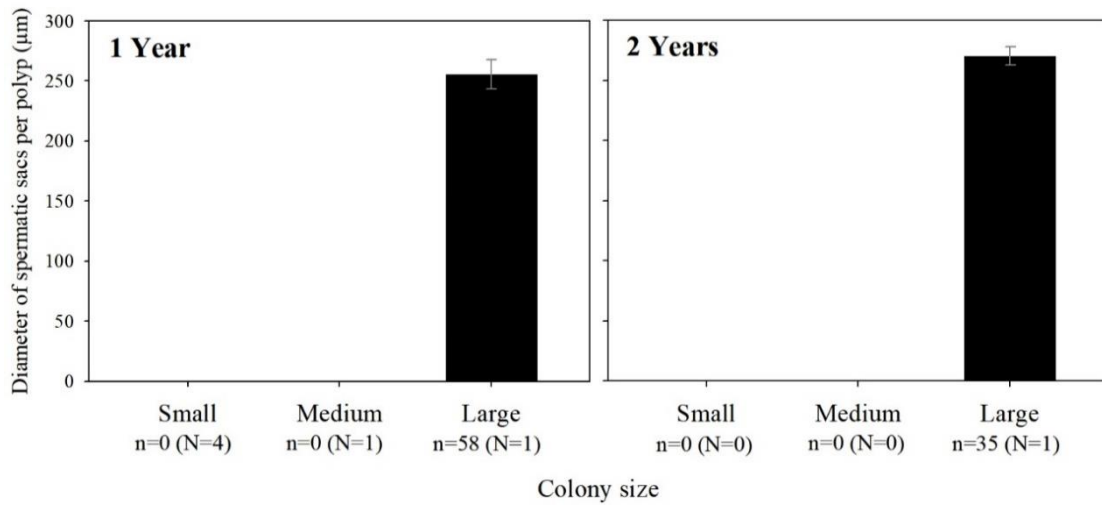
Figure 24. Diameter of spermatic sacs per polyp per colony size (mean ± SE) for 1 year and 2 years treatments; n=total number of spermatic sacs, N=number of colonies.

Number and volume of spermatic sacs per polyp in large colonies seemed to be higher in one-year treatment ($9.67 ± 0.67$, $114.96 \times 10^6 ± 142.51 \times 10^5$ μm3, mean ± SE, respectively) than two-years treatment ($5.84 ± 0.60$, $65.42 \times 10^6 ± 91.22 \times 10^5$ μm3, mean ± SE, respectively) (Figure 25 and 26).
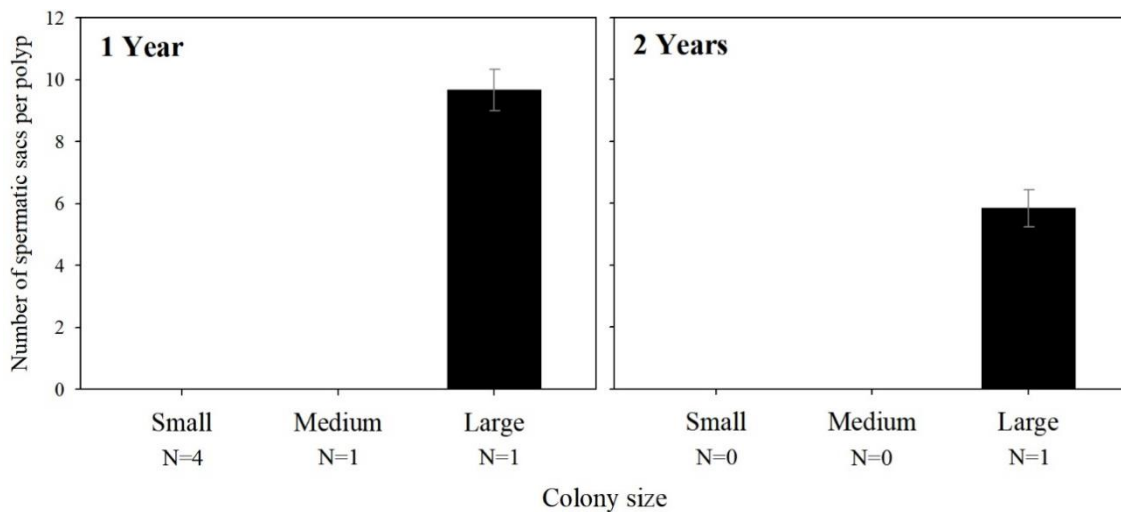
Figure 25. Number of spermatic sacs per polyp per colony size (mean ± SE) for 1 year and 2 years treatments; N=number of colonies.

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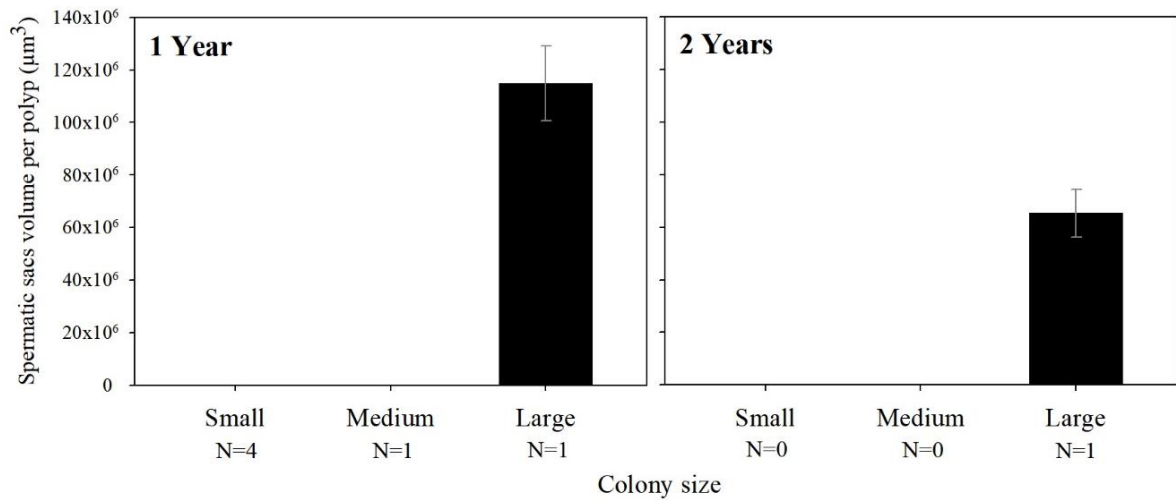
Figure 26. Spermatic sacs volume per polyp per colony size (mean ± SE) for 1 year and 2 years treatments; N=number of colonies.

DISCUSSION

This study offers for the first time a view of the reproductive capacity of *E. singularis* after restoration.

Female colonies have been reported to be more affected to disturbances than males in the Mediterranean gorgonian *Paramuricea clavata* (Linares et al., 2007). However, according to the present results, *E. singularis* showed the opposite, since female colonies presented a better reproductive capacity than males when they have been restored, but also after algal bloom event. Colony sizes of transplanted colonies has been found to be a main challenge, independently from the sex. Large transplants were the first to present less colonies recovered, probably due to their size. The large size can undergo a failure in the attachment technic as they offer more resistance to water currents, causing the detachment from the rocky bottom (Ferreira, 2020). Furthermore, small transplants were diminished over time, probably because they are more vulnerable, as have been seen (Okubo et al., 2009).

In control population, females showed the two cohorts separated in 2017, while in 2019 oocytes were found for all diameter ranges. Gori et al. (2007) and Gori et al. (2012) monitored the reproduction of *E. singularis* during a year and could saw that when maturation increases, cohorts were more differentiated. Thus, in 2017 the maturation could be finished while in 2019 could not. Both samples were taken at the beginning of June, so the same results were expected to be found. The reproduction delay could also explain the lower oocyte diameter found for both cohorts, and the highest number and volume of oocytes in the second cohort in 2019. This possible delay in female reproduction could be explained by a sublethal effect of the algal bloom, which would have caused an increase in stress and the colonies have not yet been able to recover to previous reproductive conditions. It has been seen that *E. sigularis* is more resistant (i.e. high survival) to withstand disturbances such as thermal stress, than other Mediterranean gorgonians (Fava et al., 2010), but it is known that reproduction in corals is sensitive to stress (Linares et al., 2007; Aranceta-Garza et al., 2012). Some studies suggest that the sexual reproductive output of gorgonians seemed to be related to food availability (Ribes et al., 2007). However, *E. singularis* presents a mixotrophic feeding strategy because it hosts *Symbiodinium* sp. in its tissue, what allows it not to be limited by the energy input

(Ezzat et al., 2013; Viladrich et al., 2017). In any case, there are environmental conditions that might not have been noticed and could also cause these differences between controls.

Diameter frequency distribution between treatments present gaps that do not follow any pattern. A possible idea is a different reproductive capacity between polyps of the same colony, and due to the low replicates, these gaps could be more noticed. However, it would need to be studied.

In transplanted colonies, oocytes diameter did not show differences with controls, what might mean that diameter is not determined by external factors, as food availability, however, it could not be observed in these results. It has to be highlighted that *E. singularis* is an internal brooder and larvae are lecithotrophic. This means that when larvae are released from the polyp, they can develop based solely on the maternal provisions transferred during the oogenesis (Thorson, 1950; Pechenik, 1990; Morgan, 1995).

The number of oocytes and, consequently, the volume, were very variable between treatments. Two-years treatment showed the same reproductive capacity as 2019. However, one- and three-years treatment showed a drastic decrease of these variables. If colonies could not show good levels of oocytes after one year, the results of the two-years treatment seems contradictory. Algal bloom occurred just after the transplantation of the two-years treatment, what could affect the normal response of the specie (Hughes, 1989). In this case, *E. Singularis* would be acting as a *r* strategy species, producing as many gametes as possible. Weinberg & Weinberg (1979) suggest that most corals could occupy an intermediate position between the *r* and *K* strategies, as they are long-lived and slow growing but also have a high recruitment rate. After three years, colonies showed a decreased tendency of values respect to one-year treatment, what suggest that transplantation is affecting the reproductive capacity, even the results of the two-years treatment. Indeed, Okubo et al. (2009) reported that the stress produced on *Acropora* coral by the transplantation cause a significant reduction in oocytes number and volume per polyp over time, being non-existent after 3 years.

First cohort always has more oocytes than the second. First cohort probably limits the number of oocytes that can mature, as number cannot increase after the moment they are produced (February - March) (Ribes et al., 2007; Gori et al., 2012). The lower number of

oocytes in the second cohort seems is probably due to not all oocytes matured. Indeed, Okubo et al. (2007) have hypothesized that second cohort oocytes could have been reabsorbed as a survival and adaptation strategy, getting back the resources to have additional energy to face stress of transplantation. One- and three-years treatments showed a lower number of oocytes from first cohort, suggesting that transplantation cause a sublethal effect on the production of oocytes. It has to be noted that first cohort oocytes after one year of transplantation was the only treatment showing significantly higher diameter than control 2019, but contrary, it presents lower number. It seems that one-year transplants could not produce as many oocytes as the natural population, but they provide the maximum of resources they can to those few. This could mean that next generations may be threatened, since the number and, consequently, the possibility of fertility success is getting impoverished.

The effects of colony size on coral reproduction have been suggested species-specific. Indeed, Kai and Sakai (2008) have seen a different response to transplantation in two scleractinian corals. The authors concluded that *Goniastrea aspera* could not be affected by fragmentation, while small colonies of *Favites chinensis* should again reach the size of sexual maturity for the gamete production. The present results showed that *E. singularis* fragments did not lose the reproductive capacity at any size, but the majority of oocytes were from the first cohort, again suggesting the idea of the reabsorption of second cohort oocytes. It has to be noted that small one-year colonies showed the highest number of oocytes, suggesting better adaptability. Indeed, Cerrano et al. (2005) found that small colonies of *P. clavata* were more resilient and suggest that could be due to a lower metabolism and a *Production/Biomass* ratio that decreases with age (Coma et al., 1994; Mistri & Ceccherelli, 1994; Weinbauer & Velimirov, 1995). The reduction in reproductive capacity in large colonies could also be explained by the loss of chemical defences of gorgonians while getting older (Dube et al., 2002). Indeed, it has also been seen that invasive algae have negative effects on gorgonians (Cebrian et al., 2012), producing mechanical and allelopathic interactions or even because of pathogens associated to algae (Nugues, 2004; Kuffner, 2006).

In male colonies, the very low number of samples could suggest that transplanted colonies have suffered high mortality, probably as a consequence of a combined effect of the algal bloom and the transplantation. Therefore, the lethal effects are higher than sublethal effect

and the effects on reproduction caused by transplantation cannot be conclusive. The results can only be taken as a possible response.

Male control population in 2019 showed a decrease in reproductive output compared to 2017. In 2019, spermatic sacs diameter and its distribution was narrower, what coincides with the hypothesized reproduction delay for control females. Spermatogenesis is much shorter than oogenesis (Ribes et al., 2007). Therefore, a delay in the reproduction would lead to a drastic reduction in spermatic sacs size compared to oocytes size. However, it is possible that the drastically reduction of spermatic sacs number could be caused by algal bloom occurred in NW Mediterranean on summer 2017. Gori et al. (2013) showed the same pattern on starved *P. clavata* male colonies, which present the same diameter size as the fed colonies but fewer number of spermatic sacs. Finally, the volume showed a dramatic decrease, as it combines the effect of both, the delay and the algal bloom.

Transplanted males seemed to follow the same strategy as it was suggested for control population, where they may be trying to maintain the diameter of spermatic sacs jeopardizing the number. However, spermatic sacs after three years were almost non-existent, what shows the great affectation suffered by male colonies. Moreover, it needs to me reminded the few replicates of transplanted treatments, as those results could not be reflecting the reality. It has to be highlighted that higher reproductive output for the two-years treatment than the others and the control 2019 comes from just one colony. However, the result could be underestimated, since this colony could have acquired a better adaptation or resilience capacity to perturbations as algal bloom and transplantation (Fava et al. 2010).

Small and medium colonies after one year of transplantation did not show any spermatic sac while the large colony showed it, what may evidence a size dependency on the male reproduction. Moreover, the production of spermatic sacs appeared to be decreasing over time, indicating that time of transplantation, and perhaps also the algal bloom, could be still affecting the colony by sublethal effects.

However, the previous literature about gorgonian reproduction is mainly focused on females (Guest et al., 2007; Okubo et al., 2007). Therefore, trying to explain these results is difficult and could be imprecise.

In conclusion, these results suggest that reproductive output in female colonies is very affected by transplantation, being almost non-existent after three years of transplantation, and the algal bloom could be affecting on the time of reproduction. Furthermore, male colonies appeared to be much more affected than females, contrary to what has been seen in the literature, not only by transplantation but also by environmental stressors such as the algal bloom. Both stressors seemed to create a synergy in males, what could rise concerns about the effectiveness transplantation of this sex and the long-term availability of the population.

Colony size seems to be a conditional factor on the reproductive capacity of colonies after transplantation as well. In female transplants, the small colonies appeared to show better reproductive out than large colonies. Contrary, in male transplants, large colonies showed the best reproductive capacity. This demonstrate the importance of studying both sexes, since although greater vulnerability is observed in one sex, it does not mean that it will be the same for all stressors.

Transplantation has been seen to be an effective method to restore populations (Fava et al., 2010). However, this study showed that could also lead to mortality in long term what can cause a decrease in the genetic variability population (Hare et al., 2011). To minimize the effect, an optimal colony size or a better attachment technic is needed to be found to succeed in restoration programs, considering that should improve the survival without jeopardizing the sexual products development. Moreover, more knowledge about *E. singularis* is also needed to better understand its biology and ecology and to create better restoration plans in the future.

Restoration could be a punctual solution, but not on a large time scale. Restoration is usually expensive (Bayraktarov et al., 2019; Spurgeon & Lindahl, 2000) and takes long time, not only the action of restoration itself, but also the readaptation of the colonies after the transplantation and the recovery of the entire community (Steinberg et al., 2020). Directives and laws are needed to be implemented to prevent habitat destruction, as a longer-term solution.

CONCLUSIONS

In summary, this study about transplantation of *E. singularis* shows:

- Transplantation did not supress the reporduction capacity of the colonies, however, it caused a decrease in the reproductive output on *E. singularis*.
- Female colonies showed a better reproductive capacity than males after being transplanted. Moreover, the male colonies might suffer a higher lethal effect than females.
- Response to transplantation seems to be colony size and sexe-dependent. Small female colonies showed the better reproductive capacity, contrary to males, that were the large colonies.
- During the period of this study, the transplanted colonies did not showed any sign of recovery.
- Transplatation caused mortality of large colonies at the beginning probably due to detachment produced by their size and weight, and their resistance to currents. Moreover, small colonies were also dead probably because they are more vulnerable and to a sinergy between transplantation and the non expected algal bloom.
- The unexpected algal bloom appear to cause a sublethal effect on colonies, probably causing changes in the time of sexual products production and also reducing the reproductive capacity in both sexes, creating a synergy with transplantation.

CONCLUSIONES

En resumen, este estudio sobre el trasplante de *E. singularis* muestra:

- El trasplante no suprimió la capacidad de reproductiva de las colonias, sin embargo, provocó una disminución en la producción de productos sexuales.

- Las hembras mostraron una mejor capacidad de reproducción que los machos después del trasplante. Además, los machos pueden sufrir efectos letales más pronunciados que las hembras.

- La respuesta al trasplante parece depender del tamaño de la colonia y del sexo. Las hembras mostraron la mejor capacidad de reproducción en las colonias pequeñas, a diferencia de los machos, que fueron las colonias grandes.

- Durante el período de este estudio, las colonias trasplantadas no mostraron ningún signo de recuperación.

- El trasplante provocó la mortalidad de grandes colonias en un inicio probablemente debido al desprendimiento producido por su tamaño y peso, y su resistencia a las corrientes. Con el tiempo, las colonias pequeñas murieron probablemente debido a una sinergia entre el trasplante y el bloom algal inesperado.

- El inesperado bloom algal parece causar un efecto subletal en las colonias, causando cambios en el tiempo de ovogénesis y también reduciendo la capacidad reproductiva en ambos sexos, creando una sinergia con el trasplante.

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