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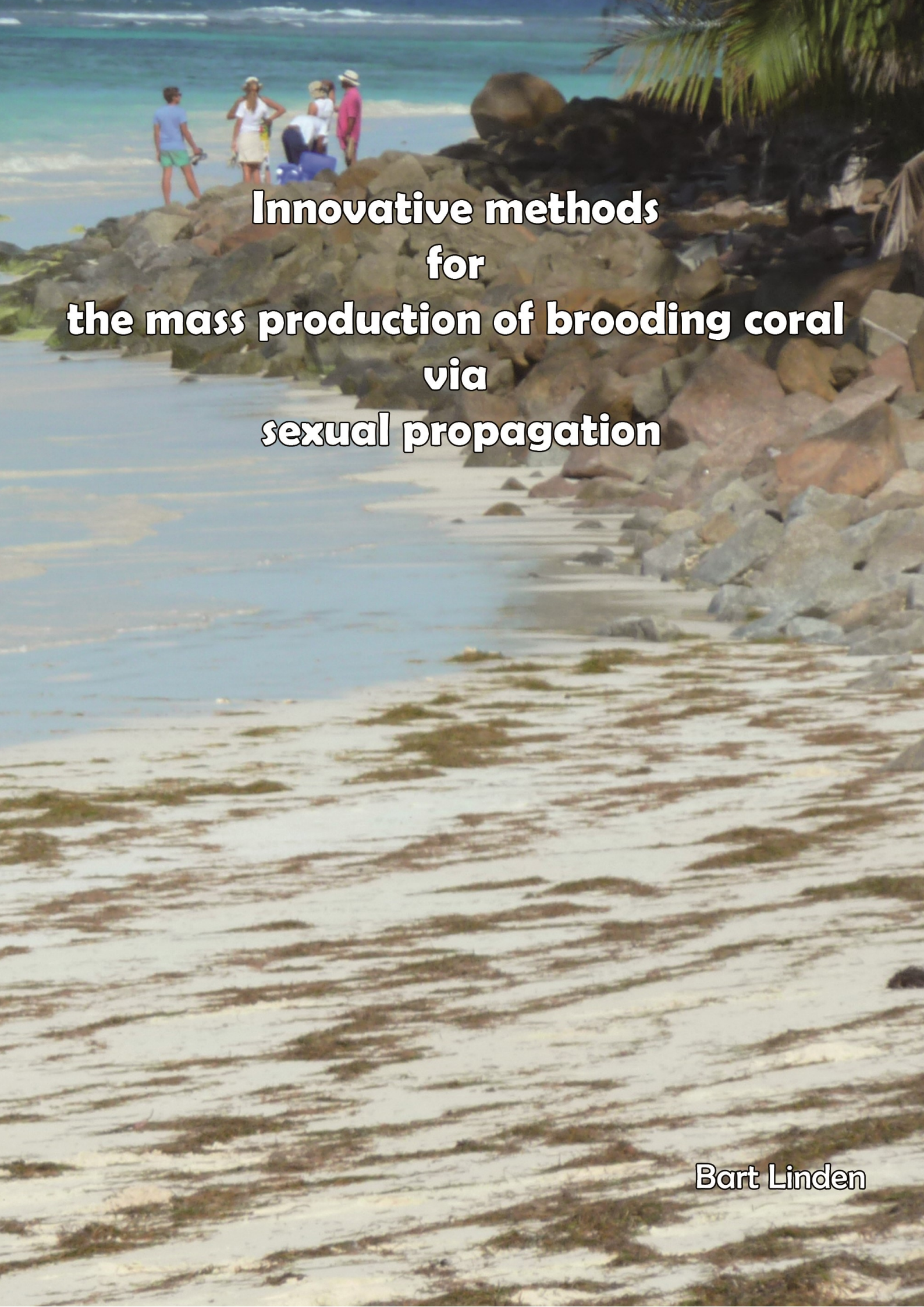

Innovative methods for the mass production of brooding coral via sexual propagation

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Innovative methods for the mass production of brooding coral via sexual propagation

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Chapter 1

Introduction

Introduction

Coral reefs cover less than 0.09% of the world's oceans (284,803 km^2) but house approximately 25% of the world's marine fish species and provide protection, nesting, feeding grounds, etc. for up to an estimated 35% of all marine species (Spalding *et al*. 2001, Knowlton *et al*. 2010). In addition to their importance as highly biodiverse ecosystems (Fisher *et al*. 2015), coral reefs are highly valued for the direct and indirect "ecosystem services" they provide (Costanza *et al*. 2014). Such services include land and coastal settlement protection from erosion and storms, but also provide food, climate regulation, recreation and potential medicine discovery (Martínez *et al*. 2007, Cinner *et al*. 2009, Costanza *et al*. 2014, Spalding *et al*. 2017). An estimated half a billion people have some form of dependency on coral reefs (Wilkinson 2008).

Over the past few decades, coral reefs have been impacted by climate change and other anthropogenic pressures. The average coral cover in nearly all biogeographic regions where reefs are found has declined by 50-75% (Aronson *et al*. 2003, Gardner *et al*. 2003, Bruno *et al*. 2007, 2009, De'ath *et al*. 2012, Jackson *et al*. 2014). The increase of CO_2 concentration in the atmosphere and the subsequent warming of the oceans has contributed to an increased frequency and duration of bleaching events and reduced the likelihood of recovery afterwards (Hoegh-Guldberg *et al*. 2018, Hughes *et al*. 2019). Bleaching is the process where coral animal host expels the algae that it normally lives in symbiosis with. This results in a loss of colouring of the coral, revealing the white skeletal structure, thus making the coral look like it has been "bleached". Bleaching is usually caused by locally extreme temperatures and light irradiation, which can result in the death of the coral if extreme conditions persist (Glynn 1983, Brown 1997, Jones *et al*. 1998, Saxby *et al*. 2003, Moustafa *et al*. 2014). The Intergovernmental Panel on Climate Change (IPCC; Bindoff *et al*. 2019) and the UN Environment (Rice & Grellier 2019) both consider coral bleaching as the greatest threat to the existence of coral reefs world-wide.

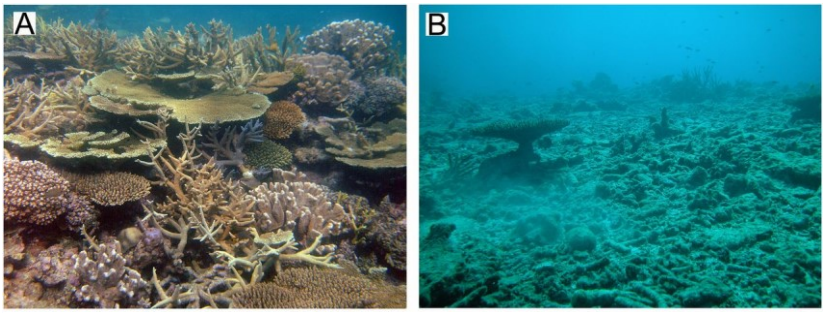

Figure 1 (A) High coral cover reef. (B) A degraded reef (Photographs and permission of use granted by: Katharina Fabricius, AIMS; Source: Knowlton (2012))

Besides bleaching, many other factors have impacted coral reefs, such as ocean acidification, declining water quality, increases in storm frequency and intensity, increased sedimentation, ship groundings, destructive fishing practices, and coral diseases, to name a few (Fig. 1; Hughes *et al*. 2003, 2019, Ayre & Hughes 2004, Bak *et al*. 2005, Donner *et al*. 2005, Hoegh-Guldberg *et al*. 2007, Rinkevich 2008, Riegl *et al*. 2009, Shaish *et al*. 2010, Bridge *et al*. 2013, Grizzle *et al*. 2016, Anthony *et al*. 2017, Rice & Grellier 2019).

Some of the above-mentioned factors that are impacting coral reefs can be managed through integrated forms of coastal management (e.g., pollution management, fishing regulations, water quality control, run off and waste management, fertilizer management and marina management; Waterhouse *et al*. 2017, Brodie *et al*. 2019), while others also require a shift in awareness (e.g., overfishing and littering; Sampaio 2010, Cinner *et al*. 2013). Factors that have a negative impact on coral reefs often exacerbate each other, for example when disease outbreaks prevent recovery after bleaching events (Bruno *et al*. 2007, Vega Thurber *et al*. 2014, Cinner *et al*. 2016, Boström-

Einarsson *et al*. 2018). The loss of hard corals (hermatypic corals) and the three-dimensional reef structures they build, will have a marked effect on the large number of organisms, including humans, that depend on them (Fig. 1; Knowlton 2012, Marhaver *et al*. 2013, Pratchett *et al*. 2014, Bruno *et al*. 2019).

One of the approaches to diminish the damaging effects of anthropogenic pressures, including climate change, on coral reefs is active reef restoration. **The aim of this thesis** is to contribute to the field of coral reef restoration by presenting new methods specifically for the mass production of a brooding coral via sexual propagation. This introductory chapter will provide the necessary background and context. Here I will highlight some of the complexities of coral reef restoration, mass production and explain why sexually reproduced corals might be a better choice for restoration than clonal reproduction via fragmentation, which is currently the favoured option. I will provide background information on the different types of sexual reproduction for comparison (brooding and broadcast spawning), how these are currently limited by natural bottlenecks and how they have the potential to provide large quantities of genetically diverse corals for restoration purposes. I will focus on a model coral species from the Red Sea, *Stylophora pistillata*, and will explain why this brooding coral species would be a good candidate to aid in coral reef restoration. Finally, I will present an outline of the thesis, beginning with our observations of the reproduction of *S. pistillata* and how I developed different methods to settle and rear the offspring both *ex situ* and *in situ*.

Reef restoration

Coral reef restoration is the process of assisting the recovery of degraded, damaged or destroyed coral reef communities in places where natural recovery is absent or too low to offset losses in coral abundance. Restoration can take many forms: it may directly or indirectly address factors that contribute to reef decline (e.g., overfishing, land-based forms of pollution); it may involve the protection of reef systems to facilitate recovery; or it may engage in activities that actively seek to facilitate recovery (e.g., outplanting of corals, removal of invasive species).

Non-invasive restoration

There are many managerial options to reduce/limit the human impact on coral reefs without any physical interaction with the coral reef. Non-invasive methods are sometimes also referred to as passive restoration practices or the traditional reef management approaches (Rinkevich 2021). An important tool to limit physical interaction with a coral reef is the designation of areas that are deemed important and require protection. Initial efforts to protect coral reefs − and associated ecosystems such as mangroves and seagrass beds − often consisted of creating Nature Reserves (NRs) or Marine Protected Areas (MPAs), where human influences can be regulated and enforced by law or society. Approximately 27% of the world's coral reefs receive some form of protection through an estimated 2,679 MPAs around the world (Burke *et al*. 2011). Many NRs or MPAs, however, lack effective enforcement and have no discernible effect, therefore, on undesirable human influences (Mora *et al*. 2006). Besides effective enforcement, the successfulness of an MPA is also determined by its size, the number of years it has been in effect and its degree of isolation (Edgar *et al*. 2014). MPAs serve an essential role in mitigating certain local anthropogenic factors such as overfishing and destructive anchoring, but many factors negatively impacting coral reefs, such as land-based forms of pollution and climate change, remain unaddressed. Like any other non-invasive method, they do not address the actual loss of corals on a coral reef, but rely on the system's natural regeneration capacity to bounce back from events impacting the coral reef. These non-invasive approaches are essential, but evidently insufficient to conserving coral

reefs, therefore many new methods are being developed. Many of these new methods actively intervene in the rehabilitation process; “active restoration” (Rinkevich 2019).

Active reef restoration

Active or assisted restoration are terms used to describe actions undertaken to physically interact with or alter an area for restoration purposes, such as removing damaging items (e.g., lost fishing nets) or re-introducing ecologically important species (e.g., corals as the main builders of complex reef habitats) that have decreased in abundance in an area. By doing so, active restoration efforts aim to halt or reverse the loss of local reef communities. Methods developed to this end are many and should be regarded as tools in a “toolbox” available to reef managers (Rinkevich 2019, 2021).

An increasingly popular method to actively restore degrading reef communities is the outplanting of corals. To restore reefs that have seen a significant reduction in the abundance of corals, it is particularly important to use hermatypic corals (i.e. those capable of reef building) in local restoration efforts (Dudgeon *et al*. 2010, Precht *et al*. 2010). To facilitate a relatively fast recovery of degraded reef communities, restoration efforts often prefer using fast-growing, branching corals from the Acroporidae and Pocilloporidae families, such as *Acropora palmata*, *Acropora tenuis*, *Acropora cervicornis*, *Pocillopora damicornis* and *Stylophora pistillata* (Rinkevich 2000, Guest *et al*. 2011, Omori & Iwao 2014, Chamberland *et al*. 2015). Slow growth rates are generally typical for massive coral species such as *Orbicella faveolata* and *Montastraea cavernosa* which are, therefore, less popular for restoration efforts. Important for these slow growing species is the development of the microfragmenting method, a technique whereby a large number of new corals can be produced by cutting existing corals into small “juveniles”, has shown potential to increase growth rates under laboratory conditions and could eventually be used to speed up the recovery of massive coral species under natural circumstances (Page *et al*. 2018).

Besides fast growth rates, mass production of corals is essential for restoration because the areas that are in need of restoration are usually very large and, therefore, require many corals to be outplanted. Creating coral colonies for outplanting currently relies heavily on the pruning, or fragmenting, of natal coral colonies (donor colonies). Traditional propagation via the fragmentation of coral relies on fragment sizes between 3-20 cm (45 or more polyps) in size in order to ensure decent survival rates after outplanting (Raymundo & Maypa 2004, Okubo *et al*. 2007). The number of fragments surviving outplanting can be drastically increased by using an “abridged phase” in which the fragments are kept in *in situ* nurseries. The abridged phase is usually in the form of a nursery such as a coral tree or mid-water nursery (Shafir *et al.* 2006a, Nedimyer *et al.* 2011). Shafir *et al*. (2001) were able to successfully grow nubbins (~5 polyps/colony) with 60% survival after 100 days in an *in situ* coral nursery.

Besides the mass production of corals via fragmentation, corals for outplanting have more recently been produced by rearing sexually produced larvae (Petersen *et al*. 2006, Baums 2008, Nakamura *et al*. 2011, Omori & Iwao 2014, van Oppen *et al*. 2017, Chamberland *et al*. 2017, Omori 2019, Pen *et al*. 2020). Using sexually produced larvae as source material for the mass production of coral signifies an important departure from the common fragmentation practice, while avoiding several potential pitfalls, such as monoculture and damaging native corals when harvesting fragments (Epstein *et al*. 2001, Baums 2008, Shearer *et al*. 2009, Baums *et al*. 2019). The sexual propagation of coral, however, is much more complex (Harrison 2011) and was proposed as a long-term approach to coral restoration by Rinkevich (1995). It was also suggested as a means to accelerate genetic restoration, especially valuable for species that are experiencing long-term failure of natural recruitment (Baums *et al*. 2019). Chamberland *et al* (2015) and Guest

et al (2014) have successfully implemented sexual propagation techniques to produce new coral colonies for outplanting purposes, although with very different *in situ* nursery survival rates.

Sexual Reproduction

There are various sexual reproductive strategies in corals (Table 1; Harrison, 2011): corals can be hermaphroditic, with male and female gametes being produced by the same colony, or gonochoric, with male and female gametes being produced by separate colonies. Most coral species are hermaphroditic (70.9%), while 26.2% of all coral species are gonochoric (Table 1). Furthermore, fertilization of gametes can occur either outside a coral colony in the water column ("spawning species") or internal ("brooding species"). These two modes of development are not mutually exclusive, and at least twelve coral species have been reported to display both modes (Harrison 2011, Schmidt-Roach *et al*. 2012). In the Indo-Pacific, the overwhelming majority of known corals (79.7%) reproduces via broadcast spawning, whereas in the Caribbean the proportions of brooding and spawning species are nearly identical (Szmant 1986, Richmond & Hunter 1990, Baird *et al*. 2009, Ritson-Williams *et al*. 2009).

The above defines the two extremes in a gradient of reproductive strategies known in corals. There are also species, for example, that release sperm while eggs remain inside the adult colony until they become fertilized, followed by the release of embryos rather than larvae as seen in brooding species (Bishop & Pemberton 2006, Marhaver *et al*. 2015). Another example of coral species that deviate from the above-mentioned main strategies are corals that produce male or female gametes in different sections of a colony, that is, species that are gonochoric at the polyp rather than the colony level (Harrison & Wallace 1990).

Table 1 Summary of reproductive strategies for 444 out of an estimated 1,500 scleractinian species worldwide (Source: Harrison (2011); https://doi.org/10.1007/978-94-007-0114-4_6)

Sexual pattern → / Mode of development ↓	Hermaphroditic	Mixed strategy	Gonochoric	Unknown reproductive strategy	Total number of species
Spawn gametes	258	6	78	12	354
Spawn gametes and brood larvae	8	1	4	-	13
Brood larvae	25	5	15	16	61
Unknown mode of development	4	-	12	-	16
Total number of species	295	12	109	28	444

Broadcast Spawning Corals

In general, broadcast spawning coral species release buoyant bundles containing eggs and sperm into the water column once or a few times per year, depending on species and geographical location (Harrison *et al*. 1984, Babcock *et al*. 1986, Nozawa *et al*. 2006, Van Woesik *et al*. 2006). At the surface, these bundles break apart temporarily resulting in slicks of gametes (Fig. 2). Fertilization occurs among gametes from different colonies resulting in embryos that develop into larvae ("planulae") within a few days and remain in the water column for days to weeks before

they settle on solid substrate (Fig. 3). Due to the extensive time spent in the water column during development, broadcast spawning coral species generally have a larger dispersal range than brooding coral species (Harrison & Wallace 1990, Nishikawa *et al*. 2003).

Figure 2 Coral slick. Accumulation at the sea surface of the positively buoyant gametes released by broadcast spawning coral species. Water movement and currents mix and disperse these gametes and resulting larvae. (Source and permission of use granted by: James Gilmour, AIMS)

Brooding corals

The sexual propagation of brooding coral species occurs via the release, dispersal and uptake of free spermatozoa. Brooding coral species brood their embryos following fertilization and releasing larvae at an advanced stage with an abbreviated pelagic phase (Jackson 1986, Pemberton *et al*. 2003, Bishop & Pemberton 2006). In some species, eggs can also develop into planulae without being fertilized, an asexual form of "brooding" that can be seen, for example, in *P. damicornis* (Harrison & Wallace 1990). Brooding species can release planulae on a daily basis for months or sometimes all year round, and they release free-swimming planulae that can start settling within a few hours after release.

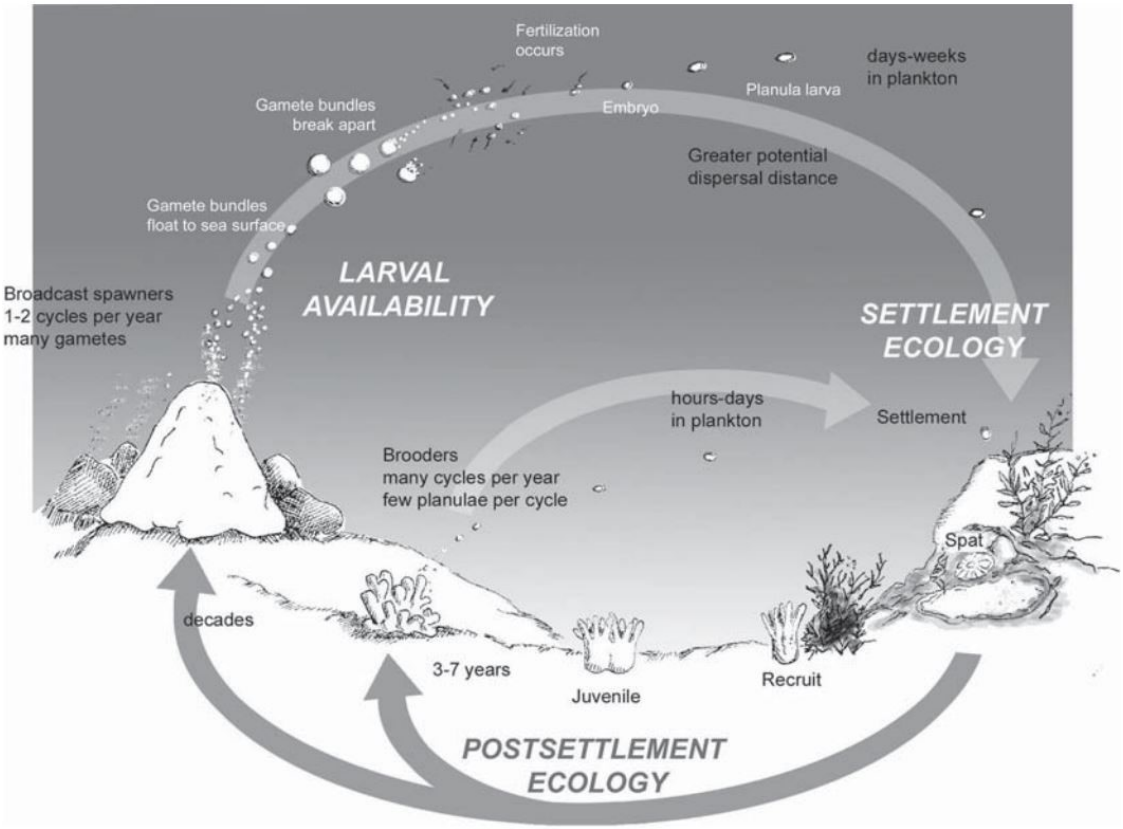

Figure 3 Three sequential phases that define successful recruitment in corals illustrated for brooding and broadcasting species; Larval availability, settlement ecology, and post-settlement ecology (Drawn by Mark Vermeij). (Source: Ritson-Williams *et al*. 2009)

Recruitment

Recruitment is defined by the successful inclusion of an individual into a population. This can sometimes be further defined using certain pre-requisites, such as size or the capacity to reproduce, which are common benchmarks used for coral offspring. Although each coral colony, regardless of sexual reproductive strategies, can produce hundreds to thousands of offspring every year, recruitment success is often low (Harriott & Fisk 1988, Gosselin & Qian 1997, Fabricius & Metzner 2004, Vermeij & Sandin 2008).

Recruitment has declined in recent decades due to major habitat altering disturbances some of which have become more frequent such as storms and cyclones, preventing recruitment and recovery between events (Bruno & Selig 2007, van Woesik *et al*. 2014, Tanner 2017). Larval recruitment has also decreased due to lower reproductive output as a consequence of reef degradation (Hartmann *et al*. 2018) and bleaching events (Hughes *et al*. 2019). Successful recruitment, therefore, is generally regarded as a major bottleneck for coral population survival and coral reef restoration. Although recruitment *in situ* has always been quite low (Hunt & Scheibling 1997), sexual reproductive strategies correspond to differences in pioneering aptitude. Brooding coral species are more likely to pioneer "new" areas than broadcast spawning coral species, though, when established, broadcast spawning coral species are competitively stronger than brooding corals when competing for space (Loya 1972, Grigg & Maragos 1974, Jaap 2000, Vermeij *et al*. 2007).

Rearing corals to attain what are normally considered recruitment benchmarks, such as size, is often relatively high under *ex situ* conditions (>20%), which contrasts with *in situ* conditions, where less than 1% of all larvae successfully settle and metamorphose into a juvenile coral (Gosselin & Qian 1997, Hunt & Scheibling 1997, Fabricius & Metzner 2004, Vermeij & Sandin 2008, Toh *et al*. 2012). Bypassing this "natural bottleneck" by rearing coral larvae under controlled *ex situ* conditions, therefore, can potentially produce the large number of coral colonies needed for restoration purposes on degraded sites. Rearing larvae and recently settled corals *ex situ* still requires labour-intensive maintenance to prevent infections by bacteria, maintain water quality, ensure optimal water temperature and minimize mechanical disturbance (Negri *et al*. 2001, Guest *et al*. 2010, Heyward & Negri 2010).

Rearing Broadcast Spawning Corals

Most methods that propagate corals via sexual reproduction have been developed for coral species that broadcast spawn (Hagedorn *et al*. 2009, Guest *et al*. 2010, Chamberland *et al*. 2015). This is not surprising as broadcast spawning is the most common reproductive strategy in corals (Table 1). Initial predictions of spawn timing for new coral species are usually based on examination of the egg development in sampled branches taken from the field (Baird *et al*. 2000). If the coral species was observed spawning during previous years, this information is used instead to predict future spawning events (Harrison *et al*. 1984, Babcock & Heyward 1986). To collect gametes, adult colonies are sometimes removed from the reef and kept in containers on shore

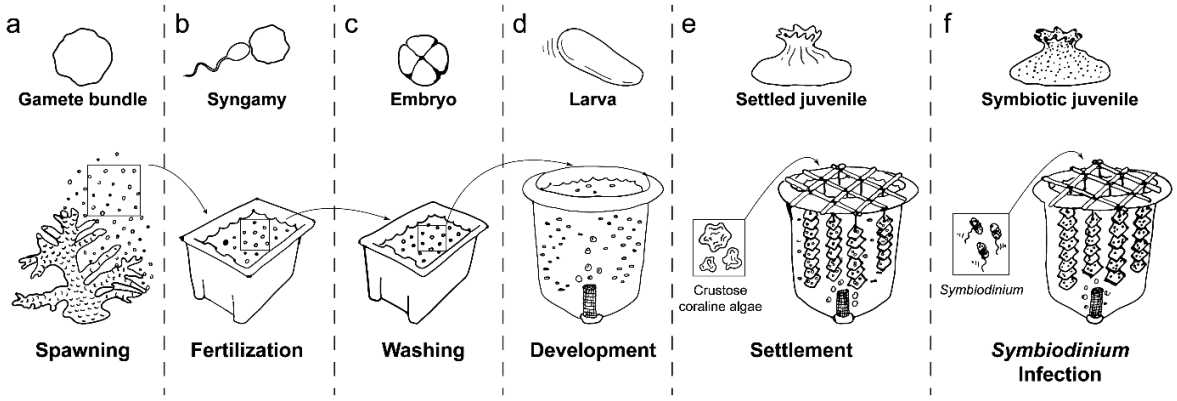

Figure 4 (a) Individual coral colonies are isolated in plastic bins right before spawning. Following gamete release, egg/sperm bundles are collected from the water surface and (b) mixed with those from other colonies in clean plastic bins to allow fertilization. (c) One hour after observation of the first embryo cleavage, embryos are processed through three consecutive washing steps to remove excess sperm and decrease polyspermy. (d) At the 2- to 4-cell stage of development, embryos are transferred to aerated flow-through larval culture tanks. Once fully developed, larvae begin exhibiting settlement competency behaviour (i.e. substratum searching), and (e) ground crustose coralline algae are added to induce larval settlement. Following settlement onto the settlement substrate (e.g., terracotta tile), (f) Symbiodinium are added to rearing tanks to initiate symbiosis. (Source: Pollock et. al. (2017);
DOI: https://10.7717/peerj.3732/fig-1)

where spawned gametes can be easily collected (Fig. 4; Joseph Pollock *et al*., 2017), after which colonies are returned to the reef (Goreau *et al*. 1981, Golbuu & Richmond 2007, Baria *et al*. 2010, Puill-Stephan *et al*. 2012, Omori & Iwao 2014).

Other methods to collect gametes for *ex situ* rearing efforts include the *in situ* use of coverings made of fine mesh equipped with a removable collection container at its top in which floating gamete bundles tend to concentrate (Omori *et al*. 2004b). Gametes are usually mixed and reared under carefully controlled laboratory conditions, though rearing fertilized embryos, or even fertilizing gametes, also occurs in *in situ* floating ponds (Fig. 5; Heyward *et al*., 2002; Omori *et al*., 2004; Omori and Iwao, 2014). The use of an *in situ* pond, subject to wave action, naturally mixes the gametes and allows the fertilized eggs to develop under more natural conditions and avoids stress resulting from handling in a laboratory.

The *ex situ* rearing process requires several steps such as the mixing of the gametes followed by several washing and rinsing steps to remove excess sperm and minimize polyspermy of yet unfertilized eggs. Embryo washing is very delicate, minimizing agitation to avoid fragmenting early stage embryos (Hagedorn *et al*. 2009, Pollock *et al*. 2017). Guest *et al*. (2014) reported 1.4% of provided motile larvae successfully settled and survived after 1 month *ex situ*. Tabalanza *et al*. (2020), using the same technique as Guest *et al*. (2014), had about 4.2% successful settlement and survival after 1 month *ex situ*.

Rearing Brooding Corals

Many of the collection methods mentioned above are also used, in somewhat modified forms, for the collection of planulae from brooders. For example, planulae can be collected by placing adult brooding coral in containers, where, due to the less predictable nature of larval release compared

to spawning, released planulae can be collected overnight in submerged nets placed in the overflow of each container (Richmond 1985). The same is true for *in situ* collection where fine mesh attached to a container has been used to collect the positively buoyant planulae (Zakai *et al*. 2006).

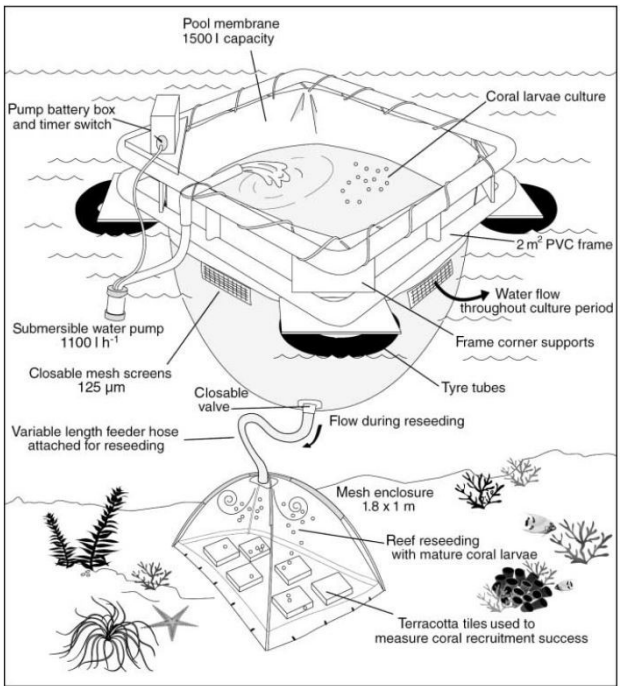

Figure 5 A schematic representation of a floating larval culture pond and reseeding system (Source: Heyward *et al*. 2002, © Inter-Research 2002)

Rearing offspring of brooding coral species generally requires less work than rearing offspring from broadcast spawning coral species. Brooding coral species release fully developed planulae that can settle within a few hours after release, thus do not require fertilisation, mixing and washing steps normally undertaken to produce broadcast spawning coral larvae (Fig. 4). Though brooding species produce small quantities of offspring at once, these can be consistently harvested for prolonged periods of time, unlike broadcast spawning corals, whose window of opportunity for harvesting is limited to only once or a few times per year. These planulae harvested also are reported to settle readily in high numbers given appropriate conditions (Lewis 1974, Nishikawa *et al*. 2003).

Very few methods have been developed so far to use the offspring of brooding corals to mass produce coral colonies for restoration purposes, e.g. Epstein *et al.* (2001) succeeded in settling *S. pistillata* (85%) but the settlers had low *in situ* survival rates (5% after 1 month). Amar *et al*. (2008) reported *ex situ* survival rates between 54-85% after 1 year, depending on colony formation. To date, very little has been achieved with growing brooding coral species to a size where they could be considered eligible for recruitment to the population (Petersen *et al*. 2006, Barton *et al*. 2017).

The use of brooding coral species in reef restoration

The development of rearing methods to generate coral colonies from larvae in brooding species such as *S. pistillata* has been very limited (Epstein *et al*. 2001). One could argue, however, that brooding coral species could be extremely useful particularly in efforts to restore degraded reef communities, given that brooding coral species often have a prolonged reproductive season, allow for flexibility in the *in situ* collection schedule, have pioneering traits, are generally fast-growing species and can settle within hours after collection. The quick and highly efficient settlement of brooding coral planulae can be used to limit both time spent *ex situ* and costs associated with facilities needed to create colonies on a large scale. In this thesis, I have developed several methods and tools to efficiently collect, settle and rear the planulae of brooding coral species *Stylophora pistillata* in the Red Sea, near Eilat, Israel.

Stylophora pistillata in the Red Sea

Figure 6 *Stylophora pistillata*. Photo by B. Linden

Stylophora pistillata (Fig. 6; Esper, 1797) is a protandrous hermaphroditic brooding coral species: both male and female gametes can be found in adult colonies, but female gametes are absent in smaller colonies and can sometimes disappear from older colonies. Male gametes generally start developing during the winter months, while female gametes start to develop during the spring/summer (Rinkevich & Loya 1979a). This species in the Red Sea is characterized by a long reproductive season, spending from December to July-October each year (Rinkevich & Loya 1979b, 1987, Shefy *et al*. 2018) Like in many other brooding species, zooxanthellae are inherited from the parental colony and are clearly visible in released planulae (Fig. 7). Some hermaphroditic species can fertilize themselves, this has not been observed in *S. pistillata* (Douek *et al*. 2011). *S. pistillata* does not require specific chemical cues, such as CCA's, to induce settlement (Loya 1976, Rinkevich & Loya 1979b, Baird & Morse 2004, Amar *et al*. 2008).

Figure 7 A *Stylophora pistillata* planula. The zooxanthellae (yellow-brown spots) are clearly visible. Photo by B. Linden

S. pistillata is one of the most studied scleractinian coral species in the Red Sea. It is one of thirteen coral species that together make up the majority (60-70%) of the total living coral cover in the Gulf of Eilat, Israel (Shlesinger & Loya 1985). *S. pistillata* is common and widespread, occurring from the northern part of the Red Sea to the middle of the Pacific Ocean (Fig. 8).

Interestingly, *S. pistillata* displays intraspecific variation in its tolerance to elevated seawater temperatures. In Australia, *S. pistillata* appears to be sensitive to high temperatures, while *S. pistillata* in the Red Sea is not (Hoegh-Guldberg & Smith 1989, Keshavmurthy *et al*. 2013, Krueger *et al*. 2017). Extreme temperature conditions occur in the Southern Red Sea, with temperatures reaching up to 34°C, which could potentially act as an evolutionary driving force to explain biogeographical differences in temperature sensitivity in this species. This is further supported by the observation

Figure 8 Global distribution of *Stylophora pistillata*. (Source: IUCN (2021) https://www.iucnredlist.org/)

that coral reefs in the Red Sea have so far not experienced large-scale bleaching events that have occurred in the Great Barrier Reef and the Caribbean (Furby *et al*. 2013, Monroe *et al*. 2018). In the Northern Red Sea, the area where the research presented in this thesis was conducted, seawater temperatures are more similar to conditions found elsewhere in the tropics where corals are found (21-27°C; winter-summer; Sawall and Al-Sofyani, 2015).

Aims and outline

The aim of this thesis is to contribute to the field of coral reef restoration by presenting new methods for the mass production of brooding corals via sexual propagation. Specifically, this thesis describes the methods I developed to generate coral colonies from larvae produced by the brooding coral species *Stylophora pistillata*. I first investigated the natural release, settlement behaviour and early development of planulae in a series of experiments and observational studies. Building on the already existing literature, our results and observations were combined to develop practical tools for creating coral stock using the larvae of brooding coral species. To achieve this, the primary focus was to efficiently reduce mortality during the phases that are identified as the bottlenecks during the earliest life stages of *S. pistillata*. These bottlenecks are identical in many marine organisms and mostly occur between the moment of planulae release to a few months after settlement (Gosselin & Qian 1997, Vermeij & Sandin 2008).

Chapter 2 discusses the planulation of *S. pistillata* from the Gulf of Eilat, Red Sea. *Stylophora pistillata* is a hermaphroditic brooding species with a long reproductive season (December/January to July/September; Rinkevich & Loya 1979b, 1987, Amar *et al*. 2007, Shefy *et al*. 2018). While long-term shifts in sexual reproduction patterns, such as reproductive seasonality and reproductive efforts, were documented for *S. pistillata* populations from Eilat over the last four decades (Amar *et al*. 2007, Shefy *et al*. 2018), the possible association of larval release with lunar periodicity has remained a subject of debate. Several authors (Rinkevich & Loya 1979b, 1987, Tanner 1996, Shefy *et al*. 2018) could not find conclusive evidence for lunar periodicity in planulation, timing and quantity of released larvae from gravid *S. pistillata* colonies, whereas other studies (Atoda 1947, Fan *et al*. 2002, Zakai *et al*. 2006) reveal a correlation between larval release and lunar periodicity. Chapter 2 aims to build on these seemingly contradictive observations on circalunar periodicity in *S. pistillata* reproduction by studying planulae release in shallow water individual colonies from the Gulf of Eilat.

Chapter 3 introduces a new method for settling and rearing coral spat of *S. pistillata*, which allows the efficient production of numerous sexually produced coral colonies. The use of sexually produced corals precludes the decrease of genetic variation and the potential harm inflicted on donor colonies to generate fragments. Previous efforts devoted to sexual propagation methods for corals have focussed on broadcast spawning coral species, so the novel *ex situ* techniques described in this thesis for the brooding species *S. pistillata* larvae, adds new “tools” that allows the inclusion of brooding species in the “toolbox” available to reef restoration practitioners.

Chapter 4 addresses major maintenance issues associated with the rearing of sexually propagated nursery-farmed corals: can 1.5-month-old *ex situ* reared coral settlers be reared successfully in an *in situ* nursery? Will limiting physical access in the form of caging provide better survival rates than colonies that are left unprotected and exposed (Nozawa 2008, Baria *et al*. 2010, Nakamura *et al*. 2011)? How does the orientation of the colony in the water column affect survival and growth inside a mid-water nursery? Does colony aggregation affect survival and growth (Buss 1982, Rinkevich 2005b, Amar *et al*. 2008)?

Chapter 5 describes the first tool specifically designed for collecting and rearing larvae of brooding coral species called the Coral Settlement Box (CSB). The CSB was developed to trap, settle and rear the larvae of the brooding species *S. pistillata* entirely *in situ*. Planulae were trapped and allowed to settle in the CSB on site, whereas the long-term rearing of settled colonies occurred in a midwater nursery. This method reduces the stress that is normally experienced by larvae and settlers when they are being handled in *ex situ* facilities.

Chapter 6 integrates the main findings from the previous chapters. In addition, this chapter points at directions for future research to further develop and improve methods for creating coral stock based on the sexual propagation of coral species. Finally, I also outline general solutions to further reduce mortality during the known bottlenecks in the mass production of coral.

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Chapter 2

Circatrigintan instead of lunar periodicity of larval release in a brooding coral species

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Chapter 2

Circatrigintan instead of lunar periodicity of larval release in a brooding coral species

Abstract

Larval release by brooding corals is often assumed to display lunar periodicity. Here, we show that larval release of individual *Stylophora pistillata* colonies does not comply with the assumed entrainment by the lunar cycle, and can better be classified as a circatrigintan pattern. The colonies exhibited three distinct reproductive patterns, characterized by short intervals, long intervals and no periodicity between reproductive peaks, respectively. Cross correlation between the lunar cycle and larval release of the periodic colonies revealed an approximately 30-day periodicity with a variable lag of 5 to 10 days after full moon. The observed variability indicates that the lunar cycle does not provide a strict zeitgeber. Other factors such as water temperature and solar radiation did not correlate significantly with the larval release. The circatrigintan patterns displayed by *S. pistillata* supports the plasticity of corals and sheds new light on discussions on the fecundity of brooding coral species.

Chapter 2

Circatrigintan instead of lunar periodicity of larval release in a brooding coral species

Introduction

Biological features and properties of coral gametes, embryos, planula larvae and coral recruitment are often considered important in addressing a myriad of ecological trajectories towards the resilience of coral reefs (Guest *et al*. 2010). Sexual reproduction in reef corals contributes to the genetic variance, population robustness and overall healthy growth of coral reefs (Harrison & Wallace 1990, Hughes *et al*. 1992, Horoszowski-Fridman *et al*. 2011). Sexual maturation, onset of reproduction, timing of spawning, biological clocks and other intrinsic traits are vital reproductive properties(Harrison 2011, Levitan *et al*. 2014, Hagedorn *et al*. 2016). In addition, sexual reproduction in reef corals is constrained by extrinsic factors such as location (Jokiel *et al*. 1985), food availability (Gori *et al*. 2013), competitive interactions (Rinkevich & Loya 1985, Tanner 1997), anthropogenic activities (Rinkevich & Loya 1979b), water temperature, solar radiation, tidal pressure (Harrison & Wallace 1990, Harrison 2011, Bauman *et al*. 2011, Torres-Pérez & Armstrong 2012, Paxton *et al*. 2016), and more. How the intrinsic and extrinsic factors determine the resultant reproductive patterns in coral colonies remains a subject of debate (Keith *et al*. 2016), but being able to accurately predict reproduction patterns of corals can be an invaluable tool for reef management and reef restoration (Jokiel *et al*. 1985, Harrison & Wallace 1990, Guest *et al*. 2005, 2010, Amar & Rinkevich 2007, Baums 2008, Sale 2008, Linden & Rinkevich 2011)

Circalunar periodicity is here defined as periodic behaviour with a ca. 29.5-day frequency (Linden & Rinkevich 2011) in which the lunar cycle acts as a zeitgeber. Circa 30-day cycles that are not influenced by the lunar phase and irradiance intensity are best characterised as circatrigintan (ca. 30-day periodicity (Halberg 1969)). Though there is no doubt about the circannual and circadian rhythm of sexual reproduction in most coral species, a convincing demonstration of lunar periodicity (Raible *et al*. 2017) in broadcasting, shedding or spawning of some corals remains elusive (Harrison & Wallace 1990, Fan Tung-Yung *et al*. 2006, Harrison 2011, Rinkevich *et al*. 2016). Populations of some brooding corals, such as *Pocillopora damicornis* (Jokiel *et al*. 1985), have demonstrated to have a circalunar reproductive pattern (~29.5 days) that is not necessarily correlated to lunar irradiance intensity. This result makes lunar irradiance an unlikely zeitgeber. In some brooding coral species (e.g., *Stylophora pistillata* (Shlesinger *et al*. 1998, Fan Tung-Yung *et al*. 2006, Gilmour *et al*. 2016)), planulae timing and releases are predicted to happen on a daily basis during certain months, revealing peaks and troughs in their reproductive intensity. These patterns are believed to be associated with environmental factors and constraints that are different from those dictating the germ line release documented in broadcasting species (Harrison & Wallace 1990, Tanner 1996, Harrison 2011). The determination of an exact spawning event for some broadcasting coral species, such as *Acropora palmata* in the Caribbean, is done with some degree of accuracy (Szmant 1986, Chamberland *et al*. 2016). This is also true for the synchronized mass spawning phenomenon of a consortium of >140 coral species in the Great Barrier Reef, Australia (Harrison *et al*. 1984, Babcock *et al*. 1986).

The common Indo-Pacific branching coral species *S. pistillata* from the Gulf of Eilat, Red Sea, is a hermaphroditic brooding species with a long reproductive season (December/January to July/September (Rinkevich & Loya 1979b, 1987, Amar *et al*. 2007)). While long-term shifts in sexual reproduction patterns, such as reproductive seasonality and reproductive efforts, were documented for *S. pistillata* populations from Eilat (Amar *et al*. 2007) in the last three decades, the possible association of lunar periodicity with larval release in this species has been the subject of some debate. Several authors (Rinkevich & Loya 1979b, 1987, Tanner 1996) could not find

conclusive evidence for lunar periodicity in planulation, timing and shed of larval numbers from gravid *S. pistillata* colonies, whereas other studies (Harrison *et al*. 1984, Babcock *et al*. 1986, Rinkevich & Loya 1987) claimed to reveal such lunar periodicity in larval release. This study investigates this deliberation of circalunar periodicity in *S. pistillata* reproduction as displayed by shallow water individual colonies from Eilat.

Methods

S. pistillata planulae were collected from a coral reef in the Gulf of Eilat, located in front of the Interuniversity Institute for Marine Sciences (IUI) in Eilat, at a depth of 3-5 metres from 1st of April to 24th of June 2011. Planulae traps were placed at nightfall covering the coral colonies and collected the following morning with their contents, as described (Tanner 1997). Eight large *S. pistillata* colonies (Fig. 1) were chosen haphazardly, situated no more than 20 metres apart.

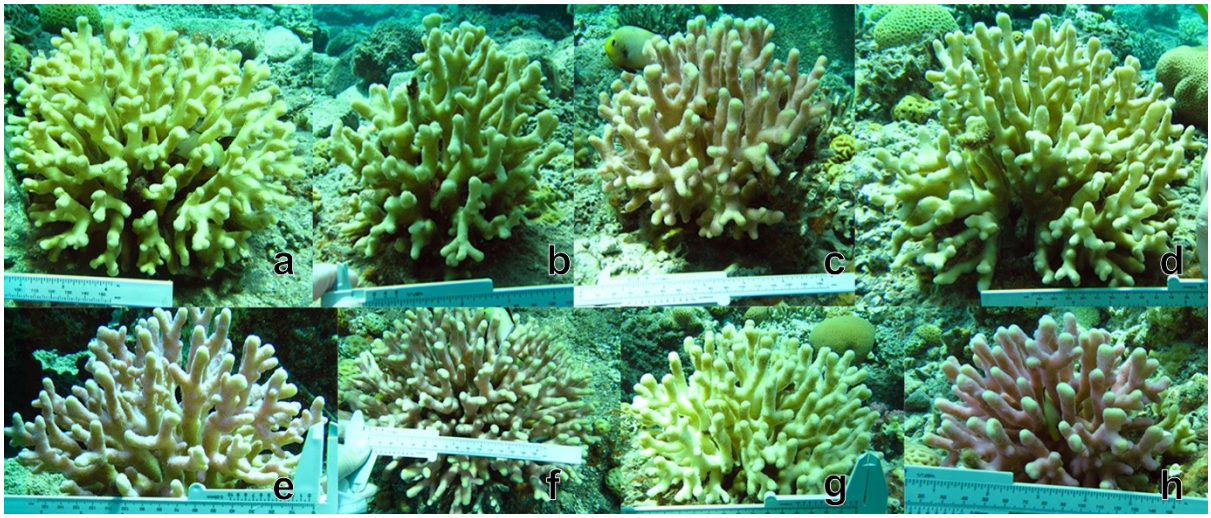

Figure 1 The eight gravid *Stylophora pistillata* colonies chosen for planulae collection. The colonies differed in size and colour, representing the morphological diversity of *S. pistillata* colonies at Eilat. A plastic ruler was used to estimate the diameter of each colony, with (**a**-**h**) representing colonies #1 to #8. The black scale bar in (**a**) indicates a length of 3 cm.

The collected nets were retrieved from the coral colonies and immediately transferred on land to a cool box on wheels with seawater. The traps were re-suspended to minimize the time exposed to air and desiccation, and transported to a wet lab situated nearby (less than 1 minute away). Each trap was rinsed separately with seawater, and the collected planulae were flushed from each trap to a separate glass container. The planulae were counted using a stereoscope and a pipette.

Auto-correlation (Lee *et al*. 1950) was applied to establish the presence of periodic patterns in larval production.

IUI data loggers collect data on site every 10 minutes and are readily available via a website (Meteorologic Services 2016). The data include water level (cm), water temperature (°C), UV irradiance (mmol m^{-2} sec^{-1}) and solar irradiance (W m^{-2}). Tidal range was calculated as the difference between high tide and low tide, maximum daily solar and UV irradiance were obtained from the irradiance data, and lunar period was calculated as:

$$y = \cos(2\pi \times \frac{Days\ since\ last\ full\ moon}{\Delta full\ moon\ to\ full\ moon\ in\ days})$$

Cross-correlation was applied to investigate relationships between fluctuations in larval production and fluctuations in these environmental variables. Furthermore, we calculated daily solar insolation (kWh m^{-2} day^{-1}), which was compared to peak reproduction timing. Images of the coral colonies were analysed using ImageJ software, and an ecological volume index was established for each colony, by approximating colonial structures to the shape of a half sphere (Rinkevich 2000). Graphs were created in Sigmaplot 12.5 and statistical analysis were performed with SPSS 21.

Results

Larvae were collected from eight colonies and counted per colony per day (during 48-59 nights per colony, over a total period of 84 days). In total, 16,586 planulae were collected during 427 sampling sessions (Table 1). The numbers of planulae released per colony per day varied greatly among the eight coral colonies (Fig. 1). Planula numbers varied between maximum peaks of 903 (colony #6) to 12 (colony #7) planulae caught on a single night from a specific colony (Fig. 2). The most gravid colony (#6) released on average 156.1 planulae per day (ranging from 5 to 903 on a collection night), whereas the least productive colony (#7) released on average 3.8 planulae per day (ranging from 0 to 12 on a collection night; Table 1). The average number of planulae collected per colony per day increased with colony volume (Pearson Correlation: r = 0.81; n = 8, p(1-tailed) = 0.007).

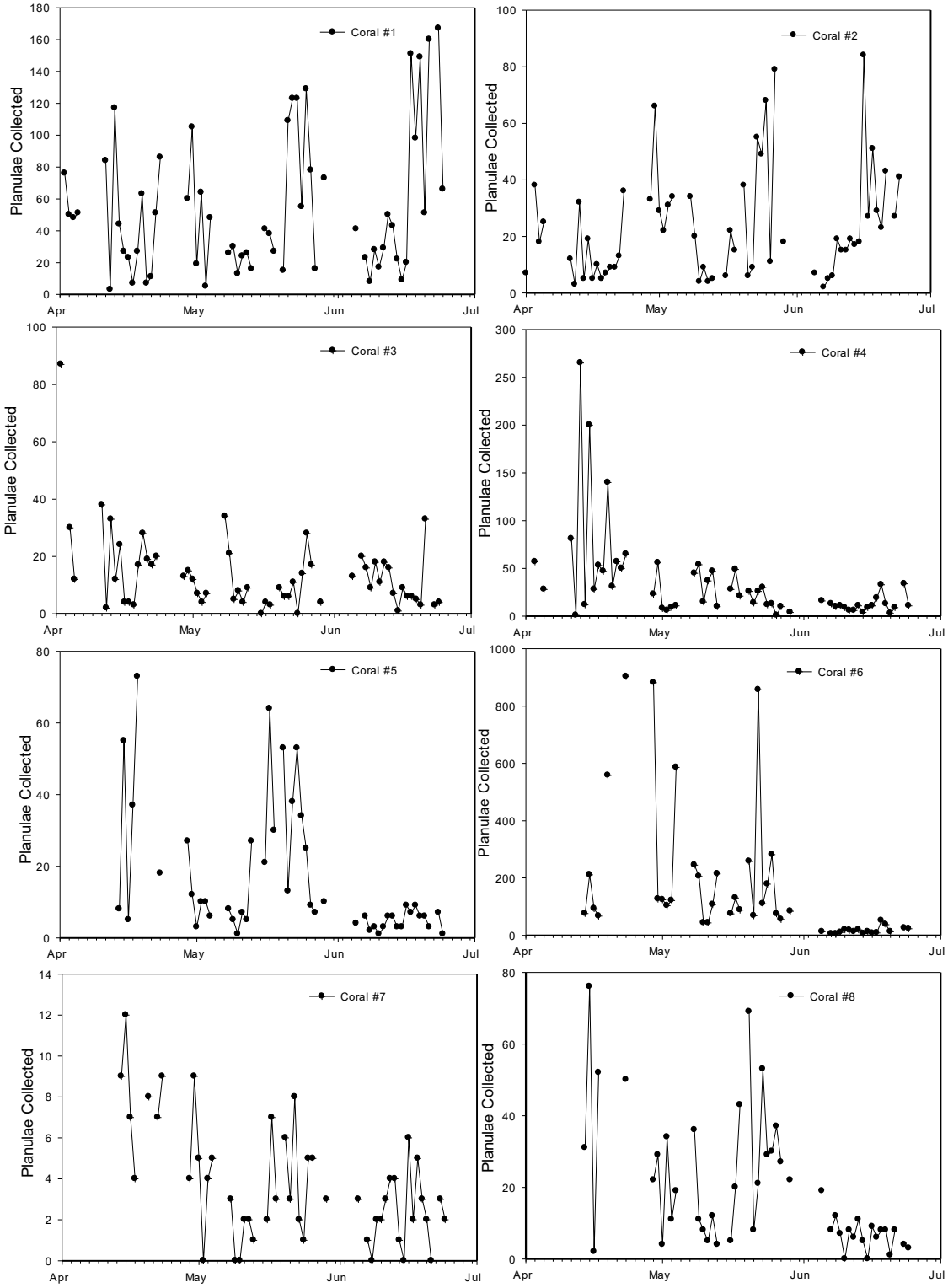

Figure 2 Daily collections of planulae for each of the eight coral colonies (**a-h**) of *S. pistillata* during April-July. Peaks are indicated with light grey highlights and are defined by number of planulae collected being greater than or equal to 50% of the maximum number of planulae collected on a single day during the study of that particular colony (see Table 1).

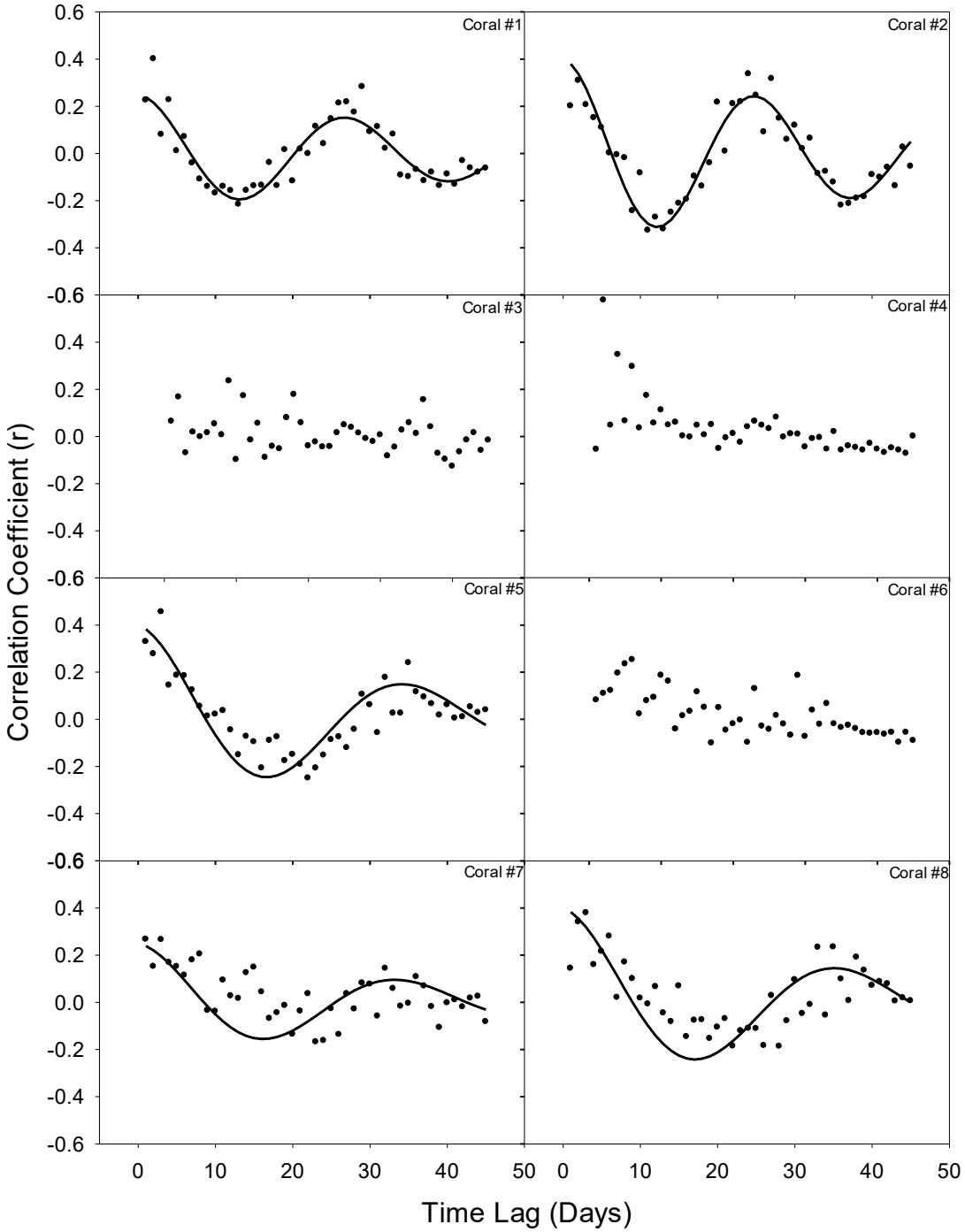

Figure 3 Auto-correlation analyses of planulae released from the eight *S. pistillata* colonies (**a-h**). Time lag was set at a maximum of 45 days. Each point in the graph represents the correlation coefficient of the data at time t+n compared to the data at time t, where n is the time lag in days. Coral colonies #1, 2, 5, 7 and 8 show a clear wave pattern, indicative of recurrence in released planulae behaviours. Trend lines were composed of damped sine waves and used to approximate period.

Table 1 Reproductive effort of eight *S. pistillata* colonies (#1-8; see Fig. 1) during the reproductive season (April-June).

Coral #	***Collecting nights***	***Average planulae/day (±sd)***	***Colony volume (cm^3)***	***Planulae total***	***Maximum planulae/day***
1	59	53.7 (±43.3)	6744.4	3170	167
2	59	23.2 (±19.3)	6864.2	1361	84
3	58	13.6 (±13.6)	7790.4	789	87
4	57	33.3 (±45.8)	12649.1	1898	265
5	48	15.8 (±18.1)	4772.4	759	73
6	48	156.1 (±226.2)	17284.4	7493	903
7	50	3.8 (±2.9)	5595.2	189	12
8	48	19.3 (±18.3)	2300.7	927	76

Auto-correlation analysis revealed three distinct patterns of peak larval release (Fig. 3). The first pattern was exhibited by colonies (#1) and (#2), which had a short peak-to-peak period of approximately 27 and 25 days, respectively. The second pattern was exhibited by colonies (#5), (#7) and (#8), which had a long peak-to-peak period of 34, 33 and 35 days respectively. The third pattern was exhibited by colonies (#3), (#4) and (#6), which did not display any repetition of planulae releasing peaks during the April-June reproductive period (Fig. 3).

Coral fecundity was high during the studied reproductive season. In April, 98.2% of the 109 collected samples contained planulae larvae, a trend that was repeated in the following months (97.1% of the 175 samples and 96.5% of the 143 samples for May and June, respectively). Coral colonies #1 and #2 released greater numbers of planulae as the season progressed, whereas the other six *S. pistillata* colonies released lower numbers of planulae as the season progressed (Table 2).

Table 2 Average number of planulae released per colony per day during the reproductive season (April-June)

Coral #	**Average planulae per day**		
	April	May	June
1	49.5	49.9	62.9
2	18.5	25.8	24.9
3	21.0	9.7	11.0
4	70.2	21.6	12.7
5	24.1	20.0	4.7
6	351.3	184.1	16.5
7	7.2	3.20	2.4
8	30.4	23.1	6.8

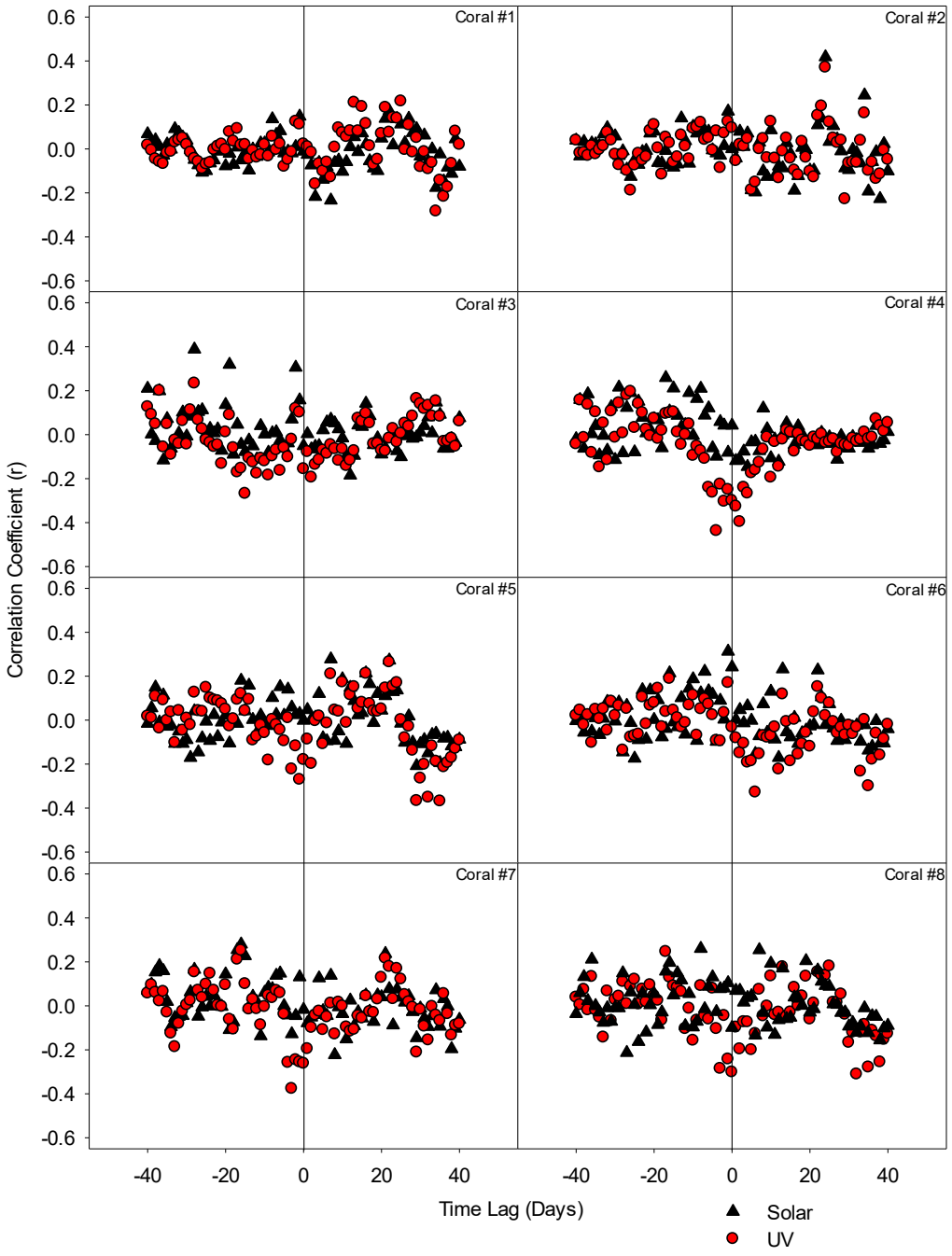

Figure 4 Cross-correlation of larval release versus daily maximum solar irradiance (black triangles) and daily maximum UV irradiance (red circles), for each of the eight coral colonies (**a-h**). Time lag was set at a maximum of ±40 days. Each point in the graph represents the correlation coefficient between the number of planulae at time t and the solar (or UV) radiation at time t±n, where n is the time lag in days. No distinct pattern was found and no period could be established.

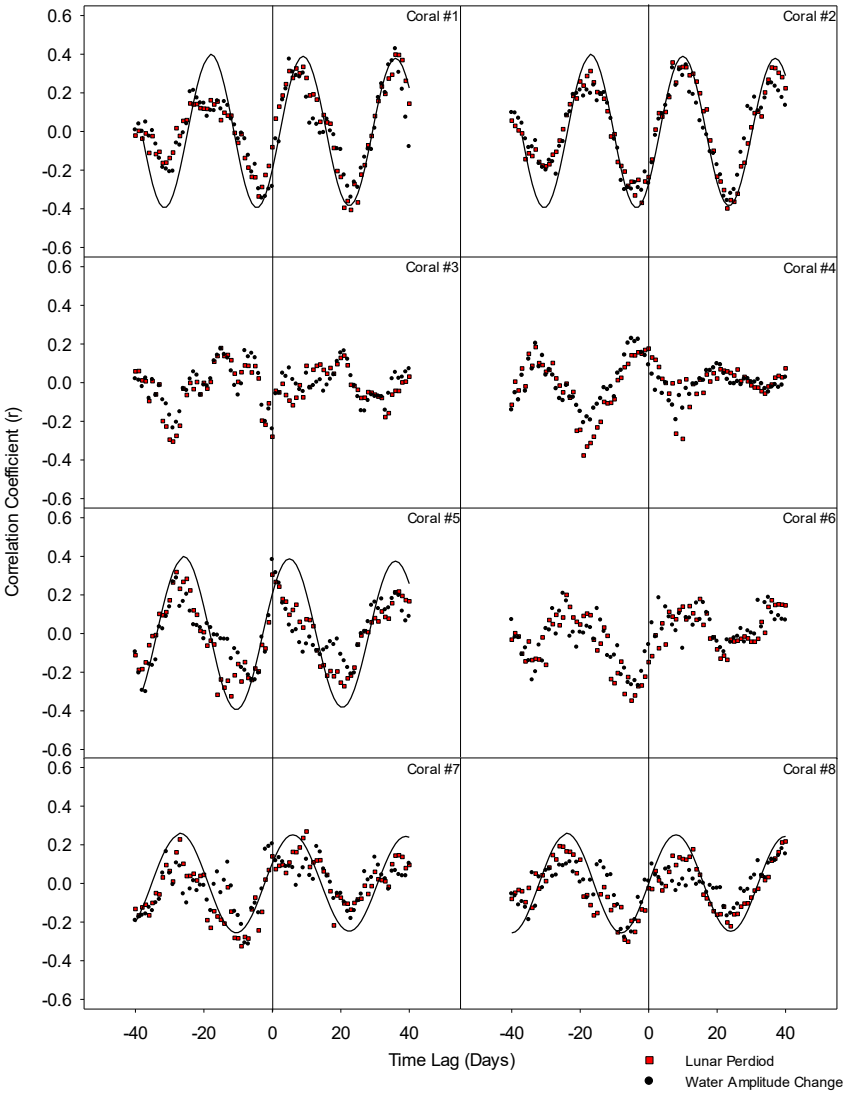

Figure 5 Cross-correlation of larval release versus lunar period (red squares) and tidal range (black circles), for each of the eight coral colonies (**a-h**). Time lag was set at a maximum of ±40 days. Each point in the graph represents the correlation coefficient between the number of planulae at time t and the lunar period (or tidal range) at time t±n, where n is the time lag in days. Trend lines were composed of sine waves and used to approximate the periodicity of coral colonies that had repeating larval spawning peaks. Coral colonies #3, 4 and 6 did not show periodicity (see Fig. 3), and were therefore left out of the cross-correlation analyses.

Cross-correlation analysis revealed no tangible link between *S. pistillata* larval releases and daily maximum solar irradiance or UV irradiance (Fig. 4). Cross-correlation analysis of the 5 periodic colonies showed coherent oscillations of *S. pistillata* larval releases with the lunar period and tidal range, characterized by a variable periodicity of 27 to 33 days and a variable time lag of 5 to 10 days after full moon depending on the colony (Fig. 5).

In total, peaks in larval release were not related to water temperature or solar irradiance. They were loosely associated with the lunar cycle, but occurred during both neap and spring tide, and both before and after full moon (Fig. 6).

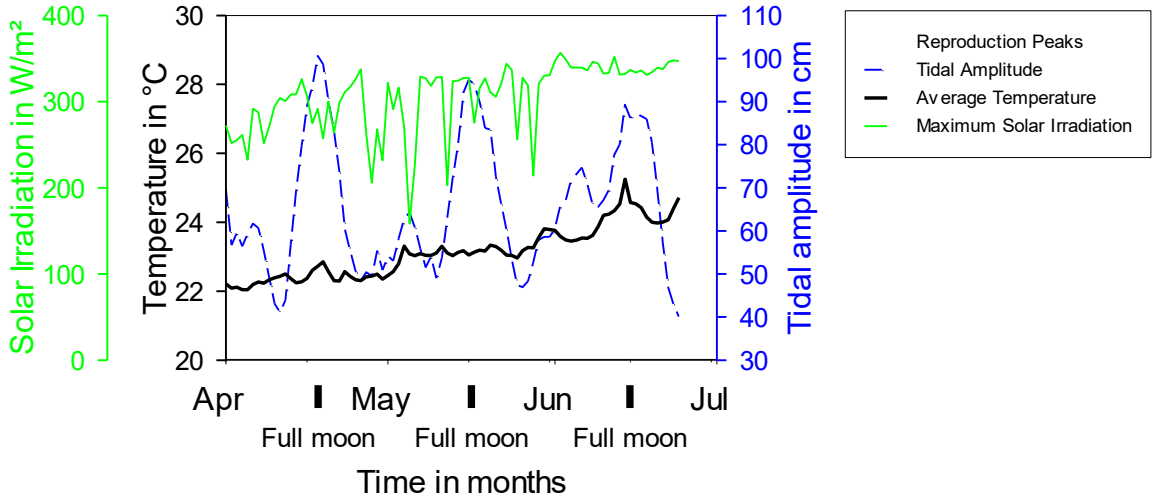

Figure 6 Compilation of reproduction peaks and concomitant changes of tidal range (green line, solid), average water temperature (black line, solid), daily solar insolation (red line, solid) and full moon phases (black line, dashed). The light grey areas comprise the reproductive peaks of all eight *S. pistillata* colonies (see Fig. 2). Full moon occurred on the 18th of April, 17th of May, and 15th of June.

Discussion

Of the eight coral colonies sampled, five had consecutive peak larval releases during the sampling period; two coral colonies had a period of 25 and 27 days, while the other three coral colonies had periods of 33-35 days. This variation in the timing of planulae release does not support population-wide synchronization to a zeitgeber. Despite earlier suggestions that water temperature and UV or solar radiation might be potentially important environmental drivers of larval release periodicity (Harrison & Wallace 1990, Tanner 1996, Mercier *et al*. 2011, Brooke & Järnegren 2013, Keith *et al*. 2016), our results did not reveal such a relationship with these variables.

Cross-correlation analysis showed a relation between the reproductive patterns of the periodic coral colonies and the lunar cycle (~29.5 days) as well as between reproductive patterns of the periodic coral colonies and the spring-neap tidal cycle (as captured by the variation in tidal range). The periodicities of these cross-correlations varied from 27 to 33 days, with a time lag of 5 to 10 days depending on the colony, and none of the coral colonies actually showed a ~29.5-day periodicity in larval release. Consequently, peaks in larval release gradually shifted phase with respect to the lunar cycle, and occurred during both spring tide and neap tide, and both before and after full moon (Fig. 6).

It could be argued that *S. pistillata* is loosely circalunar, having approximately 29.5-day reproductive cycles when averaged over all periodic colonies, but the large lunar phase variation

(from full to new moon and spring to neap tide) of peak larval release (Fig. 6) implies that the entrainment with an external zeitgeber required for the presence of circalunar periodicity (Raible *et al*. 2017) is lacking. At the moment this ca. 30-day cycle would therefore be more aptly described with the term circatrigintan (Halberg 1969) and within that definition the reproductive cycle still shows large variation among the colonies (25-35-day periodicity).

The presence of some sort of free running endogenous clock, controlling the reproductive output of shallow water *S. pistillata* in the northern Red Sea, might be a vestige from earlier times when the lunar cycle did act as zeitgeber. The intense light pollution at night in Eilat (Tamir *et al*. 2017), potential impacts of global changes causing shifts in reproductive seasonality (Tanner 1997) or perhaps a low evolutionary need for entrainment with the lunar cycle in the Red Sea could be possible reasons why the reproductive cycle of this brooding species has been decoupled from the lunar cycle. This would also explain why some of the colonies do not have a pronounced periodicity in their reproductive output. The lack of a zeitgeber and the variation in circatrigintan patterns displayed by *S. pistillata* colonies sheds new light on the discussion on coral plasticity and fecundity, and the possible implications for other brooding coral species that have previously been thought to display lunar periodicity.

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Chapter 3

Creating stocks of young colonies from brooding-coral larvae, amenable to active reef restoration

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Chapter 3
Creating stocks of young colonies from brooding-coral larvae,
amenable to active reef restoration

Abstract
Coral reefs are declining worldwide, even though traditional reef practices continuously underlie reef protection. This calls for exploration and integration of novel restoration techniques and tools, such as the "gardening" concept. The gardening approach, which has been successfully applied in various reef sites worldwide, is based on farming coral stocks in mid-water nurseries. To date, the farming of asexually produced coral material has chiefly been studied. Here, we test the performance of a novel spat-stocking tool for planulae of *Stylophora pistillata*, a brooding coral species. Two prototypes of a new settlement apparatus and one original apparatus made of Petri dishes lined with preconditioned transparency (Mailer's paper) disks had been stocked with >3730 planulae. After 96 h, only 95.3% of >2080 settlers were found on the Mailer's paper provided. One-month-old survivors (80.8% of initial settlements) that were kept *ex situ* in a flow through seawater table were detached from the papers, "transglued" onto plastic pins, and transferred to mid-water coral nursery, where the trays were covered with fitted plastic nets (1 cm^2 mesh) to prevent predation and detachment. Four months later, more than 89% survivorship was documented, with colonies starting to form 3D structures. We estimate that 676 person-hours would be required to create 10,000 5-month-old genotypes of equal size to small branch fragments. This novel methodology allows farming of large quantities of colonies originating from sexually produced planulae and may enhance local populations' genetic variability within a short period. This method is inexpensive and easy to perform in remote places for incorporation in coral reef management practices.

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amenable to active reef restoration

Introduction

The Global Environmental Facility (GEF) has recently estimated that 19% of worldwide reef area has already been lost, 15% of the reefs are under serious threats of loss within the next 10−20 years, and 20% will be lost in 2-4 decades (Wilkinson 2008). Peer-reviewed studies published in the last two decades have dealt at length with the various agents (i.e., climate change, pollution, ocean temperature and acidification, coral bleaching and disease) responsible for the wide-scale decline of coral reefs and the consequences of biodiversity loss (Hughes *et al*. 2003, Ayre & Hughes 2004, Hoegh-Guldberg *et al*. 2007, Rinkevich 2008, Riegl *et al*. 2009, Shaish *et al*. 2010). The establishment of Marine Protected Areas (MPAs), a 'passive' management measure (Rinkevich 2006, 2008), has increased rapidly over the past three decades, aiming to reduce reef degradation and assist natural recovery of degraded reefs. While MPAs currently cover 18.7% of the total global reef area (98,650 km^2, Mora *et al*., 2006), they have been inefficiently applied and many MPAs have often failed to meet their goals (Mora *et al*. 2006, Sale 2008).

Integrating 'active' restoration tools into the currently employed MPA management strategies may be an improved approach to alleviating rates of reef decline. In most cases, it is unlikely that a system that has shifted away from the coral dominated phase will spontaneously revert back without involving active restoration acts (Rinkevich 2008). Clearly, as ecosystems are naturally dynamic and subject to changes in time (Jackson & Hobbs 2009), restoration should be applied or employed to reestablish the function and not necessarily focus on the conservation of one or two the species. Where possible, eco-engineering tools should be given the opportunity to restore ecological services and reef resilience and reconstruct the food webs that support ecosystem functions (Byers *et al*. 2006). This is the reason why we use *Stylophora pistillata* from Eilat as a model system. This is a robust, fast growing, and dominant species in the northern Red Sea coral reefs (as compared to populations from other localities; Marshall and Baird, 2000; McClanahan *et al*., 2004). It can tolerate high temperatures and is a target species for large numbers of reef dwelling fish and invertebrates. These characteristics make *S. pistillata* an excellent bioengineering candidate for restoring ecosystem functions and denuded coral reefs in Eilat.

The latest active restoration tool employed, is based on the 'gardening concept' (Epstein *et al*. 2001, 2003), mirroring the well-established scientific discipline of terrestrial reforestation in the form of a two-step protocol (Rinkevich 2005a, 2006, Edwards & Gomez 2007). The first step entails rearing coral ''seedlings" in underwater nurseries to transplantable size, and the second step involves transplanting nursery-reared colonies onto damaged reef areas. Most corals used in active reef restoration originated from asexual source material, coral branches, fragments and nubbins (Shafir & Rinkevich 2008), taken from field growing colonies or stray coral fragments (Nagelkerken *et al*. 2000, Bowden-Kerby 2001, Lindahl 2003, Yap 2004, Putchim *et al*. 2008, Shaish *et al*. 2008, Levy *et al*. 2010, Mbije *et al*. 2010, Guest *et al*. 2011; and literature therein)). Notably fewer studies have explored the use of sexually generated coral material for reef restoration, by collecting eggs and sperm from broadcaster coral species, or planulae from brooders (Nozawa, 2008; Okamoto *et al*., 2008; Omori, 2005; Petersen *et al*., 2005a).

Clearly, restoring a coral reef via fragmentation may not provide the same genetic diversity that is acquired by planting sexually produced corals (Rinkevich 2005a), though maintenance of high levels of genetic diversity in coral populations is crucial in terms of resilience against disturbance (Van Oppen & Gates 2006). Because coral planulae and spats have a much higher chance of survival *ex situ* than in the wild (Keough & Downes 1982, Fabricius & Metzner 2004), successful

maintenance of coral spats requires the development of complex inland facilities (Petersen *et al*. 2005a, Okamoto *et al*. 2008), which are not always available near coral reefs. However, it was shown that planulae could easily be transported from collecting sites for several days with high survival rate. One study (Petersen *et al*. 2005a) recorded >90% survivors transported for 10 days in 10 µm-filtered seawater and 15 or 50 ml tubes (with planulae concentrations ≤3.3 larvae ml^{-1}). It is possible, therefore, to collecting larvae in remote areas, transport them, and settle them in inland facilities.

The use of coral larvae for reef restoration measures calls for the concurrent development of untraditional settling methodologies, using a substrate type that is attractive to coral larvae, recyclable, and replaceable. This constituted a major drawback in previous studies, in which spats settled permanently on first-available solid substrates (Nozawa, 2008; Okamoto *et al*., 2005; Omori and Iwao, 2009; Petersen *et al*., 2005a). These spats could not be spaced for individual growth and, due to intraspecific competition, had therefore reduced spat viability and growth rates.

Here, we introduce a new stocking method for coral spats, which allows the efficient production of numerous sexually produced coral colonies with the same attachment versatility as asexual fragments or nubbins, avoiding decrease of genetic variation and the potential harms inflicted on donor colonies through fragmentation. This novel technique was performed on *S. pistillata* larvae, adding new facets to the research as most previous efforts had been devoted to planulae of broadcasters (Harii *et al*. 2001, Okamoto *et al*. 2005, Omori 2005).

In the established gardening technique (Epstein *et al*. 2001), fragments of corals are grown on plastic pins, which are used for transplantation. Shafir *et al.* (2006) calculated the time required to create stocks of 10,000 colonies per species, from five species, through fragmentation using unskilled personnel. In congruence with this idea, we calculated the time needed to produce an equal stock of corals on the same type of plastic pin, using planulae as source material. The resulting stock of colonies can be used as needed in the second step of the 'coral gardening' protocol (Rinkevich 2005a, 2006).

Materials and Methods

The study was conducted in front of the Inter University Institute for Marine Science (IUI) in Eilat, Israel (northern Red Sea). Planulae were collected *in situ* from 15 gravid *S. pistillata* colonies, ranging between 11.5-29.8 cm in diameter, residing at a depth of 3-5 m. All planulae were collected at night using simple planulae traps, as described in Amar and Rinkevich (2007).

As settlement apparati, we used aseptic disposable Petri-dishes (90×15 mm) that were covered with preconditioned polyester papers, double-sided matte (Mailer's paper; manufactured by Jolybar, Israel), cut to fit the top (9 cm diameter) and bottom (8.5 cm diameter) of the Petri-dishes. Preconditioning was performed by submerging the Mailer's paper disks in a flow through seawater table for at least two months, as according to Petersen *et al*. (2005), during which natural biofilm developed. After biofilm development, the papers were glued to the top (lid) and the bottom of the Petri-dishes. When put together, the Petri-dishes created a closed vessel in which planulae could settle on the top and the bottom.

We inserted 1-69 sister planulae (same day cohort, released from a single mother colony) per dish, depending on collection yields. Most of the Petri-dishes were initially stocked with 60 planulae. Three apparatus prototypes (treatments) were established (Fig. 1). The first (A) was a Petri-dish without a lid, which was placed in a humidity chamber, a system similar to that used previously (Amar *et al*. 2007) to settle planulae. The second and third treatments (B and C) tested settlement in seawater-filled Petri-dishes with lids. In addition, the third prototype (C) was fitted with silicon plugs, perforated for air to escape. The two closed apparati (B and C) were placed in a flow through seawater table for stable ambient temperature and kept submerged, tightly closed by placing a weight on top of the lid. All air was removed from the submerged Petri-dishes, either via the opening between dish and lid (treatment B) or through the silicone plugs (treatment C), to allow the planulae to settle on the top lid, which would otherwise be obstructed by air. We counted larval settlements in the first four days after their release, a period consisting of two 48-hour periods. The Mailer's paper disks carrying recruited spats were removed after the first period and replaced with new pre-treated paper disks. Then all free-swimming planulae were returned to the same Petri-dish for an additional 48–hour period. Leaving the planulae in stagnant water for periods of three days or longer resulted in increased mortality rates (pers. obs.).

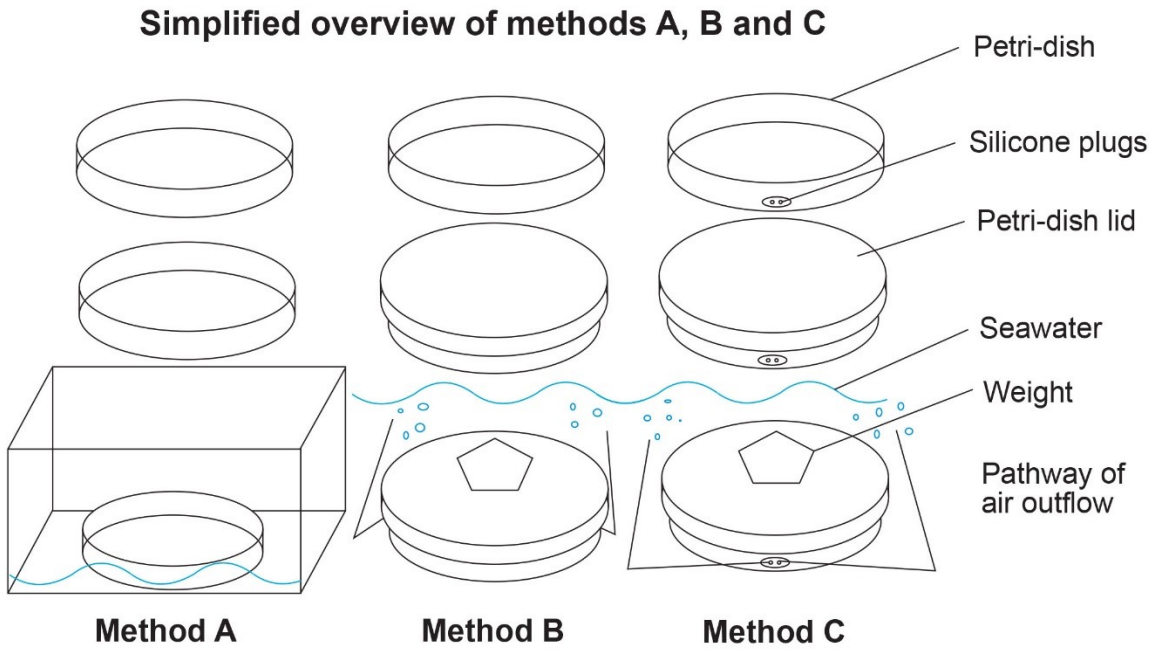

Figure 1 A simplified outline of treatments A, B and C. In the first step, each Petri dish is stocks with ~60 planulae. The second step shows Petri dishes for treatments B and C covered by their lids. The third step is the placement of the three treatments in respective environments; A is in a humidity chamber, B and C are submerged in a flow-through seawater table.

Where possible, the planulae of a single mother colony were distributed evenly over treatments and observed simultaneously in the three treatments. In total, 162 Petri-dishes were observed. For the first settlement, 89 Petri-dishes (A = 29, B = 30 and C = 30) were used; for the second settlement 73 Petri-dishes (A = 24, B = 24 and C = 25) were required. As mentioned, in treatments B and C, each Petri-dish was fitted with two Mailer's paper disks (top and bottom), whereas treatment A used only one (bottom). The settlement rate in our apparati was not influenced by the initial number of planulae or available settlement area (Amar *et al*. 2007). In total, 271 Mailer's paper disks with coral spats were produced. The first settlement period yielded 149 and

the second 122 settled Mailer's paper disks. We calculated larval mortalities during the 96-hours period as total planulae at day zero, minus the sum of settled and free-swimming larvae.

A 'flypaper' system (Morse *et al*. 1994) was adopted for handling Mailer's papers with settled planulae. First, adhesive (Super Glue 3, Loctite, Ireland) was applied to scratched parts of a glass slide (7.5×5 cm) on which an unconditioned square of Mailer's paper was then glued. This intermediate substrate was used because previous attempts at gluing paper disks with attached spats directly onto the glass slides failed to hold permanently (pers. obs.), probably because of the developed biofilm. The Mailer's paper disks with settled planulae were then glued onto the clean paper squares (Fig. 2a).

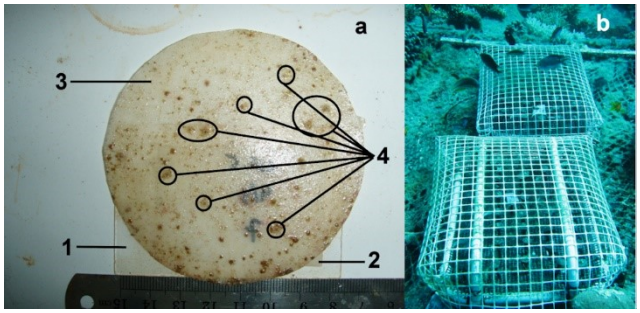

Figure 2 *Ex situ* and *in situ* components of the spat stocking protocols. (a) *In situ* planulae settlement apparatus. '1' glass slide, '2' square Mailer's's paper, '3' circular Mailer's's paper and '4' *Stylophora pistillata* spats. (b) Two trays covered with 1 cm^2 plastic nets in the Coral Nursery, Eilat, Israel at a depth of 9 meters.

Sets of four glass slides were placed vertically in a Polypropylene staining rack (7×9 cm) and returned to the flow through seawater table. Using only four of the 20 slots available in the staining rack allowed free water flow, easy access for small herbivores that graze on algal assemblages, and reduced particle accumulation (Fig. 3). Randomly selected Mailer's disks (n=42) were used to calculate survival after a month by comparing photographs of the Mailer's paper disks at t=0 and t=1 month.

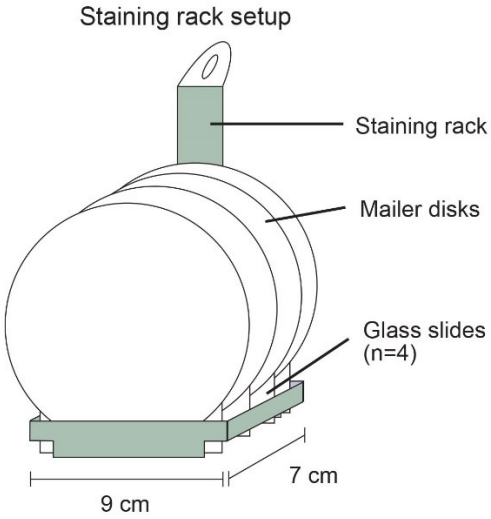

Figure 3 The placement of glass slides in a staining rack. A maximum of four glass slides per staining rack. The glass slides are evenly spaced, leaving space between for water flow through and access for grazing by small herbivores.

After one month in the flow through seawater table, the surviving settlements were easily detached from the papers, 'transglued' (Super Glue 3, Loctite, Ireland) onto plastic pins (Red-Sea Corals LTD., Israel.; Shafir *et al*., 2006) and then moved from the *ex situ* conditions to the our mid-water coral nursery located *in situ* at the North Beach area of Eilat (29°32.4'N, 34°58.40'E). In the nursery the trays (45×32 cm) were fitted with plastic net covers of 1 cm^2 mesh size (Fig. 2b). The trays were made of PVC pipes forming a frame and black plastic net with 0.5 cm^2 mesh size covering the frame. Holes were drilled into the PVC pipe to allow air to escape, and the mesh was kept in place by cable-ties (KSS nylon cable ties 203×2.5 mm, Kai Suh Suh Enterprise CO., LTD., Taiwan). We used the term 'transgluing' in order to avoid confusion between coral transplantation onto reefs and spats' translocation to other substrates.

The transfer of the colonies from the lab to the nursery was done by boat, and, during transportation, prepared trays carrying pins with colonies were submerged in seawater-filled containers. Using SCUBA, we took the trays from the boat to the nursery and attached to the nursery using two cable-ties, at opposite points. Each tray was covered by plastic covers of 1 cm^2 mesh size (Fig. 2b), attached to the tray on one side with a plastic cable-tie and, on the opposite side, with a wire that could be released when needed, for easy access.

The trays with settled young corals were monitored *in situ* for four months. Routine maintenance was performed once a month by SCUBA, including the removal of covering nets and clearing away algal assemblages and major encrusting invertebrates (tunicates and other sessile filter feeders) by brushing the trays and nets, using gloves and tweezers for fine work. Sea urchins (*Diadema setosum)* and other herbivorous invertebrates that had settled on the trays were not removed. Each tray was photographed on a monthly basis to assess growth and mortality rates. Maintenance of *in situ* farmed colonies was identical with farmed nubbins (Shafir *et al*. 2006b).

We analyzed the treatments for significant differences in settlement rates, orientation, and survival. For these analyses, we used one-way ANOVA and Tukey's Honestly Significant Difference (HSD) tests available in SPSS 16.0. Photo analysis for survival *ex situ* was done using an Olympus Stylus 720 SW on self-timer suspended above the Mailer's paper on a laboratory stand using Macro option at highest resolution of 7.1 megapixels availability. By setting up a laboratory stand with camera, the high resolution and self-timer yielded sharp pictures, allowing settlements of any age be easily spotted. *In situ* photographs were done with Sony Cybershot DSC-W7 7.1 megapixel. Mortality (through lack of growth a 4-month period or detachment) was determined using freeware UTHSCSA Image Tool, Version 3.0.

Results

We collected 1236, 1307, and 1278 planulae for the A, B and C treatments, respectively, from the 15 coral genotypes. Seven genotypes, which released over 86% of all planulae collected, shed >180 planulae per colony. Yields of ≥180 planulae/coral were subjected to all three treatments and observed simultaneously.

In the A treatment 45.4±21.9%SD (n=510) of the planulae settled. Settlement rates of B and C treatments (63.2%±24.7%SD, n=828 and 60.0%±22.2%SD, n=747 spat, respectively) did not differ significantly from each other but were higher ($p < 0.05$; Tukey's HSD) than A treatment outcomes (Table 1). In the first 48-hour period, most planulae settled on the Mailer's disks placed in the lids (Petri dish tops) of B and C treatments ($p<0.05$; one-way ANOVA). During the second 48-hour

period, most planulae settled at the bottom of the Mailer's papers ($p<0.05$; one-way ANOVA; Fig. 4). Of the 2082 recorded settlements, only 97 (4.7 %; discarded from follow-up experiment) settled on the side of the Petri-dishes where no Mailer's paper was present, the majority (n=67) of which were recorded in the A treatment. None of the settlements recorded were found underneath the paper surface, between the Petri-dish surface and the Mailer's paper. Randomly chosen Mailer's disks (n=42 Mailer's disks, with n=229 coral spats) were further monitored *ex situ* for one month. During the first month in the flow through seawater table, 80.8% of the spats survived (n=185 coral spats).

Table 1 Settlement rates and mortality of *Stylophora pistillata* planulae in treatments -A-, -B- and -C-. 1st and 2nd indicate the results for the first and second 48 h settlement periods after release, respectively. * indicates a significant difference between treatments, Tukey's (HDS) $p<0.05$

Treatment/ Period	Planulae (N)	Settlement rates (%) ± SD	Mortality (%) ± SD
A, 1st	1236	35±23	18±16
B, 1st	1307	50±29*	15±22
C,1st	1278	47±25*	15±20
A, 2nd	523	22±20	16±23
B, 2nd	474	31±28	13±15
C, 2nd	489	29±25	18±23
A, total	1236	45±22	27±23
B, total	1307	63±25*	19±23
C, total	1278	60±22*	23±21

In the first 48 hours, 47.2-49.6% of *S. pistillata* planulae settled in the B and C treatments, as compared to 34.5% in treatment A. The second settlement cycle increased total settlements by 10.9-13.5%, demonstrating that the competence of the planulae to settle decreases beyond the first 48-hour period (Table 1). Four days post-release, 16.3-23.1% of the initial numbers of planulae were still in free swimming phase.

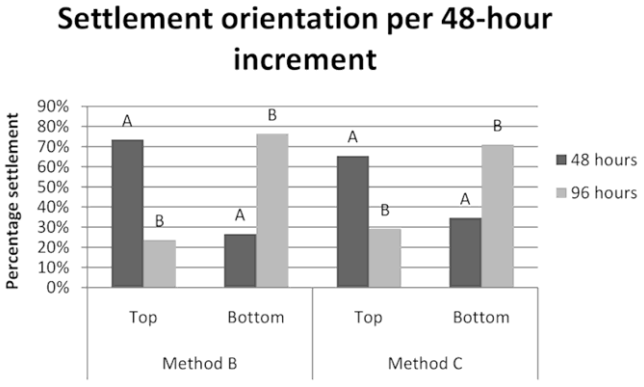

Figure 4 *S. pistillata* larval settlement preference, along two 48-hour periods after their release. *S. pistillata* showed a significant preference ($p<0.05$, one-way ANOVA for both method B and C) for the top and bottom Mailer's paper during the first 48-hour period (A) and second 48-hour period (B) respectively.

The difference in settlement rates could not be explained by the initial number of planulae (1236, 1307 and 1278) used per treatment (A=29, B=30 and C=30 Petri-dishes, respectively; $p=0.722$; Tukey's HSD). Similarly, mortality or loss of planulae could not be attributed to any of the

treatment (p=0.810; Tukey's HSD; Table 1). It could not be discerned if planulae settlement rates were affected by the number of planulae per Petri-dish (1 to 69 planulae), because group sizes were too small and distributed unevenly to allow proper statistical analysis.

At the age of 1-2 months, 480 spats were easily peeled off the Mailer's papers, by bending the papers. Spats were individually transglued to plastic pins previously used for attaching coral nubbins and fragments (Shafir *et al*. 2006b). The colonies were then placed *in situ* within the floating coral nursery near Eilat, covered with a 1 cm^2 white plastic net to protect against fish predation and accidental detachment. After four months *in situ*, 89% of the colonies (n=428) were alive, starting to form 3D structures (Fig. 5).

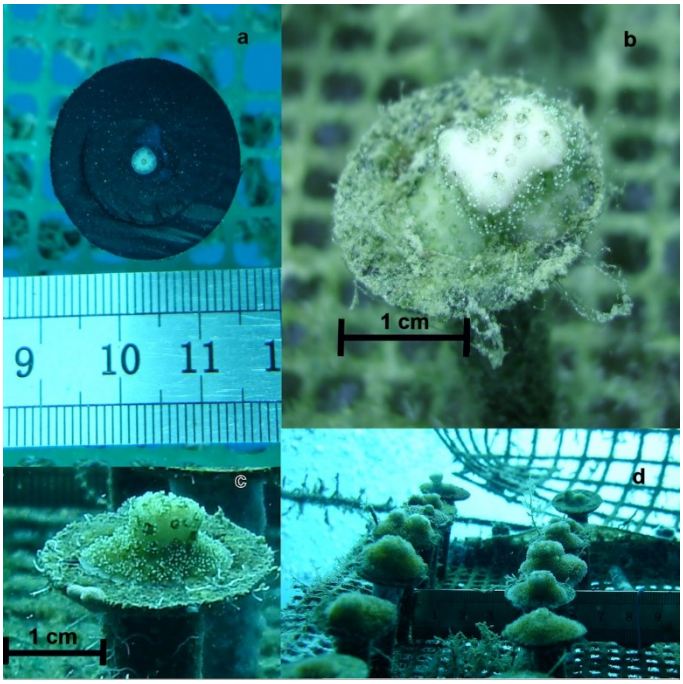

Figure 5 Young *Stylophora pistillata*: colonies on a plastic pin. (a) A one-month-old colony on a plastic pin *in situ* immediately after having been brought to the nursery via SCUBA. (b and c) Colonies at age of five months, farmed for four months *in situ* in the coral nursery. (d) Five-month-old colonies farmed in the coral nursery on pins inserted into a tray. All pins are uniform in size.

Settling and retrieving planulae traps took 10 min per colony, harvesting, on average, about 100 planulae/night/coral colony during the peak of the reproductive season (pers. obs.). Setting Petri-dish, including preparation and gluing of the Mailer's papers, took 5 min per dish. Transferring planulae from the trap to the Petri-dishes took 10 min per 60 planulae. An additional 10 min per Petri-dish was required for replacing the Mailer's papers (for the second settlement period) and for transferring the settled Mailer's papers onto glass slides. Transgluing each one-month old colony onto a plastic pin took approximately 2 min per colony.

Spat stocking for 10,000 5-month-old *S. pistillata* colonies in the Gulf of Eilat (taking into account losses incurred by planulae mortality, larval incompetence, and *ex situ* and *in situ* spat mortalities) necessitates the collection of 23,000 planulae by inserting of 230 traps and 40 person-hours during the peak of the reproductive season. Assuming an average 60% settlement rates, 130 person-hours would be required for the first and second settlement periods (97 and 33 person-hours respectively, assuming 60 planulae per Petri-dish during both settlement periods), creating a total of approximately 14,000 spats. With 80% survival rates, about 11,200 coral spats were

transglued onto plastic pins (an additional 370 person-hours). It took 40 diving-hours to place trays with coral spats on pins in the nursery (50 corals per tray, 240 trays). Following the 90% survival for the first four months *in situ*, 10,000 small coral colonies remained alive after five months. Maintenance of nursery-farmed corals required 24 diving-hours per month.

In total, 676 person-hours (4 person months) were needed to produce 10,000 small, 5-month-old colonies (size of each is equal size to a small branch fragment) in Eilat's nursery. This time investment is comparable to other methodologies used for different types of coral material, but with better results (Table 2). Preparing and growing 10,000 fragments requires between 317-547 person-hours (Shafir *et al*. 2006b); rearing and growing 10,000 sexually produced coral spats derived from broadcasting species requires between 968-1,210 person-hours (pers. com. Omori). Different coral species or environmental conditions can influence these numbers.

Table 2 Overview of time requiered to develop a stock number of 10,000 new colonies at age of 5 months. * = Brooders; ** = Fragments; *** = Spawners. Comparison of stock creation and time requierement.

		This study*	**Shafir *et al*** (2006)**	**Omori*** (pers. com.)**
Preliminary step	Collection of eggs/larvae	40 h	N.A.	19 h
	Settlement	130 h	N.A.	119 h
	Predicting spawning	N.A.	N.A.	20 h
First Step	Preparing corals for nursery/transgluing	370 h	100-200 h	24 h
	Placing of corals in nursery	40 h	40 h	30 h
	Maintenance per month	24 h	24 h	12,5 h
	# colonies at the age of 5 months	10,000	6,580-8,200	2,000-2,500
	Total time needed/10,000	676 h	317-547 h	968-1,210 h

Discussion

In view of the worldwide decline of coral reefs and the failure of currently used management methods (Sale 2008) to reach their set goals, we must continue to work towards improving the tools and methods for reef rehabilitation. By applying 'active' restoration methods to the already existing management measures, we may be able to restore otherwise denuded reefs or encouraging reefs towards natural recovery. This study enhances 'active' restoration methodologies by using sexually produced new coral colonies that can be exploited as reefs' eco-engineers or as boosters in corals' genetic variation and reef resilience. We offer two apparati (treatments B and C) that are more efficient than the previously used apparatus (treatment A) for settling planulae of brooding coral species. This settlement efficiency stems from the inclination of *S. pistillata* planulae to settle top substrates in the first 48 hours, a preference that is absent from treatment A. The settlement rate in treatment A was comparable to results of a previous study (27.1-36.1% within 14 days; Amar *et al*., 2007) performed on *S. pistillata* larvae released from the same site population.

The B and C treatments are highly efficient in establishing coral spat stocking (about 40% of released *S. pistillata* larvae reached the stage of 5-month-old small colony) following a short settlement period (96 h). Most larvae settled on the provided substrate (>95%), and only a minority settled on the Petri-dishes' side. Planulae mortality was low in all treatments (21-23%). Therefore, the flexible Mailer's paper serves all needs, allowing high settlement rates with minimal pre-treatment procedures and high survival rates of settled larvae. It is a recyclable

substrate from which spat can be easily detached undamaged and 'transglued' to any other permanent substrate of choice.

Another benefit of the spat stocking methodology is the reduction of chimerism (fusion between different spats of the same species) and competition to which coral spats are exposed after collective settlement. Chimerism is a common outcome in spat-aggregates and reduces the number of available individuals (Amar *et al*. 2008). Competition (rejection, overgrowth, etc.; Amar *et al*., 2008; Amar and Rinkevich, 2010) between young settled conspecifics may cause another problem. Planulae that settled and metamorphosed on 'permanent' substrates, such as the 'coral pegs' (62.5 settlements per peg; Omori and Iwao, 2009) start competing for space shortly after settlement, which results in only 1-2 surviving colonies/peg and increased loss of valuable material. This outcome is also evident in other cases of settlements on permanent substrates, e.g. Petersen's pyramid and flat-tiles (Petersen *et al*., 2005b), Nozawa's micro-crevice tiles (Nozawa 2008), and Okamoto *et. al*. coral settlement device (CSD; Okamoto *et al*., 2005). Moreover, larval settlements within substrate crevices, commonly recorded in all former methodologies, offer limited prospects for successful transfer to other substrates because of damages inflicted during their detachment. An inherent characteristic of our new spat stocking tool for *S. pistillata* is its simplicity. Past studies (Morse *et al*. 1994, 1996, Heyward & Negri 1999, Iwao *et al*. 2002) suggested using specific chemical cues for improving planulae settlement rates, which can be achieved by the simple pre-conditioning protocol of the Mailer's papers. The same applies to the recommendation for rough-textured substrates because larvae not only prefer to settle within crevices but also show increased *in situ* survival rates as compared to flattened substrates (Omori *et al*. 2004b, Okamoto *et al*. 2005, Nozawa 2008).

In our new system, the application of a short (one month) *ex situ* farming protocol prior to transferring the spat to nursery conditions yields not only high survivorship (89% in 4-month period) and fast growth rates (enhanced rates as compared to *ex situ* conditions; unpublished) but also reduced maintenance efforts. This high survivorship is similar to the survivor values of *S. pistillata* nubbins under nursery conditions (65.8-82.0%, 100d *in situ*; Shafir *et al*., 2006). In addition, the short *ex situ* period is inexpensive and easy to operate. A density of approximately 800 spats/m^2 table surface area is obtainable by using staining racks, each containing four glass slides covered with Mailer's papers. This high-density does not reduce survival (80% within the first month after settlement), and preliminary results revealed that the one-month *ex situ* period could be curtailed. Young *S. pistillata* colonies develop wide basal plates almost immediately after settling, making the spat easily removable as early as two weeks post-settlement (pers. obs.).

Transgluing each 1-month-old colony onto a plastic pin takes approximately two minutes, similar to gluing an *Acropora* nubbin. Shafir *et al.* (2006) calculated 191 to 415 person-hours, depending on the coral species used and level of skill, to create 10,000 plastic pins carrying coral fragments and 120 person-hours for five months maintenance period. In Palau, Omori and Kimura (pers. com) calculated 768 to 1,010 person-hours for establishing 8,000-10,000 coral spats (broadcasting species), and 200 more person-hours (maintenance) for five months old spat. These spats are subject to further intraspecific competition because they cannot be spaced.

We estimate about 676 person-hours for 10,000 5-month-old sexually produced coral colonies. It should noted, that maintenance of the mid-water floating nursery and farmed coral colonies could differ greatly per location and seasonality; e.g., algal blooms require more time cleaning. Although our procedure for creating 10,000 5-month-old corals is more time-consuming than

Shafir's method with asexually produced coral colonies and requires more skill to perform, each of the resulting corals is genetically unique, thus preserving local genetic variance, and no corals are damaged in the process of creation. Furthermore, creating a stock of 10,000 asexual produced corals necessitate fragmentation of 10 to 50 corals (212-1054 ramets per coral; Shafir *et al*., 2006), which makes our method particularly attractive for endangered or threatened species.

Therefore, the above results refute the general assumption that sexually produced material, while being an invaluable tool for active reef restoration, is difficult to use in large restoration projects because it is time-consuming and costly to grow coral colonies from the larval stage. Whereas we successfully used a model brooder, hermaphroditic species (Harrison & Wallace 1990), the applicability of the novel methodology on other brooder species has yet to be demonstrated. Additionally, populations of *S. pistillata* in the Gulf of Eilat are particularly robust compared to *S. pistillata* in other reef areas (Marshall & Baird 2000, McClanahan *et al*. 2004). We would suggest that, together with farming of fragments and nubbins, it is possible to farm large quantities of sexually produced *S. pistillata*, for restocking genetic variation of local populations within a short period.

We here present part of the first step of a methodology for active restoration with sexually produced corals. As 5-month-old small colonies as grown in this study are too small for transplantation, they should be kept in the nursery until they reach sexual maturity, about three years in the field and much less under nursery conditions (Amar & Rinkevich 2007). Rearing young coral spats in mid-water nurseries to large colony sizes, however, has yet to be studied thoroughly.

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Chapter 4

Elaborating an eco-engineering approach for stock enhanced sexually derived coral colonies

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Chapter 4

Elaborating an eco-engineering approach for stock enhanced sexually derived coral colonies

Abstract

Despite all traditional conservation management efforts, coral reefs worldwide continue to face a future of degradation and destruction. Current studies call for the augmentation of coral reef management with restoration practices, particularly alternative reef management approaches, such as the 'coral gardening' tenet of active reef restoration. This includes the stock creation of sexually derived coral colonies and their mariculture in mid-water coral nurseries. In this study, single and aggregated (resulting in non-fusion and chimeric entities) spats of the branching coral *Stylophora pistillata* were studied. Planula larvae were settled and spat were reared *ex situ* for 50-75 days before they were transplanted to the *in situ* mid-water nursery in Eilat, Israel, where they were followed for up to two years, showing a very low (<2%) detachment rate. Spats were cultured in horizontal and vertical orientations, in caged (for the first 9 nursery months) and non-caged scenarios, and on two nursery beddings (nets and ropes). Caging of horizontally situated young spats on the net substrate resulted in the highest survival (>80% after 2 years). Corals farmed in a vertical orientation had the lowest survival rate of the caged experiments (36.7%) but showed that chimeric/aggregated entities performed significantly better than the single colonies. The uncaged experiments had low (32.7%) to zero surviving spats after two years *in situ*. The surviving colonies reared under the uncaged conditions were significantly smaller than the caged colonies after the 9 months *in situ* 'caging period'. Generally, the placement of the young spats in the mid-water nursery resulted in high growth rates: After two years *in situ*, coral colony diameter increased from 0.1 cm to 8.16 ±1.58 cm for the vertical caged scenario, and 7.08 ±1.72 cm, 7.02 ±1.48 cm for the two caged horizontal designs (HN and HR). This is nearly twice the growth rate observed in natal colonies. The mid-water coral nursery is a much cheaper solution for growing corals compared to *ex situ* water tables, which require high maintenance and expensive facilities to mimic *in situ* conditions. Non-caged coral stocks showed reduced survival and growth rates, similar to previously published results. The culturing of caged spats in a horizontal position in a mid-water nursery exponentially augment survival and growth rates, thus enhancing stock creation yields. This offers new possibilities for creating stocks of sexually derived spats from eco-engineering coral species such as *S. pistillata*.

Chapter 4
Elaborating an eco-engineering approach for stock enhanced sexually derived coral colonies

Introduction

Coral reefs throughout the tropics continue to deteriorate (Wilkinson 2008, De'ath *et al*. 2012), even with all currently implemented conservation management practices in place (Miller & Russ 2014, Rinkevich 2014). Many of these practices fail to prevent damages to the coral system, or to rehabilitate damages that are already widespread (Hughes & Tanner 2000, Rinkevich 2008, Ritson-Williams *et al*. 2009, van Woesik *et al*. 2014). On top of this, global change impacts and the expected increase in anthropogenic activities will augment reef degradation. This increase in pressure on the global ecosystem is expected to lead to a loss of about 70% of the coral biodiversity in the next four decades (Bruno & Selig 2007). The shrinkage of global reef structural complexity is unprecedented, and many reefs experience various degrees of phase shift phenomena (Dudgeon *et al*. 2010).

Taking into consideration these unfavourable prospects and attempting to counteract reef-degradation trends, the literature has made suggestions for alternate reef management approaches (Risk 1999, Sale 2008, Graham *et al*. 2013, Micheli *et al*. 2014), including propositions for active reef restoration (Omori 2005, Putchim *et al*. 2008, Shaish *et al*. 2008, Edwards *et al*. 2010, Shafir & Rinkevich 2010, Mbije *et al*. 2013, Rinkevich 2014). The methods and practices that have been put forward, all aim either to complement or to substitute currently employed conservation efforts in order to halt or reverse the coral reefs' degradation trajectory.

In order to retain the natural genetic diversity of corals (Shearer *et al*. 2009), active reef restoration attempts have further explored the use of planula larvae for the creation of coral stocks (Okamoto *et al*. 2005, Omori 2005, Baria *et al*. 2010, Nakamura *et al*. 2011). Planulae are available in endless supply (Hughes *et al*. 2010), can be caught without damaging adult reef corals, and, if properly handled, can be utilized to create genetically polymorphic stocks that may be used to actively increase the numbers and genetic heterogeneity of reef corals in an area or as "ecologically engineered" tools for coral recruitment.

Enhanced coral recruitment is one of the main instruments for assisting the "resilience" of reefs. "Resilience" is defined as the ability of reefs to absorb recurrent disturbances and rebuild coral-dominated systems (Hughes *et al*. 2010). Coral recruitment is one of the most important factors driving the ecology of coral reef assemblages and is critical for maintaining viable coral populations (Hughes *et al*. 2010, Lukoschek *et al*. 2013). Low recruitment rates may further lead to reduced coral population sizes/genetic properties, low fertilization rates in gravid corals, and higher pre- or post-settlement mortality (Dizon & Yap 2006, van Woesik *et al*. 2014). High coral recruitment rates, in contrast, may offset detrimental impacts of anthropogenic and natural disturbances, as local and global threats continue to decrease coral coverage and the current prognosis is that they will continue to do so (Lukoschek *et al*. 2013).

Through their ability to change biogeochemical services of reef habitats and to build reefs, reef corals function as a key autogenic reef engineering species and, as they modify the environment by modifying themselves, also altering the environment as allogenic reef engineering species (Wild *et al*., 2011). Pioneering, opportunistic, protandrous hermaphrodite coral species, such as the Indo-Pacific branching species *Stylophora pistillata* (Rinkevich & Loya 1979b, Fadlallah 1983, Richmond & Hunter 1990), are particularly well-suited for active reef restoration.

Table 1 Literature documentations on juvenile coral survival *in situ* and *ex situ.* The header "Juvenile state" depicts the size that was classified as "juvenile" in the study. "Period" is the duration of the experiment, and "% survival" is the survivals at the end of the study (the presented order corresponds to the list of species). "Settlement" refers to the initial settlement of the coral colonies used in the described experiments, either *in situ* or *ex situ* and if/when the corals were transferred to *in situ* or *ex situ* during the experiment.

Publications	Species	Juvenile state	Period	% survival	Settlement
Sato, 1985	*Pocillopora damicornis*	3-day old spats	6 months *in situ*	16%	*In situ*
Babcock and Mundy, 1996	*Platygyra sinensis,* *Oxypora lacera*	Just settlement	4 months *in situ*	0.5 and 3.9%	*In situ*
Epstein *et al*., 2001	*Stylophora pistillata*	Settlement and 3 months	1 month *in situ*	0% and 5%	*Ex situ,* then moved *in situ*
Raymundo and Maypa, 2004	*Pocillopora damicornis*	1,3,5,6 month old spats	1 year *in situ*	0, 2.5, 13, 49%	*Ex situ* for 1,3,5,6 months, then moved *in situ*
Wilson and Harrison, 2005	*Acanthastrea lordhowensis,* *Goniastrae australiensis,* *Montastrea curta*	Few day old spats	7-9 months *in situ*	1%, 2.8% and 0.2%,	*Ex situ*, then moved *in situ*
Baria *et al*., 2010	*Acropora tenuis*	1,5-2 month old spats	3 months *in situ*	33%	*Ex situ* for 1,5-2 months, then moved *in situ*
Linden and Rinkevich, 2011	*Stylophora pistillata*	Just settlement	1 months *ex situ*	80.80%	*Ex situ*
Nakamura *et al*., 2011	*Acropora tenuis*	Just settlement	10 months *ex situ*	59%	*Ex situ*
Boch and Morse, 2012	*Acropora hyacinthus*.	Just settlement	9 days *ex situ;* 1 year *in situ*	Site #1- 25%, Site #2- 16%	*Ex situ*
Nozawa *et al*. 2006	*Alveopora japonica,* *Acropora solitaryensis,* *Cyphastrea serailia,* *Favia favus*	1,5 month old spats	3 months *in situ*	3%, 15%, 0% and 2%	*Ex situ* for 1,5 months, then moved *in situ*
Toh *et al*., 2014	*Pocillopora damicornis*	Just settlement	6 months *ex situ*, 6 months *in situ*	45-58%, 38-63%	*Ex situ* for 6 months then moved *in situ*

This study addresses three major issues associated with the creation of nursery farmed coral stocks from ~1-mm^2 coral spats: spat mariculture protocols, survival rates in the nursery, and nursery periods. The specific aims were to test conditions at the nursery phase (Shafir *et al*. 2006b, Baria *et al*. 2010), using designs that could influence the growth and survival of spats *in situ*. The overall goal was to develop more effective and efficient methods for creating sexually derived coral stocks for reef restoration (Table 1). This study tested the effect of spat aggregations (Buss 1982, Rinkevich 2005b), the effect of substrate and culture orientation (Nozawa 2008, Enochs 2012), and the effect of presence/absence of caging in the nursery. These parameters were correlated with survival and growth (Nozawa 2008, Baria *et al*. 2010, Nakamura *et al*. 2011).

Methods

The *in situ* study was conducted at the mid-water nursery located adjacent to the Israel Oceanographic and Limnological Research (IOLR) branch at Eilat (Shafir *et al*. 2006b, Linden & Rinkevich 2011). The mid-water nursery bedding was constructed by repurposing the 14-meter diameter buoyant ring of a floating fish cage. The buoyant ring was submerged, brought to a depth of 10 meters, and attached to the substrate by a central anchor at a depth of 24 meters. The mid-water nursery is situated at about 10-12 m depth away from the reef, 12 m above a sandy bottom. All young *Stylophora pistillata* colonies were created from planulae that had been collected *in situ* from 15 gravid colonies, each ranging between 11.5 and 29.8 cm in diameter, residing at depths of 3 to 5m in front of the Interuniversity Institute for Marine Sciences (IUI) near Eilat, Israel. Planulae were collected at night using simple traps and were settled on Transparency Film as described in Linden and Rinkevich (2011).

After a minimum of 1-2 months growth *ex situ* in an outdoor running facility, as described in Linden and Rinkevich (2011), corals spats were moved from the Transparency Film to plastic pins, 1-4 sibling (originating from the same maternal colony) spats/pin. Spat aggregates composed of 2-4 settlers formed either naturally within a few hours of settlement (n=42), or artificially, 30+ days after settlement (n=104;Amar *et al*. 2008, Linden and Rinkevich 2011). All plastic pins (n=249) were randomly divided over five plastic trays (size 50x35cm; 50 pins per tray). In total, 116 pins with a single spat/pin and 133 pins with aggregated colonies/pin (consisting of histocompatible and incompatible combinations) were transferred to the mid-water coral nursery at Eilat by boat and placed on the nursery bedding using SCUBA. Trays were covered with plastic mesh cages (4 cm^2 mesh size; dimensions: 50x35x10cm) that were removed 267 days (9 months) later. The trays were distributed over two different types of nursery bedding: a recycled fish net (3.25 cm^2 mesh size) and 4-6 mm diameter ropes placed horizontally with a 20 cm spacing, parallel to each other (Fig. 1). The trays were placed on the substrates in either vertical or horizontal orientation to the seawater plane. The trays were set up, therefore, using the terms HN for “horizontal position” on the fishnet (n=99; single=47, aggregates=52), HR for “horizontal position” on ropes (n=50; single=30, aggregates=20), and VR for “vertical position” on ropes (n=100; single=39, aggregated=61) (Fig. 1). This experiment lasted for 678 days.

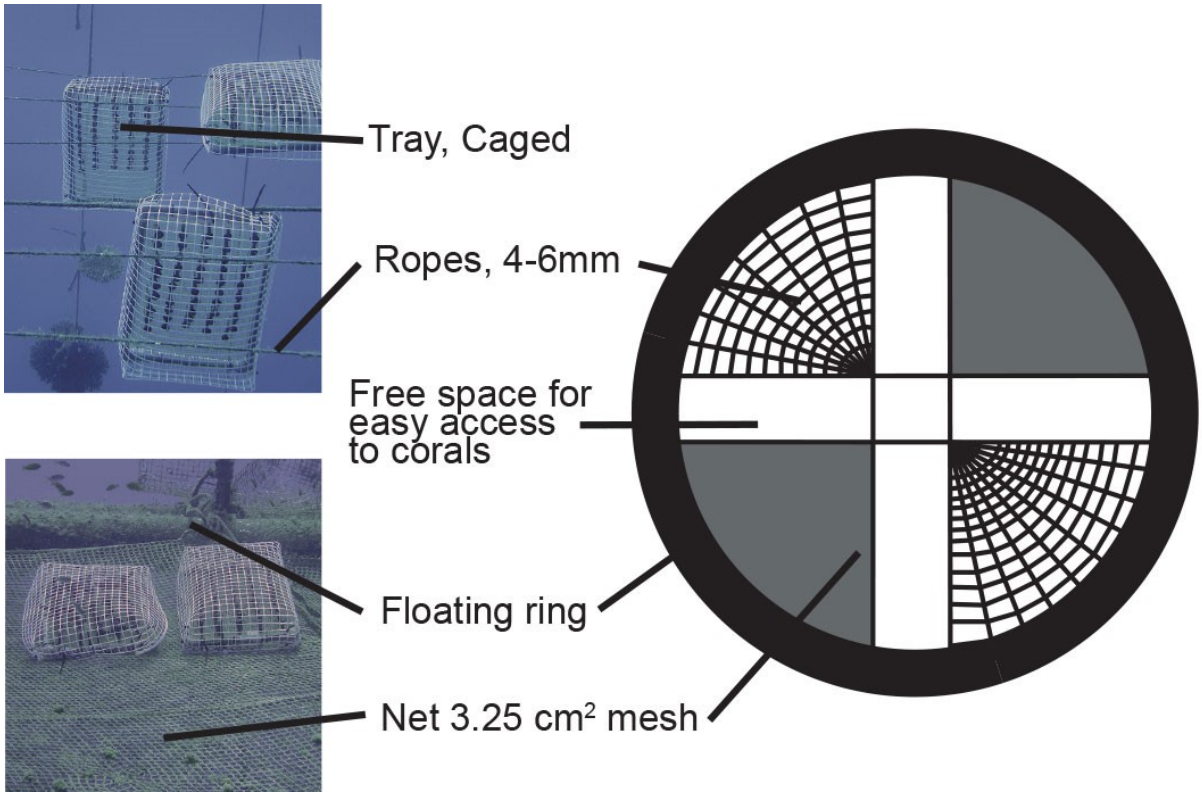

Figure 1 Nursery top view. A schematic illustration of the mid-water nursery top view ($\emptyset = 14m$) and the division of substrates ("net" and "ropes"). The bottom left panel depicts the HN (horizontal, net position). The top left panel depicts the HR (horizontal, rope position) and the VR (vertical, rope position).

To test the effect of caging, two non-caged trays were installed two months after the first experiment began. One tray was positioned hanging from the ropes similar to the VR design, and one tray was positioned similar to the HN design (n=39; single=33, aggregates=4 and n=55; single=45, aggregates=10). This experiment lasted for 605 days.

The trays were cleaned on a monthly basis. All nursery-farmed colonies were monitored for 22 months *in situ* through 10 surveys sessions. The colonies were tracked individually for 14 months, after which they were spaced by moving them from original trays onto new trays. Spacing corals to new trays was necessary to allow continued unimpeded growth. As a result of this spacing, tracking could no longer be verified on an individual level and was, therefore, discontinued.

The two-dimensional growth of the spats (termed the "aerial surface area" [ASA], calculating from the maximal width and length dimensions of the growing corals) was recorded by camera photography and calculated by calibrating the photos in ImageJ software, using the diameter of an empty plastic tip (2cm) as the reference size. Diameter (Ø) was calculated by averaging the width and length measured for ASA. Close visual inspections of the photos were used to determine injuries, damages, allogeneic rejections, and colony mortalities. Obscured colonies were excluded from the calculations of growth for that specific time frame. "Lost" colonies that "appeared" again in the following monthly census were treated as living colonies with missing size parameters. All statistics were performed using SPSS 21, and graphs were created using SigmaPlot 12.

Results

On the day of transfer to the nursery (75, 50, and 50-day-old spats for "horizontal position" on the fishnet [HN], "vertical position" on ropes [VR] and "horizontal position" on ropes [HR], respectively), all spats revealed 2D structures characterized by flat basal plates, but after 125 days *in situ* they started to develop the 3D pattern formation characteristic of *Stylophora pistillata* (Fig. 2, 3). Spats were monitored individually for a period of 411 days, during which small colonies developed to an average ASA (±SD) of 941.9 ±442.0 mm^2 for single genotype spats and 1078.4 ±590.5 mm^2 for aggregates (Table 2) after which all single and aggregate young *S. pistillata* entities were pooled per experimental set-up. At transfer, HN single spats (0.12 ±0.05 mm^2) were significantly larger than the single spats in the other designs (0.09 ±0.04 and 0.08 ±0.03 mm^2 for VR and HR respectively; $P<0.05$ for single spats, Fisher's Least Significant Difference [LSD]). HN aggregate spats (0.24 ±0.11 mm^2) were significantly larger than HR (0.18 ±0.07 mm^2) aggregate spats. After 40 nursery days these differences were no longer significant (Single spat size, 0.15 ±0.07, 0.17 ±0.10, and 0.14 ±0.05 mm^2; Aggregate, 0.29 ±0.13, 0.28 ±0.16, and 0.21 ±0.08 mm^2 for HN, VR and HR respectively; $P>0.05$, Fisher's LSD). Results further revealed that the aggregate ASAs in each of the three caged experimental designs (HN, HR, and VR) were significantly larger than those of the single spats ($p<0.05$, Mann-Whitney U Test; Table 2) for the first 4, 2 and 2 nursery months, respectively. After that, there was no significant difference in size between single and aggregated colonies within the caged experimental designs ($p>0.05$, Mann-Whitney U Test; Table 2).

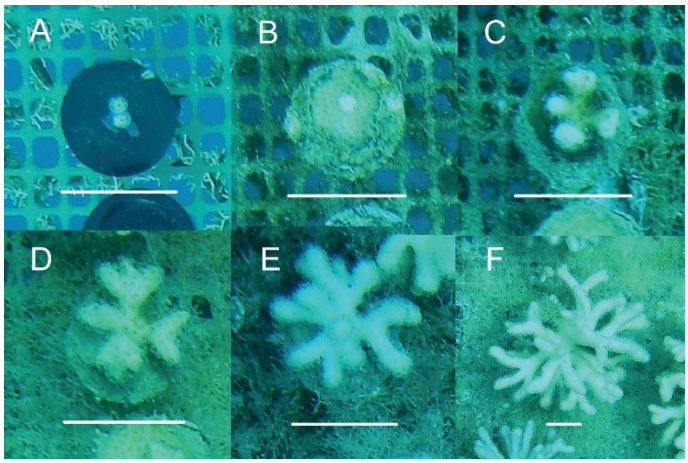

Figure 2 Timeline of a nursery farmed chimeric colony (HN design). The white bar represents a 2 cm scale. Pictures are in chronological order, reading left to right and top to bottom. A) Chimera (n=2) at 1-2 months old, on the day of transfer to the nursery. B) Four months old. C) Five months. D) Nine months. E) Eleven months. F) Twenty-two months; branching has wide openings and multiple side branches.

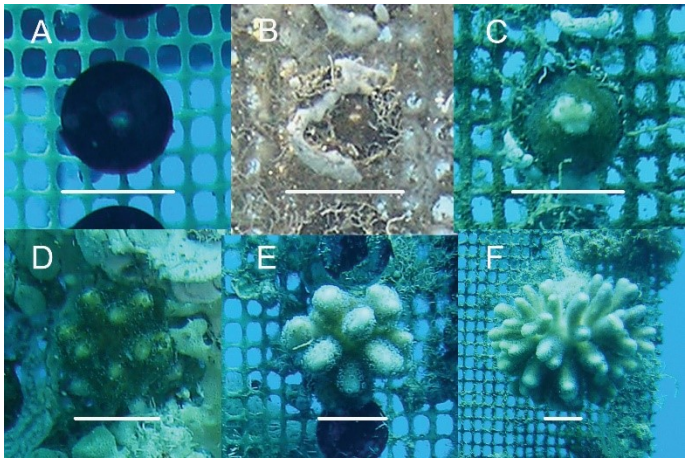

Figure 3 Timeline for a nursery-farmed single spats (VR design). The white bar represents a 2 cm scale. Pictures are in chronological order, reading left to right and top to bottom. A) A single colony at 1-2 months old, initial size, on the day of transfer to the nursery. B) The 2-month-old colony surrounded by an invading colonial tunicate, which were removed with tweezers and a toothbrush. C) Colony at 4 months. D) A 9–month-old colony. E) An 11-month-old colony. F) A 22-month-old colony; branches are thick with polyps, less open space, and less side branching than in the HN design.

The aggregates (n=146) were photographed and inspected for allogeneic fusions (chimeras) and rejections (tissue damages, partial mortality; Fig. 4). Possible chimeras were identified by eliminating those that suffered from any sign of allogeneic response (Rinkevich & Loya 1983). Of the 146 aggregates, 27 showed signs of locality secured points/lines of rejection (Fig. 4D and 4E), and 47 showed signs of advance allogeneic responses in the form of partial mortality. After 14 months, a total of 89 aggregates (60.3%) survived, 43 of which had experienced at least some histoincompatibility, having rejected the neighbouring colony, suffered from partial mortality, or both (Fig. 4), while the remaining 46 aggregates were labelled as chimeric entities (Fig. 2; Frank *et al*., 1997). The aggregates were formed either naturally by co-settling planulae, or experimentally by creating aggregates from larvae settled separately on the Transparency Films (Linden and Rinkevich 2011). The survivorship of chimeric entities (age 14 months) was not dependent on either methodology (13 out of 42 naturally settled and 33 out of 104 of the experimentally made; Mann-Whitney U, p=0.927). This study recorded significantly higher numbers of rejections in naturally formed aggregates than in experimentally made aggregates (Mann-Whitney U, p=0.047). "Partial mortality" and "mortality" did not differ significantly between natural and experimentally made aggregates (Mann-Whitney U, p=0.078, Mann-Whitney U p=0.822, respectively). At 14 months *in situ* (n=89 total), the aggregate sizes did not differ significantly between natural (n=25) and experimentally made aggregate entities (n=64; Mann-Whitney U, p=0.391). The entities that had shown no sign of histoincompatibility in the previous 14 months *in situ*, and were therefore labelled as chimeras (n=46), were significantly larger than the histoincompatible aggregates (n=43; Mann-Whitney U, p=0.012).

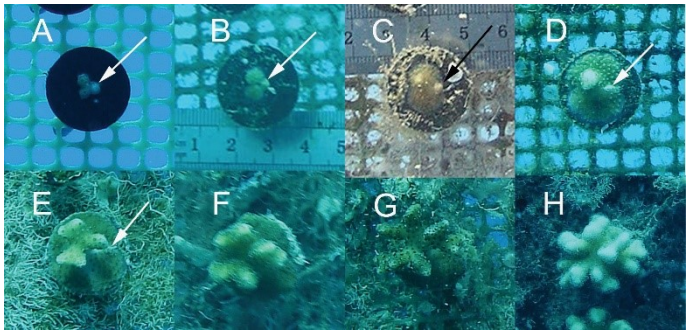

Figure 4 A colonial aggregate (n=3) showing allogeneic rejection and fusion in a single aggregate (VR caged). A) Three colonies in an aggregate, of which the right colony (arrow) is paler, day 1 in the nursery. B-E) One to six months in the nursery; the process of allogeneic overgrowth of tissue and skeleton on the paler colony (arrows) and the creation of a narrow rejection line in touching areas. The tissues/skeletons of other two spat fused, forming a unified entity. F-H) Seven to eleven months in the nursery; the continued development of the aggregate without the display of further allogeneic rejection.

The detachment of spats from their pins due to their translocation into the nursery was minimal (2 out of 249, <2% detachment rate for all settings). Most of the recorded mortality for the caged design after 22 months *in situ* (72.5%; 74 of 102 recorded deaths) developed within the first 4 months of placing the colonies in the nursery, revealing 86.9% and 90% survival for the caged horizontal orientation on the net and on ropes (Fig. 5A and 5C, respectively), and only 46% survivorship for the caged vertical orientation (Fig. 5B). Survival of the colonies during the first 14-month nursery phase (Fig. 5A-C) did not differ between the single colony entities and the aggregates in the caged horizontal designs (Pearson χ^2, p=0.591 for HN and p=0.533 for HR), whereas it was significantly higher for the aggregates (9 singles, 26 aggregates, 12 of which were chimeras) in the caged vertical design (for the 4-14-month period; Pearson χ^2, p<0.046). After 22 months, survival had further gone down to 80.8%, 70%, and 36.7% for HN, HR, and VR, respectively (Fig. 5).

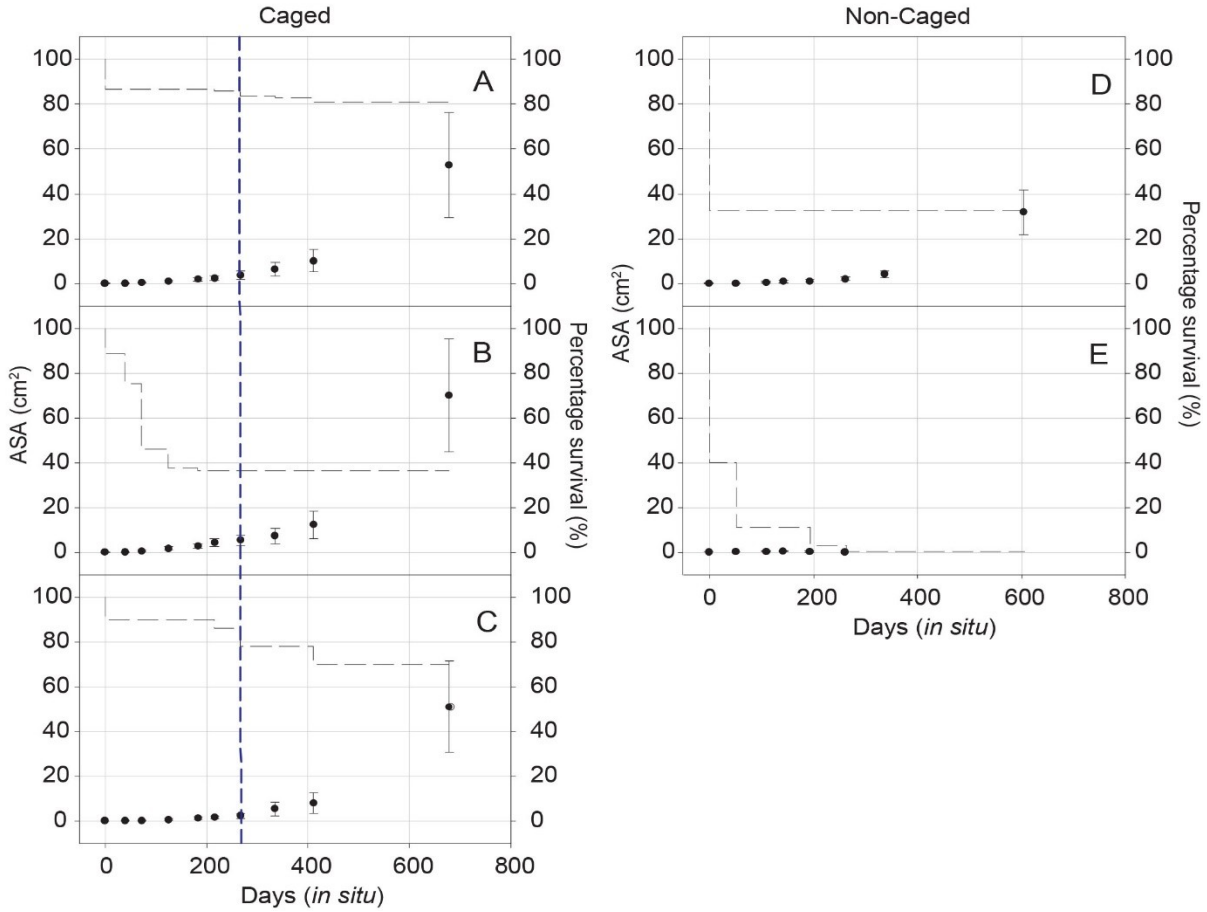

Figure 5 Resultant survivorship and growth rates for all designs over the length of time of the experiment. Survivorship (black dashed lines; %, right vertical axis) for the Horizontal Netted (HN; A and D), Vertical hanging from Ropes (VR; B and E), and Horizontal on Ropes (HR; C) designs. The left graph shows the "caged" results, and the right graph shows the "non-caged" results. Growth (points marking the mean ASA in cm^2 with standard deviation bars; left vertical axis) for the Horizontal Netted (HN), Horizontal on Ropes (HR) and Vertical hanging from Ropes (VR) designs. The removal of the cages in the "caged" experiments is indicated by the vertical (blue) dashed line (267 Days *in situ*) per design.

After 22 months *in situ*, the average spat sizes (ASA ±SD) for the caged designs had increased from 18.6 ±10.5, 12.0 ±7.0 and 16.3 ±10.8 mm^2 at instalment to 5290.4 ±2334.9, 5119.5 ±2056.9, and 6997.3 ±2510.8 mm^2, for HN, HR, and VR, respectively (Figs. 2, 3, 5A-C; Table 2). The nursery bedding of a net (allowing physical connections between trays, HN, Ø=7.08 ±1.72 cm) did not affect growth and colony sizes when compared with the “trays on rope” setting (without physical connections between individual trays, HR, Ø=7.02 ±1.48 cm, p=0.857, Fisher’s LSD) while colonies in the vertical orientation (Ø=8.16 ±1.58 cm) were significantly larger than the colonies in horizontal orientations (p=0.01 and p=0.04, Fisher’s LSD, HN and HR, respectively).

Survivorship of HN and VR caged spats was 86.87% and 88.78%, respectively, after the first 40 days. In the non-caged trays, survivorship was dramatically reduced to 32.7 % in the HN and 39.5% in the VR designs within 52 days (Fig. 5). This was 84.7% of the total mortality experienced after 20 months *in situ* by the non-caged colonies (61 of 72 recorded deaths). Survivorship in the HN design remained constant at 32.7% for the next 553 days until the end of the research period.

Survivorship in the VR design was reduced to 0% at day 338 (Fig. 5E), revealing altogether lower survival of non-caged colonies when compared to their caged counterparts (Fig. 5A-C).

At the end of the "caging period" (9 nursery months), the surviving colonies of the non-caged HN (18 of the 45 single spats [40%]; none of the 10 aggregates) were significantly smaller (n=18, 416.7 ±169.4 mm^2) when compared with the caged single spats of HN (n=40, 597.9 ±255.5 mm^2) and VR (n=9, 695.0 ±301.5 mm^2; Table 2; ANOVA, F=9.382, P<0.000; T-test HN, p=0.014; T-test VR p=0.008). The recorded survival rates following the prolonged caging conditions were 85.86%, 86.00%, and 36.73% (HN, HR, and VR, respectively) for the first 267 days. The removal of the cages resulted in minimal added mortality (2%, 8%, and 0% for HN, HR and VR, respectively) after one month. This is unambiguously different from the non-caged designs, resulting in 32.7% and 2.6% survival after days 267 *in situ* without cages (Fig. 5).

Table 2 Average sizes (ASA) of single and aggregated/chimeric spats in nursery-farmed corals in the three caged designs here notated as "horizontal position" on the fishnet (HN), "horizontal position" on ropes (HR) and "vertical position" hanging from rope (VR). Statistical significance (*, p<0.05; Mann-Whitney U Test) between single colonies and aggregates

Days in situ	HN Single		HN Aggregate			HR Single		HR Aggregate			VR Single		VR Aggregate		
	n	ASA (mm^2)	n	ASA (mm^2)	p	n	ASA (mm^2)	n	ASA (mm^2)	p	n	ASA (mm^2)	n	ASA (mm^2)	p
0	47	12.24	52	24.29	0.000*	30	8.06	20	17.82	0.000*	38	8.80	60	21.03	0.000*
40	43	15.14	44	29.04	0.000*	27	13.92	18	21.24	0.039*	33	16.61	54	27.87	0.000*
73	43	32.50	44	45.54	0.017*	27	24.82	18	27.37	0.516	26	54.42	48	71.70	0.063
125	43	100.91	44	115.29	0.144	27	80.60	18	79.85	0.616	14	183.92	32	187.90	0.060
183	42	187.11	44	196.17	0.556	27	151.12	18	140.08	0.348	11	299.34	27	287.42	0.657
216	41	229.88	44	228.54	0.635	27	181.60	18	166.28	0.570	9	381.20	26	385.29	0.828
267	41	340.49	44	401.69	0.287	26	236.46	17	268.00	0.405	9	515.20	26	556.68	0.440
335	40	597.94	43	670.03	0.494	23	531.03	16	619.71	0.300	9	695.04	26	755.23	0.477
411	39	981.82	43	1051.07	0.788	23	809.96	16	809.50	0.989	9	1105.67	26	1288.92	0.521

Discussion

The "gardening the reef" tenet, which is primarily created for the farming of new colonies created via fragmentation (Rinkevich 2014, 2015), provides a wide range of eco-engineering approaches with regard to the use of sexually derived colonies in reef restoration acts. First, it was documented that mid-water nurseries provide an important platform for growing large stocks of coral colonies *in situ*, eliminating the use of expensive on-land facilities, and that nursery-farmed *Stylophora pistillata* colonies, originating from coral fragments, produce fitter planulae than wild grown colonies (Shafir *et al*., 2006b; Amar and Rinkevich, 2007). Furthermore, Horoszowski-Fridman *et al*. (2011 and unpublished) provided ample evidence that, years after the transplantation of nursery-farmed *S. pistillata* colonies, these coral colonies still release a greater number of planulae than natal colonies, even though they are exposed to the same local disturbances. Other eco-engineering approaches have suggested using stock colonies grown in

mid-water coral nurseries as coral larvae-producing hubs in areas where the supply of planulae is low and as a supplementary measure to active rehabilitation via direct coral transplantation (Amar & Rinkevich 2007).

The findings in this study add to the aforementioned ideas for using sexually derived entities in reef restoration by revealing the practicality of the mid-water nursery as an efficient tool for coral spat farming in a way that lowers the costs associated with very low survival rates (Cooper *et al*. 2014). As shown in this study, about 84% of coral spats that are transferred after 1-2 months, of *ex situ* settlement and growth, to caged *in situ* nursery conditions will reach the age of 1 year, and 80% will survive the following 2 years in the nursery. These results deviate dramatically from Sato (1985) and Babcock and Mundy (1996), documenting very low survival rates in the first few months when the young corals are directly grown on the reef (16% and 0.5-3.9%, respectively, within 4-6 months *in situ*; Table 1). Short *ex situ* periods for settlement and growth of isolated branches/coral fragments, 1 to 5 months, followed by *in situ* deployment on the reef also resulted in low transplant survival rates (Epstein *et al*. 2001, Wilson & Harrison 2005, Baria *et al*. 2010, Boch & Morse 2012). Longer *ex situ* periods, ≥6 months, generally resulted in higher transplant survival (Raymundo & Maypa 2004, Toh *et al*. 2014).

For all purposes, the mid-water nursery, therefore, functions as an improved "*ex situ*" platform for coral farming (80.80%, *ex situ*; Linden and Rinkevich, 2011). This is reflected by the high survival percentages and fast growth rates of corals that need extra care (spacing, cleaning, and regular maintenance), while providing improved conditions for free, including but not limited to; current flush, increased food supply, and optimal natural light regimen. This makes coral farming in a mid-water nursery a much cheaper option than any land-based nursery.

This study also reveals that initial caging of developing spats for several nursery months resulted in augmented survival when compared with the non-caged status (80.8% and 36.7% for HN and VR with cages, versus 32.7% and 0% for HN and VR without caging, respectively), and that caged colonies had grown significantly larger than the non-caged colonies after 11 months *in situ*. Caging, therefore, not only provided a higher survival chance for recently settled spats but also allowed for faster growth rates, even when compared to natal colonies. Under the current nursery conditions, small *S. pistillata* spats grew to colonies of an average of 7-8 cm in diameter (7.08 ±1.72 cm [NH], 8.16 ±1.58 cm [VR] and 7.02 ±1.48 cm [HR]) at the age of 23-24 months (22 months *in situ* and 1-2 months *ex situ*), nearly twice as large as natal colonies (22-month-old naturally settled *S. pistillata* colonies measured 4 cm in diameter; Loya, (1985)). This confirms previous results regarding the high performance of the nursery in Eilat (Shafir & Rinkevich 2010). Rinkevich and Loya (1979) found that *S. pistillata* with a colonial diameter of 5 to 8 cm corresponds with 69.2% of sexually reproducing colonies and in sizes of >8 cm diameter, 90% reproductive probability is attained. The farmed corals, therefore, reach sexual maturity faster than their natal counterparts.

The phenomena of planulae settling in aggregations and the follow-up fusions between spats have been documented in hard and soft coral species (Raymundo & Maypa 2004, Amar *et al*. 2008) as well as other marine invertebrates, such as sponges and tunicates (Rinkevich & Weissman 1992). The benefits of aggregated settlement, fusion, and chimerism between conspecifics may include enhanced survivorship, increased body size, an earlier onset of reproduction, and reduced predation risk at the cost of competition between cell lineages (Buss 1982, Rinkevich & Weissman 1992, Amar *et al*. 2008). Apart from the first 4 nursery months, the results of this study did not document colonial size benefits for aggregated entities (containing

both compatible and histoincompatible partners) compared to solitary entities. The aggregated colonies set for the vertical (VR) design exhibited higher survival rates, whereas colonies in the horizontal designs (HN and HR) did not reveal significant survival differences between aggregated and single spat colonies, which is probably due to the high survival rates recorded in all designs, making the initially significant size benefits of aggregation obsolete under caged conditions.

Engineering species such as *S. pistillata* may be promoted as excellent candidates for enhanced reef restoration. The inexpensive method of creating stocks of nursery farmed colonies via sexual reproduction in large numbers with high survivorship/growth rates, as shown in the present study, and their use in coral reef restoration will lead to more resilient coral populations with higher genetic variation (Shearer *et al*. 2009). Creating stock colonies from sexually produced planulae is a selective process, in which local colonies can be used to supply the stocks (Coles & Riegl 2013), thus keeping current population genetics repertoires unaffected. Taking into consideration the threats of global change to coral reefs worldwide (Wilkinson 2008, De'ath *et al*. 2012), selection of corals for rehabilitation should be based on qualities that can cope with these pressures, sustain the ecosystem, and provide invaluable ecosystem services. Selection processes should further rely on donor colonies have adapted for generations to environmental conditions (Guest *et al*. 2014). Colonies that have survived bleaching events, for example, or that grow in areas with higher water temperatures, such as shallow lagoons, could be possible candidates for creating large sexually derived coral stocks that could be specifically well suited for the rehabilitation of frequently bleached reefs in that area.

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Chapter 5

The coral settlement box: A simple device to produce coral stock from brooded coral larvae entirely *in situ*

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Chapter 5
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Abstract

Coral reef restoration requires efficient, effective and scalable techniques and methodologies to counteract the continued decline of coral reefs. Here we tested an *in situ* method to collect and settle fully developed planulae shed by the brooding coral species (*Stylophora pistillata*). Three devices called Coral Settlement Boxes (CSB; L x W x H: 50 x 40 x 6 cm; V: 4.5 L) were built from transparent Plexiglas and designed to be easily assembled and disassembled. Each CSB contained two integrated biofilm-covered nets (0.5 x 0.5 cm mesh size), which functioned as settlement substrate. The trap container of a traditional planulae trap was replaced by the CSB, and this new construction was used to collected planulae over 4 consecutive days. The CSBs were then transported to a mid-water coral nursery at 12 m depth. One CSB was disassembled immediately, the two settlement substrates were removed and each was placed in a protective cage (mesh size 4 cm^2). The other two CSBs were opened after a 4-month period, leaving the four settlement substrates attached to the Plexiglas plates and covered by protective cages. None of the settlement substrates were cleaned of fouling organisms in the nursery. After 5 months in the mid-water nursery, a total of 120 healthy juvenile coral colonies had resulted from the estimated 2045 planulae initially trapped. This inexpensive and simple approach to producing sexually propagated stocks of colonies entirely *in situ* may enhance the efficiency, effectiveness and scalability of restoration activities that include brooding coral species.

Chapter 5
The coral settlement box:
A simple device to produce coral stock from brooded coral larvae entirely *in situ*

Introduction

The ongoing decline of reef ecosystems has led to the recent development of active restoration approaches (Edwards *et al*. 2010, Rinkevich 2014, 2015). Active restoration methods aim to produce corals to restore degraded reefs through asexual (Bowden-Kerby 2001, Okubo *et al*. 2005, Shafir *et al*. 2006a, Levy *et al*. 2010, Shafir & Rinkevich 2010) or sexual propagation methods (Rinkevich 1995, Linden & Rinkevich 2011, 2017, Okamoto *et al*. 2012, Guest *et al*. 2014, Chamberland *et al*. 2017).

Asexual propagation, also known as fragmentation, is currently the most commonly used approach for reef restoration (Barton *et al*. 2017). Fragmentation allows for the creation of large quantities of coral colonies, but this is done at the expense of wild colonies, which are wholly or partly fragmented in the process (Rinkevich 1995), and results in clonal offspring potentially limiting genetic novelty and variation in restored areas (Shearer *et al*. 2009). These asexually produced coral colonies are transplanted to the reef within 6 months to 2 years after fragmentation and can have high survival rates, depending on factors such as species' growth rates, method of attachment and initial fragment size (Bowden-Kerby 2001, Raymundo & Maypa 2004, Yap 2004, Shafir *et al*. 2006a, Dizon *et al*. 2008, Levy *et al*. 2010, Shafir & Rinkevich 2010, Chamberland *et al*. 2016).

Recent studies have shown that, unlike asexual propagation methods, sexual propagation methods can produce very large quantities of coral colonies, which are all novel genetic combinations (Guest *et al*. 2014, Chamberland *et al*. 2016, Linden & Rinkevich 2017, Cruz & Harrison 2017). Currently, different concepts and methods are being tested, such as settling corals in land-based facilities and directly transplanting young settlers to the reef (Yap 2004, Guest *et al*. 2011, Chamberland *et al*. 2015), direct settling on the reef by confining larvae in the area (Cruz & Harrison 2017) or using spawning pools to direct the settlement of coral larvae (Heyward *et al*. 2002). There have also been suggestions to use genetic manipulation methods (van Oppen *et al*. 2015, Cleves *et al*. 2018) to create more robust corals for future restoration purposes. The use of coral larvae for restoration efforts has a much lower impact on the reef ecosystem than fragmentation because adult colonies do not need to be moved or damaged (Barton *et al*. 2017), and the gametes and larvae shed in the wild normally suffer extremely high natural mortality before and directly after settlement (Fabricius & Metzner 2004, Nozawa 2008).

Currently, most larvae for restoration purposes are settled *ex situ* and require a land-based facility, reliable electrical systems and labour to maintain the coral cultures, and as a result they regularly suffer from human errors and system failures. Disease outbreaks can further increase the costs to produce new corals in such facilities (Chamberland *et al*. 2015, Barton *et al*. 2017). Time spent rearing corals in an *ex situ* facility is expensive, and these costs can be reduced by transporting recently settled planulae to an *in situ* nursery, where the coral colonies can grow in a semi-controlled environment and have high survival rates, according to the rationale of "coral gardening" (Rinkevich 1995, 2014, 2015, Epstein *et al*. 2001).

Here we tested the first advanced *in situ* larval settling device specifically designed for brooding coral species. Our novel and simple prototype, called the "Coral Settlement Box" (CSB), was developed to trap, settle and rear the larvae of the brooding species *Stylophora pistillata* entirely *in situ.* The planulae were trapped and allowed to settle in the CSB on site, while the rearing of the settled colonies continued in a midwater nursery.

Methods

A clear 1.5 cm thick Plexiglas plate was used to construct a simple box (Fig. 1a; L x W x H: 50 x 40 x 6 cm). Two plates of 1.5 cm thick Plexiglas (L x W: 50 x 40 cm) formed the two outer walls of the box. Two Plexiglas frames (also 1.5 cm thick and 3 cm wide each) were sandwiched in between these plates, creating 4488 cm^3 of open space inside the box for coral planulae to move around. Plates and frames were held together with stainless-steel carriage bolts and stainless-steel wing nuts, which also held and stretched the settlement substrate, i.e., two black polypropylene plastic nets (Aquaculture netting; thickness: 1.6 mm, mesh size: 0.5 x 0.5 cm; Manufacturer: P.O.R., HaHotrim, Israel, Category #: 130033110501) over the front and back plate of the box (Fig. 1a). We chose to use a net substrate because it could be cut to produce surfaces harbouring single settlers for transplanting, a property that differs from most designs for transplanting recently settled corals (Okamoto *et al*. 2008, Omori & Iwao 2009, Guest *et al*. 2014, Chamberland *et al*. 2017). The nets covering the Plexiglas plates accounted for 3394 cm^2 of surface area available for settlement, which is 53% of the total surface area (6386 cm^2) inside the CSB.

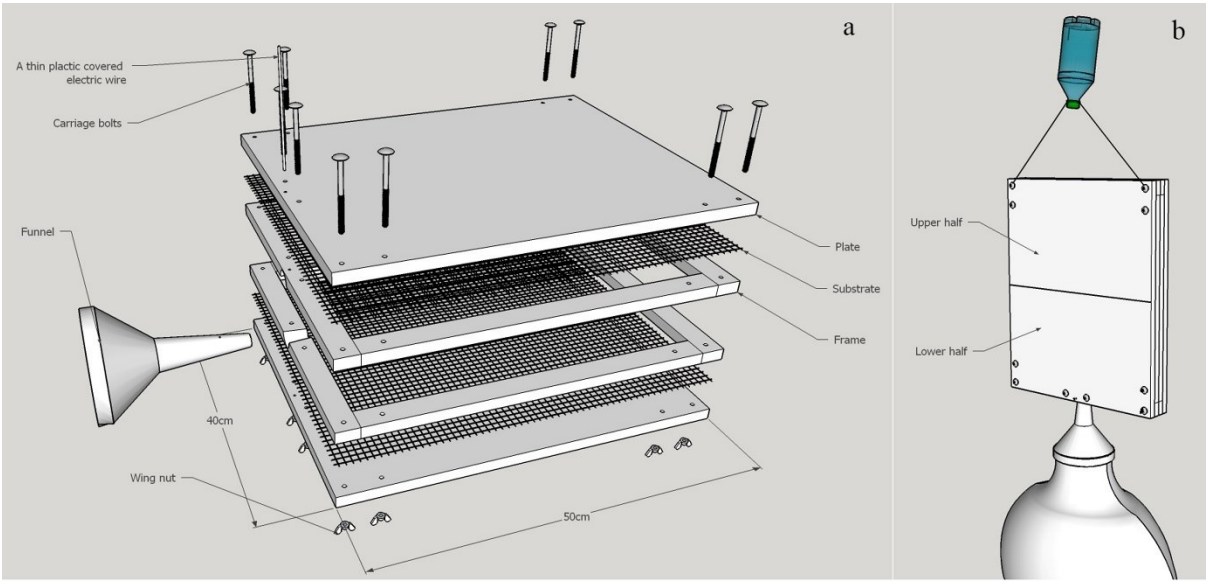

Figure 1 (a) An exploded view of a Coral Settlement Box (CSB). Components consist of two large Plexiglas plates (40x1.5x50), two 3-cm-wide frames with an opening for a funnel (40x1.5x50), two 1-cm^2 mesh settlement substrates (40x50), ten winged nuts and complimentary carriage bolts, one thin plastic covered electrical wire, one funnel and one conical 120 µm plankton net. The small opening for insertion of a thin plastic-covered electrical wire that goes through the narrow part of the funnel ensures that the plankton net and funnel stay attached to the bottom of the CSB. The two settlement substrates were clamped and stretched onto the (front or back) plate with the carriage bolts and were then situated between the plates and the frames. Tightening the screws squeezes the two settlement substrates between the plate and the 3-cm-wide frames and seals the CSB. (b) The assembled CSB in vertical position (also denoting upper and lower half) with a plankton net attached to the funnel and a simple buoy at the top to keep the CSB afloat.

Plastic net substrates were conditioned for 6 months prior to the experiments by attaching them to the mid-water nursery (depth: 12 m) in front of the IOLR (Israel Oceanographic and Limnological Research) in Eilat, Israel, to allow the formation of biofilms (Harrison & Wallace 1990, Harrington *et al*. 2004). The plastic net substrates were inspected before being inserted into the CSB, but they contained no filter feeders or coral settlers prior to CSB assembly. Plexiglas

components of the CSB were not conditioned and had accrued no biofilm prior to *in situ* instalment. A funnel was attached to the CSB by inserting a thin plastic-covered metal wire through a small hole drilled into the funnel and the funnel intake of the CSB (Fig. 1a). A nylon plankton net (mesh size 120 µm) was attached to the funnel forming a conical net, which could be put over an adult colony to function as a "classic" planulae trap (*sensu* Amar *et al.*, 2007).

Planulae were collected from gravid *Stylophora pistillata* colonies, in front of the Interuniversity Institute for Marine Sciences (IUI) in Eilat, Israel. The CSBs (n=3) were placed over adult *S. pistillata* colonies (diameter: 14 to 28 cm) *in situ* (3-5 m depth) in April, 2011, i.e. the middle of the reproductive season of *S. pistillata* (Rinkevich & Loya 1987). Actively reproducing colonies suitable for seeding the CSBs were identified based on prior random placement of planulae traps (*sensu* Amar *et al.*, 2007). The estimated number of planulae collected by each CSB was based on the daily average number of planulae collected by these regular planulae traps, from 3 days before to 1 day after the period during which the CSBs were deployed (i.e., n=4 per CSB). Our goal was to collect at least 100 planulae per trap prior to their being used in subsequent experiments. Lastly, CSBs were brought into vertical position and kept afloat by adding air to an attached buoyancy device (Fig. 1b). Due to their size, the use of the CSBs was restricted to areas and periods with slow-moving currents to reduce drag-borne stress on the gravid coral colonies.

CSBs were placed over coral colonies at dusk and removed at dawn to collect larvae released at night (Rinkevich & Loya 1979a) over four consecutive days. A knot was tied in the plankton net after each night of collection, and the CSBs were tied to a nearby anchor during the day. The four-day collection period was chosen to account for the large variability in the number of planulae released per night by individual *S. pistillata* colonies in the northern Red Sea (Zakai *et al*. 2006, Shefy *et al*. 2018, Linden *et al*. 2018).

After the four-day larval collection period, the CSBs were once again closed by tying a knot in the plankton net and were positioned horizontally *in situ* (IUI, 3 m depth) for 72 hours. They were rotated every day, in the vertical plane, to improve the even distribution of planulae inside the CSBs. CSBs were subsequently transported in seawater inside an open plastic container to the mid-water nursery at North Beach (~29˚32'18" N, 34˚58'12" E; 6.8 km from IUI, 12 m depth). Upon arrival, the first CSB (1) was opened under water, and the flexible net-substrate was removed and hung vertically from a horizontal rope in the mid-water nursery, with no rigid Plexiglas plate support. The net-substrates (n=2) of CSB1 were placed in a cage made of white plastic mesh (4 cm^2) to protect the nets from larger herbivores in the nursery *sensu* Linden and Rinkevich (2011).

When the opened box (CSB1) revealed that many of the planulae had settled on the Plexiglas instead of on the available net-substrate, we decided to not open the other CSBs (2 and 3) in an effort to limit the loss of settlers by allowing them time to grow from the Plexiglass onto the net-substrate. The second and third CSBs (2 and 3), therefore, were not disassembled, but they were hung vertically from a horizontal rope attached to the nursery. Two small openings (ø 2-3 mm) on opposite sides of the CSBs were created by inserting a wooden wedge between the Plexiglas frames to increase water exchange between the contents of the CSBs and the surrounding water column. The downward-pointing funnel opening (Fig. 1a) was also left open to allow for the exchange of water.

Immediately after instalment in the mid-water nursery, the number of settlers inside one of the CSBs was quantified from photographs made through the transparent Plexiglas with an Olympus

Stylus 720 SW. After 4 months *in situ*, these two CSBs (2 and 3) were also opened, but the net substrates (n= 4) were not removed from the Plexiglas plate as in CSB1 (Fig. 1a, 2) but secured in place with small plastic-covered wires and covered with the same type of mesh (1 cm^2) used to construct cages around the nets of CSB1. After 5 months in the nursery, photographs were made of all nets (n= 6) to quantify the size and abundance of all settlers using ImageJ 1.50b software and the program Home Paint (Windows 10). The technical drawing of the CSB (Fig.1) was created with SketchUp 2016 (free version).

Results

Deployment of the CSBs did not cause visible damage to the colonies over which they were placed. The average number of planulae released per day per colony prior to the deployment of the CSBs was 119 (SD: 180, n=9) and varied between 2 and 558. The estimated number of planulae caught inside each CSB ranged between 133 (CSB2) and 1774 (CSB1) (Table 1).

A total of 161 individual settlers were identified on one side of one of the CSBs that was photographed when first introduced to the midwater nursery. Of the 161 settlers, 137 (85.1%) had attached to the Plexiglas and 24 were attached to one side of the net-substrate. Forty-eight settlers (~29.8%) had settled in aggregations (n>2), and a total of 14 aggregations were present (mean number of individuals per aggregation: 3.4, SD: 1.1). Most of the colonies (n=137, 85.1%) had settled in the upper half (Fig. 1b) of the CSB.

Table 1 Planulae counts for the three coral settlement boxes (CSB) with estimated initial number of planulae based on n=4 samples per CSB, number of juvenile colonies and their sizes, after 5 months *in situ*.

CSB	Estimated number of planulae (±SD) inside the CSB	Nursery deployment status	5 months *in situ*		
			Number of colonies		Average size
			Net substrate #1	Net substrate #2	settlement (mm^2 ±SD)
1	1774 ±735	Open	49	7	38.3 ±14.1
2	133 ±59	Semi-open	3	11	38.0 ±16.3
3	138 ±46	Semi-open	24	26	62.1 ±31.1

After 5 months in the mid-water nursery, without any maintenance or cleaning, a total of 120 live colonies remained on the 6 net-substrates (Table 1). The CSB1 net-substrates (n=2) harboured a total of 49 and 7 colonies resulting from the estimated number of 1774 planulae initially trapped, i.e., 3.2% of the larvae in CBS1 had settled and survived after 5 months. A total of 14 and 50 juvenile colonies were found attached to the net-substrates of CBS2 and CBS3, respectively, i.e., 10.2 % and 36.2% survival for CSB2 and CSB3, respectively (Fig. 2). The average size of the 5-month-old colonies was 48.8 mm^2, SD: 26.1 mm^2, with an average density of one colony per 85 cm^2 of substrate surface and only 5 settlers remained on the Plexiglas of CSBs 2 and 3, i.e., on average 1.3 settlers per Plexiglas plate.

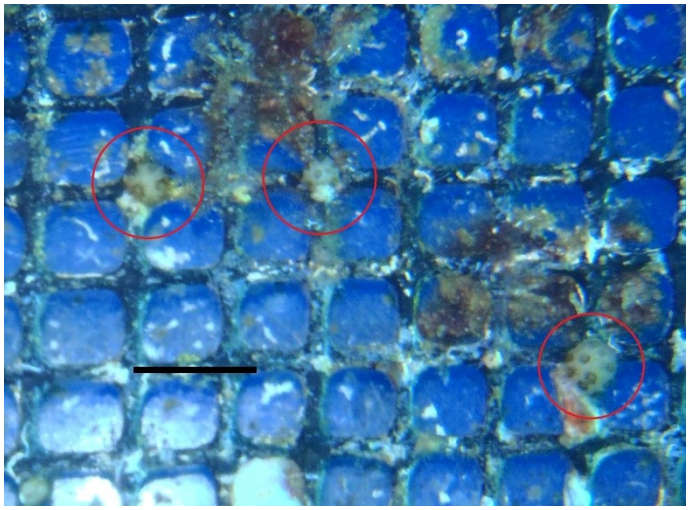

Figure 2 Five-month-old *Stylophora pistillata* (marked by red circles), each consisting of several polyps. Settled corals of this age are predominantly found on the plastic mesh and not on the underlying Plexiglas (Black bar = 1 cm).

Discussion

The main bottleneck for coral colony survival occurs during the earliest life stages when planulae and recent settlers are extremely vulnerable to predation, competition and mechanical damage (Fabricius & Metzner 2004, Nozawa 2008). For brooding and broadcasting coral species, *ex situ* rearing conditions can improve settlement and survival rates (Linden & Rinkevich 2011, Chamberland *et al*. 2017) but also may result in high rearing costs (Barton *et al*. 2017). A combination of *ex situ* and *in situ* methodologies generally reduces costs associated with coral restoration. By conducting some of the *ex situ* work in mid-water coral nurseries, reduced mortality rates can be achieved compared to methods whereby recently settled corals are transplanted directly onto the reef (Levy *et al*. 2010, Guest *et al*. 2014, Linden & Rinkevich 2017). Coral rearing whereby larvae are caught, settled and grown entirely *in situ*, therefore, has potential to reduce costs even more and makes it possible to rear corals in areas where there are no land-based facilities available for settling planulae.

After 5 months *in situ*, survival rates using CSBs are higher than rates reported for direct placement of *ex situ* settled colonies on the reef, as reported in the literature and reviewed in Linden and Rinkevich (2017). Plexiglas devoid of biofilms initially proved a better settlement substrate than biofilm-covered net-substrate, though settling on the Plexiglas drastically reduced settler survival compared to the net-substrate. After 5 months *in situ*, very few settlers remained on the Plexiglas, and we expect that the rigid, smooth surface of the Plexiglas in combination with the constant motion of the CSB in the water column caused settled corals to become dislodged at some point in time.

Unfortunately, we were unable to quantify the initial number of settlers for each of the CSB's. Our current CSB prototypes produced a total of 120 colonies from 3 traps after 5 months and were never taken out of the water, i.e., no *ex situ* steps were included and no time was invested in cleaning. Our only active involvement was trapping the planulae in the CSBs and opening the CSBs (at 0 days or after 4 months). The most labour-intensive aspect of this method involves the

construction of the CSBs and the midwater nursery. Using CSBs as larval traps and settling devices, therefore, will simplify and reduce the production cost of large stock of settlers from larvae of brooding coral species for restoration activities. They will also be useful in remote reef locations, where *ex situ* facilities are often absent. Little to no training is required to successfully implement the CSB method, although its effectiveness may vary among locations and species.

The CSB can easily be adapted to address the particulars of working with other species, for instance by using variable settlement substrate types (Harrington *et al*. 2004, Omori & Iwao 2009, Chamberland *et al*. 2017), by increasing surface areas available for settlement and by adding microhabitats preferred by the targeted coral species (Nozawa 2008). We believe, therefore, that this device or advanced prototypes have the potential to become a valuable addition to the available toolbox for reef restoration aimed at restoring large areas in remote locations in a short period of time.

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Chapter 6

Synthesis and future directions

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Introduction

The mass production of new corals through sexual propagation is an attractive option for coral reef restoration compared to more commonly used methods whereby corals are fragmented. A key advantage of the sexual propagation method is that it yields large numbers of offspring representing novel genetic combinations, potentially better capable of dealing with environmental conditions at present day. Initiatives focussing on sexual propagation of corals focus almost exclusively on broadcast spawning species. Here I continue with a discussion of the results presented in previous chapters to provide directions for future research on methods to mass produce corals with a brooding reproductive strategy via sexual propagation.

Mass production of brooding corals for eco-engineering

I developed methods to mass produce coral using sexually created offspring of the brooding species *S. pistillata.* These newly created corals can be used to restore degraded reef communities. Reef restoration is known to have positive effects on fisheries, tourism and coastal protection, and offers a potential source of new medicines (Spurgeon 1992, Barbier *et al*. 2011, Narayan *et al*. 2016, Hein *et al*. 2019, Zhao *et al*. 2019, Abdelhafez *et al*. 2020). Research on mass producing corals through sexual reproduction has foremost focused on species with broadcast spawning reproductive strategies (Babcock *et al*. 1986, Hayashibara *et al*. 2004, Omori 2005, Omori & Iwao 2014, Edwards *et al*. 2015, Chamberland *et al*. 2017, Cruz & Harrison 2017), in particular species whose populations experienced a drastic decline in previous decades in response to anthropogenic factors. Examples include the Caribbean Acropora species *A. palmata* and *A. cervicornis* (Boström-Einarsson *et al*. 2020, Cramer *et al*. 2020).

Coral species with a brooding reproductive strategy are often considered “weedy”, because they grow fast and are short-lived (Loya 1976, Szmant 1986, Knowlton 2001, Glynn & Colley 2008, Doropoulos *et al*. 2015). Although the annotation “weedy” might seem undesirable, it highlights the capacity for such species to grow in environments that are unsuitable for many other hermatypic corals, including those with a broadcast spawning reproductive strategy. These brooding species can transform such otherwise harsh environments and create suitable conditions for the subsequent establishment of many other species. In other words, they represent perfect candidates to “jumpstart” the community development on degraded reef habitats and engineer the structures that will attract other species. Using brooding corals in reef restoration does not only have the advantage of fast growth, but also implies that fully formed planulae can be directly collected from adult colonies, avoiding the many risk-prone procedures (e.g., gamete mixing, rearing developing planulae) typical for raising broadcast spawning species. At the moment, brooding coral species are generally an overlooked option for reef restoration practices.

Most of the knowledge on succession within coral communities remains anecdotal, but generally shows that brooding corals are the first hermatypic corals establishing themselves in open areas. This is supported by for example observations made during the colonization of submerged lava flows in Hawai’i (Grigg & Maragos 1974), where *Pocillopora meandrina*, a brooding coral species, was an early colonizer of lava flows, that was eventually outcompeted by broadcasting coral species such as *Porites lobata* and *Montipora verrucosa*. Loya (1976) as another example, observed that *S. pistillata* eventually became outcompeted by broadcasting species including, e.g., *Favia favus* and *Platygyra lamellina* in the deeper areas (20-50m depth) near Eilat. The creation of a pioneering coral population on a denuded reef seems therefore a natural “first step”

to promote the natural development of a coral community that will eventually be taken over by competitively stronger K-selected coral species.

Many coral species with a brooding reproductive strategy ("r-selected, pioneering species") could serve as suitable candidates to restore lost coral communities on reefs that have become denuded of living hard corals. Assuming local factors responsible for the initial death of corals and the lack of natural recuperation are attended to first, active restoration using pioneering corals species on denuded reefs will rapidly create reef complexity, alter the environment and create additional niches. This particular approach assumes that the brooding species represent a natural foundation from which a local coral community can develop over time through natural succession. If there are still opportunities for dispersal by other species from nearby coral habitats, this may potentially result in a coral community resembling the original that was lost. This approach whereby communities establish themselves through natural succession may ultimately result in a more robust coral community than the active outplanting of end-successional (K-selected) species. The success of restoration efforts using pioneering species should consequently not be measured as the long-term survival of the initial transplants, but as the degree to which r-selected species become replaced by K-selected species in terms of density and abundance.

Entrained planulation by the lunar cycle is exceptional in brooding coral species

Using the sexually produced planulae from brooding coral species, it is possible to create a vast number of corals, while retaining the genetic diversity of the population. Although planulae can be directly collected from brooders, predicting when planulation will occur is essential in order to effectively use brooding corals for restoration purposes. Being able to accurately predict when planulation will occur requires insight into the mechanisms that control the reproductive process. Many organisms in the world possess endogenous clocks that control biological processes in response to rhythmic changes in environmental factors such as light and temperature. Endogenous clocks also allow organisms to adjust their behaviour in anticipation of upcoming environmental changes or states (Levy *et al*. 2007). An important behavioural display that requires impeccable timing is the simultaneous release of egg and sperm cells into the water column by broadcast spawning corals. Broadcast spawning events can be predicted very precisely for many coral species in Minutes After Sunset (MAS) and days After Full Moon (AFM). The exact mechanisms that control this highly synchronous release of gametes are still poorly understood, but changing nightly light regimes by artificially providing "moonlight" at different cycles than naturally occurring has been shown to inhibit spawning (Kaniewska *et al*. 2015). It is therefore likely that nightly irradiation and changes therein influences the timing of spawning in many broadcasting coral species.

Brooding corals release planulae that no longer require fertilisation and can settle within a few hours after being released. Successful reproduction of brooding corals is therefore not reliant on the synchronization of planulation. As a result, unlike broadcasting coral species, brooding coral species release planulae over many days, weeks, or even months instead of during short "peak-events". Correlations between larval release and lunar cycles have been reported for certain brooding species, though the synchronization is less dramatic compared to gamete release in broadcast spawning species (Atoda 1947, Fadlallah 1983, Richmond & Hunter 1990, Tanner 1996, Fan *et al*. 2002). Many authors have assumed a strong relationship between larval release and lunar cycles (Atoda 1947, Lewis 1974, Stimson 1978, Fadlallah 1983, Szmant-Froelich *et al*. 1986, Richmond & Hunter 1990, Soong 1991, Tanner 1996, Vermeij *et al*. 2003, Zakai *et al*. 2006),

despite the small number of studies that (experimentally) demonstrated the influence of nightly irradiance on the reproductive timing in brooding corals.

To date, only Jokiel *et al.* (1985) provided convincing evidence for lunar entrainment of the reproductive cycle in a brooding coral (seasonal planulation cycles in *Pocillopora damicornis*). They showed that manipulating lunar irradiation resulted in a shift in the planulation rhythm that matched the shift in lunar timing, for two types of *P. damicornis*. This supports the notion that changes in nightly irradiation (lunar periodicity) act as Zeitgeber for the planulation cycle in *P. damicornis*.

Although broadcast spawning and brooding are generally referred to as sexual reproduction, there are species (e.g., *P. damicornis*) that seem to be able to produce planulae asexually as well as sexually (Stimson 1978, Stoddart 1983, Ayre & Miller 2004, Sherman *et al*. 2006, Medina-Rosas 2011, Schmidt-Roach *et al*. 2012, Crowder *et al*. 2014, Rinkevich *et al*. 2016). An even rarer characteristic of *P. damicornis* is its ability to reproduce not only as a brooder, but also via broadcast spawning. (Schmidt-Roach *et al*. 2012). Unfortunately, the results provided by Jokiel *et al.* (1985) are widely used to support the notion of circa-lunar planulation in brooding species in general, regardless of sexual or asexual reproduction (Fan *et al*. 2002, Zakai *et al*. 2006).

Like *P. damicornis, S. pistillata* shows periodic planulae release in Eilat, Israel (Chapter 2, Shefy *et al.*, 2018; Zakai *et al.*, 2006), where the highest numbers of planulae are released between December/January and March-June (Rinkevich & Loya 1979b, Zakai *et al*. 2006, Amar *et al*. 2007, Shefy *et al*. 2018). Unlike *P. damicornis*, all *S. pistillata* planulae are produced via sexual reproduction (Douek *et al*. 2011) and do not display the same consistent lunar periodicity of planulation as described in Jokiel et at. (1985). Similar to our findings in Chapter 2, where individual coral colonies have different planulation periods (25 or 35 days), extrapolation of the data provided by Fig. 5 in Zakai *et al*. (2006) shows that planulation peaks of *S. pistillata* are initially longer than a lunar month but change to shorter than a lunar month over a period of 4 lunar months. This results in an average period of approximately a lunar month, but the large variation in timing, especially when compared to the precision of planulation displayed by *P. damicornis* (see Fig. 1 in Jokiel *et al*. 1985), suggests that the data provided by Zakai *et al*. also undermines the idea that lunar irradiation functions as a Zeitgeber for *S. pistillata*. As a circa-lunar periodicity implies that the system is regulated by lunar irradiance, I therefore proposed to use the term “circatrigintan periodicity” to describe the planulation pattern of *S. pistillata*, thus removing the implied effect of lunar irradiation. Based on these findings, I also hypothesise that the planulation of sexually produced planulae by “true” brooders (e.g., corals not capable of spawning) in general is not influenced by lunar irradiation.

We can only speculate on the main drivers of a circatrigintan planulation cycle, what Zeitgebers exist, or if the process is initiated by e.g. water temperature and remains “free-running” until the water temperature drops below a certain threshold. Minimum and maximum temperature thresholds for coral reproduction have been suggested, not only for *S. pistillata* (Jokiel & Guinther 1978, Prasetia *et al*. 2017, Shefy *et al*. 2018). If the process is regulated by ambient temperature, it could explain several observations. For example, it could explain the observed increase in the length of the circannual reproduction period of *S. pistillata* over the past several decades as a consequence of the rising temperature of the Red Sea (Shefy *et al*. 2018). The tendency to release planulae around the full moon could be the result of a thermocline reaching to lower depths during neap-tides, thus locally elevating the temperature to levels that allow for the reproductive process to move forward or encourage planulation. This would also offer an explanation why the

planulation period is longer at the start of the reproductive period (intermittent temperature increase as the depth reached by the thermocline increases and decreases in accordance to the tides), but becomes shorter as the ambient temperature increases to a point where other factors such as ova maturation, fertilisation and planula development become limiting factors. To gain a better understanding of the underlying mechanisms, one would need to control the environment (and changes therein) experimentally, as done by Craggs *et al.* (2020).

Settlement behaviour

The early stages of the coral's life are perhaps the most consequential and dynamic of all its life stages (Vermeij & Sandin 2008). Fabricius and Metzner (2004) estimated that 99% of all planulae *in situ* fall prey to the reef's many inhabitants. After successful settlement of the remaining few, additional factors such as sedimentation and exposure to grazing further contribute to high mortality rates (Harriott & Fisk 1988, Nozawa 2012, Trapon *et al*. 2013). Not surprisingly, the entire process from broadcasting or planulation up to and including the first few month's post-settlement is a major bottleneck to coral community development as mortality rates often approach 100%.

The survivorship of planulae and spats *ex situ* is generally higher compared to that of conspecifics that remain *in situ*. For *S. pistillata*, we found that the majority of planulae (45.4-63.2%) settled when placed inside a sealed Petri dish (i.e., dish + lid), which was subsequently fully submerged in a water-table. The dish was fitted with Mailer's paper on its bottom and underneath the lid. The Mailer's paper was covered with naturally developed biofilm and used as a settlement substrate (Chapter 3). We found that during the first 48 hours, most planulae settled on the downward-facing side of the substrate (i.e., in an upside-down orientation) underneath the lid of the Petri dish, rather than on the upward-facing side at the bottom of the Petri dish. While the preference to settle in an upside-down fashion resulted in high settlement rates (Chapter 3), the remaining planulae shifted their preference towards settling on the bottom of the Petri dish after the first 48 hours. This change in settlement orientation could be due to the planulae's loss of buoyancy over time, as described by Szmant and Meadows (2006), indicating that ongoing physiological processes (e.g., use of lipid reserves) during the earliest larval stages likely affect settlement patterns *in situ* to some degree. Current *ex situ* sexual propagation methods often provide settlement substrates near the bottom of the containment and thus rely on planulae to "sink" or swim to the bottom to settle. Granted that most brooded planulae can settle within a few hours after release and that buoyancy might play a role in the settlement preference, I suggest changing protocols to include settlement surface located near the water surface as well as at the bottom, if the goal is to efficiently settle planulae.

Our goal was to maximize the efficiency and cost effectiveness of mass-produced brooded coral offspring. Some losses are always expected, because a fraction of "unsettled" planulae is naturally too unfit to survive (e.g., due to malformations). Handling planulae in a wet lab (using e.g., pipettes) likely further reduces their viability. To maximize the fraction of planulae that successfully settles, efforts should focus on 1) reducing the overall time that planulae are handled and 2) providing settlement substrates in a 3-dimensional environment as pointed out above.

Many of the benefits of settling larvae *ex situ*, such as reduced predation and controlled growth conditions, can also be achieved *in situ* despite losing some control in the form of limited access and exposure to natural variability (of, e.g., temperature and light). To collect and rear larvae of *S. pistillata in situ*, we created a setup where the buoyant planulae that are released were trapped in a container, thus creating a semi-enclosed environment while remaining *in situ*. The container,

named the Coral Settlement Box (CSB), also contained a settlement substratum (Chapter 5) and was placed above the colony. The temperature inside the container was assumed to be relatively stable as it was permanently submerged and some water exchange still took place between the contents of the container and the outside. Many of the planulae settled quickly inside the container and most of the settlement happened near the top of the container confirming earlier findings of a preference to settle on downward-facing substrates. Accordingly, the designed container could greatly benefit the mass production of new *S. pistillata* colonies as it greatly reduces the time needed to handle the planulae. However, a non-invasive method to count planulae that go into the container would be needed to confirm actual settlement rates.

Caging

Long-term enclosures and caging have been extensively used in coral reef studies to investigate effects of herbivore exclusion on reefs. The results show that exclusion of herbivores generally enhances algal proliferation, which in turn negatively impacts both adult and settling corals (Lewis 1986, Lirman 2001, Hughes *et al*. 2007). Prolonged caging without any form of herbivory, particularly of small invertebrate herbivores (Omori & Iwao 2014, Craggs *et al*. 2019), can result in high mortality of coral colonies due to algal competition (Knoester *et al*. 2019). However, short-term caging studies have shown some success by 1) enclosing motile larvae *in situ* and thereby limiting larval mobility, resulting in higher local settlement rates within specific areas on reefs (Heyward *et al*. 2002, Edwards *et al*. 2015, Cruz & Harrison 2017) and by 2) protecting recently settled coral spat from accidental grazing by larger indiscriminate herbivores (Baria *et al*. 2010, Nakamura *et al*. 2011, Penin *et al*. 2011).

Our findings for *S. pistillata* showed that at least 9 months of caging, with a 1 cm^2 mesh, improved initial survival of coral settlers, in the nursery, due to reduced accidental grazing and dislodgement by larger fauna (Chapter 4). In these experiments, we did not remove naturally settled herbivores from the cages nor did we exclude the contribution of small herbivores that could have entered the cages. The experiments were conducted on a mid-water nursery that was not located next to a coral reef and therefore only reachable for many reef inhabitants via the pelagic. All the herbivores that settled on the tray containing corals, such as snails and sea urchin, arrived from the water column. It is likely that caging will have protected smaller herbivores that arrived from e.g., the plankton that also may have contributed to reduced algal build up inside the cages and increased survival rates of young coral spat.

Since prolonged caging of a coral reef can greatly reduce coral performance (Hughes *et al.*, 2007), the time that recently settled corals spent encaged in nurseries should be carefully considered. However, our results clearly show that protection of recently settled spat by caging reduces otherwise high mortality rates *in situ*. In addition, the optimal time to remove a cage might also provide an indication of what size and/or age of settled corals are best suited for outplanting and survival success.

The Coral Settlement Box

To make use of the high settlement rates of *S. pistillata* we designed and tested a new tool, the Coral Settlement Box (CSB), to collect and settle planulae and rear them entirely *in situ*. This method is specific to brooding coral species with buoyant larvae and allows for the mass production of corals in their own environment. Accordingly, it does not require expensive *ex situ* land-based facilities and supporting personnel. We found that through the use of a CSB (Chapter 5) many planulae were caught directly in the field that subsequently settled in large numbers.

Although the survival in CSBs after a few months was lower compared to the success of previous methods (Chapter 3 and 4), with a few adjustments this CSB-concept should be capable of producing better results compared to *ex situ* methods. Initial methods for collecting and rearing *S. pistillata* larvae required *ex situ* steps, transplantation from Mailer's paper to the final settlement substrates, and transportation to a midwater nursery (Chapter 3 and 4). Using the CSBs larvae settled directly on final substrates, in this case consisting of mesh that could be cut to size and attached to the benthos where needed. This approach no longer required an *ex situ* step and could transport the CSBs to a midwater nursery directly after settlement.

The previous findings, in Chapters 3 and 4, provide some insights on how the initial design and efficiency of the CSB can be improved. There was a nonuniform settlement pattern throughout the contraption as most settlers were found in the upper half of the box reflecting their preference to settle higher in the water column as described in Chapter 3. The loss of colonies in the mid-water nursery was likely due to the vertical orientation of the substrate in the water column (See Chapter 4). The majority of settled-planulae chose to settle on the CSB's unconditioned Plexiglass sides instead of the preconditioned substrate provided. *S. pistillata* is known to be an eager settler, but previous observations in Chapter 3 reflected that substrate with biofilm was preferred over the exposed plastic sides of the Petri dish. The substrate used in the CSB was conditioned in a different location and of a different material than the substrate that was used in the Petri dish experiment, which could be the cause for the discrepancy found. Each of these points can be addressed in future versions of the CSB, and there are many more aspects that can be changed and improved upon (e.g., ease-of-use of the CSB or recyclability of settlement substrate). For example, the Plexiglass could be replaced by another material that discourages settlement (unstable settlement surface such as fine mazed flexible mesh bag), or coated with settlement discouraging chemical compounds. The settlement surface area in the top of the container can be increased by using small positively-buoyant settlement substrate units. Another option is to fill the entire open space with many loose settlement substrate units. This creates a maze of spaces for the planulae to roam and settle in, instead of the single open space where planulae can only settle on the sides of the container.

Research directions for mass production of brooding coral species

While current methods for rearing sexually propagated larvae for specific coral species are being developed, the actual application of such techniques in reef restoration has only recently been implemented and remains small in scale. Most restoration programs mass-produce corals via fragmentation and tend to focus on species that can be easily reared. Such selection is often a poor reflection of the natural diversity found on local reefs. Programs using sexual propagation methods focus on broadcast spawning corals, while brooding coral species are arguably just as important for reef restoration and much less complex to propagate sexually. Besides the advantages that brooding coral species have to offer, developing methods for sexual propagation of an increasing number of coral species will greatly benefit reef restoration. A greater range of available species could cover a wider range of niches and increase the effectiveness of the tool as well as its applicability. Finetuning existing methodologies to cater to the specific life history needs of each species will additionally contribute to the effectiveness of restoration efforts, and further reduce costs, which is one of the main obstacles limiting current restoration efforts via sexual propagation.

Some brooding corals have pioneering qualities that are an overlooked asset in the restoration of degraded reefs. Pioneering corals are often sturdier and could provide a foundation for further

coral community development in places where all, or nearly all, coral has disappeared. An important consideration, as coral reefs continue to degrade, is whether restoration of coral species that have failed to prevail is even sustainable. Although untested, I propose that the outplanting of pioneering corals is a promising, but often overlooked option, especially during the initial restoration efforts on strongly degraded reefs where most corals have died.

Substrate innovation

The settlement substrate is the coral's foundation from settlement to final outplanting. Having the means to directly influence this environment, via the composition and shape of the substrate and its placement and orientation, allows for a wide scale of interventions aimed at increasing settlers' survival rates. Acute dangers directly after settlement such as indiscriminate grazing, sedimentation, desiccation and competition can all be controlled *ex situ*, but potentially also through the specific design of settlement substates.

Settlement substrate design has been mostly limited to simple formats such as pegs, tiles, and recently tetrapods (Fig. 1). Several aspects need to be considered when choosing a settlement substrate such as settlement preference, portability and ease of underwater attachment (e.g., on a reef flat).

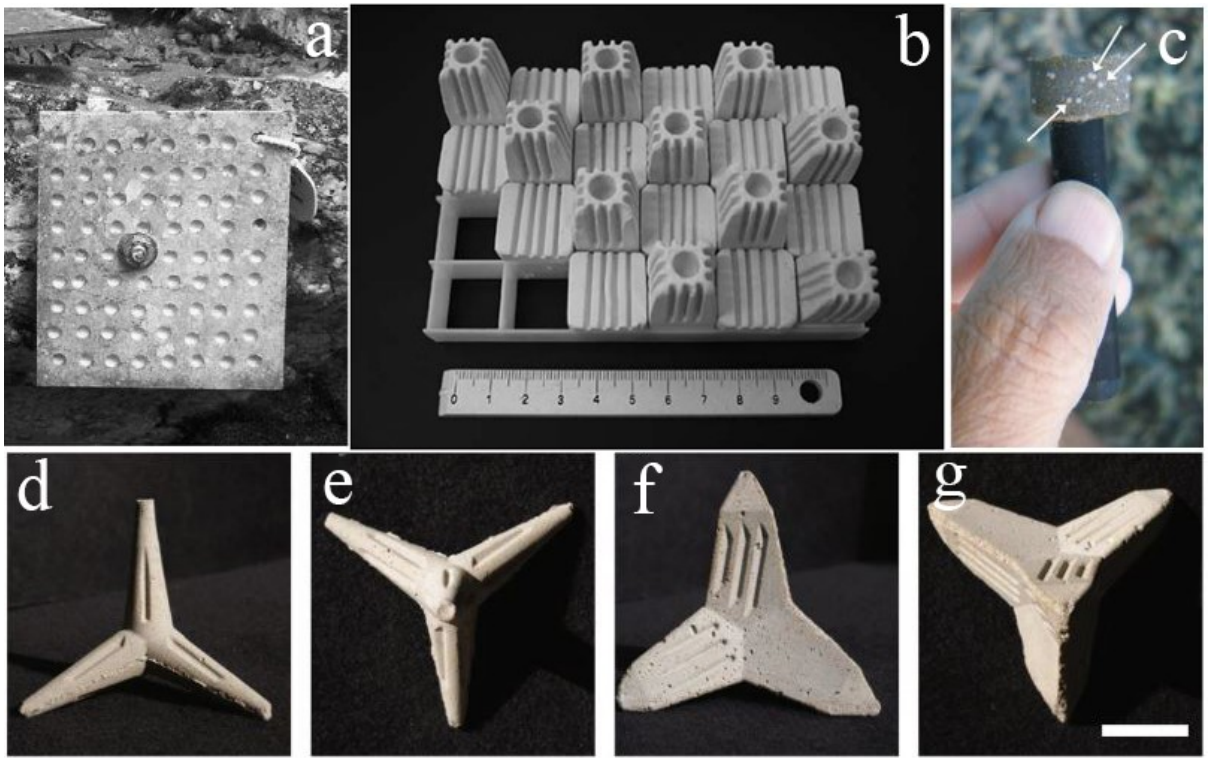

Figure 1 A few examples of innovative settlement substrate used for settling coral larvae. a) A tile with micro-crevasses to provide protection from grazers (Nozawa, 2008; but see also Doropoulos *et al.*, 2016). b) Tiles that provide horizontal and vertical niche space (Petersen *et al*. 2005b). c) The Coral Peg used to settle *Acropora tenuis* (Omori & Iwao 2009). d-g) Two tetrapod designs used for outplanting *F. fragum* (Chamberland *et al*. 2017).

Controlling the settlement substrate size gives a means to control the density of settlement per substrate unit, e.g., smaller substate units result in less settlements per unit, which is especially of interest for species that aggregate their settlements. For such aggregating species, closely packed substrate units at the size of pins could provide a means to separate the aggregates into

smaller/individual settlements. Planulae preferences for different types of physical structure and orientation for settlement, regardless of chemical cues, have only been tested in limited cases for a few species (Petersen *et al*. 2005b). Improvements on placement, structural complexity and substrate size will contribute to higher settlement rates and more efficient mass production of new corals.

Local discouragement of the settlement of planulae can be very useful for species that settle gregariously. Covering areas, like the anchor of the settlement substrate or parts that will be inserted into the reef with metamorphosis deterrents or "blockers", would reduce the number of settlers that settle in such areas and that would thus be lost upon outplanting. Partial covering of specific areas on the settlement substrate could also be used to limit the number of settlers per unit area, in order to decrease the number of colonies lost due to intraspecific competition on the same settlement substrate (Rinkevich & Loya 1983, 1985).

One of the key issues relevant for substrate choice is the attachment of the substrate to the reef during outplanting. Chamberland *et al.* (2017) showed that tetrapods (Fig. 1) can be randomly distributed over the reef area, whereby most of them get "stuck" somewhere on the reef during the next 6 months. This is a fast way to distribute settlement tiles as one does not need to attach each substrate unit to the reef by hand. However, it does require a certain amount of rugosity in the reef topography to be successful. Other common methods for attaching coral substrates are screws, nails, drilling & gluing, tying, and cementing. Unfortunately, all of them are labour intensive, creating a need to find more efficient ways to attach coral substrates under water. The most efficient method would be some sort of rapid *in situ* self-attachment of the substrate to the outplanting area, preventing high energy currents and storms from dislodging and destroying the transplant and its surroundings. We are unaware of such methods and therefore propose a few alternatives that may decrease time spent on restoring a denuded coral reef. Firstly, although untested, light cured silicone-based glue that hardens when exposed to ultraviolet light is potentially a faster attachment method than any of the previously mentioned. Cementing would take between 3-30 seconds depending on the bond strength. Light cured silicone-based glue could really speed up outplanting to rival that of Chamberland *et al.* (2017) and would be less dependent on rugosity of the reef. It would be faster and less invasive than some of the other methods mentioned above. Another option could be to create a piece of transplantable reef (*sensu* Golomb *et al.*, 2020) in a nursery that can be outplanted as is. It might even be possible to create such ready-transplantable units from settlement in a containment like the CSB. For example, an artificial reef unit (a frame) with 100 newly reared colonies could be cemented to the reef and would drastically reduce time compared to attaching and removing individual settlement substrates with a similar number of corals. Clearly, more research will be needed to advance and compare these methods.

Mass production of corals *in situ*

Relocating all land-based practises of coral mass production to coastal waters nearby potential transplant candidates (denuded reefs) removes the necessity of expensive land-based facilities and reduces transport logistics. At the same time, it opens up a new frontier for establishing coral production with high connectivity and potential mobility without ever leaving *in situ* conditions. The construction, labour, and maintenance of infrastructure needed to house coral stock *in situ* is a fraction of the costs of land-based facilities. The stock required to restore degraded reefs is much greater than the current production capacity, which makes in *situ* rearing even more attractive. Natural sea water temperatures and currents provide, under normal circumstances,

cost-free environmental stabilisation and water movement needed for e.g., oxygen supply and waste removal. *In situ* approaches, however, do increase the risk of e.g., disease outbreaks and competitive overgrowth by other organisms. Finding a balance between such “hands on” and “hands off” aspects need to be considered to achieve the most efficient sexual propagation method for each species.

Besides the economic and logistic advantages for propagating corals entirely *in situ*, there are other important aspects that can be better addressed *in situ*. For example, local settlement under *in situ* conditions will allow better acclimatization to final outplanting conditions. Furthermore, in situ approaches may enable the use of a much wider range of species for reef restoration, and may enhance genetic diversity to create more robust coral communities on a local scale.

Further development of this technique and techniques like it, that focus on *in situ* rearing of coral colonies (Heyward *et al*. 2002, Omori *et al*. 2004b), should be paramount. Local stake holders can easily be involved to rear corals for the restoration of local reefs. Involving the local community has been shown to result in a better awareness and acceptance, which contributes greatly to the success of reef restoration efforts (Christie & White 2007, Cinner *et al*. 2013, Hein *et al*. 2019). Capturing and settling planulae *in situ* will contribute to such ambitions by reducing costs and providing a means to restore a natural resource to communities that do not have access to expensive land-based facilities. These techniques and methods are capable of providing genetically diverse generations of corals and potentially have an even higher production capacity than possible via commonly used present-day approaches that rely on fragmentation for mass production and approaches that rely on land-based coral nurseries.

Summary

Innovative methods for the mass production of brooding coral via sexual propagation

Many methods are currently being developed to restore coral reefs from the damaging effects of climate change and other anthropogenic pressures. In this thesis, I contribute to coral reef restoration by the development of new methods for the rearing of corals by sexual propagation. One of the major objections to sexual propagation of coral species is its high costs. The time and finances required to catch coral planulae, raise these planulae under (semi)-artificial conditions and finally transplant juvenile coral back on the reef often hinder the applicability beyond scientific purposes. Most research on sexual propagation of corals concerns species that reproduce via broadcast spawning, where corals release large numbers of gametes during short periods at (semi)predictable times. In this thesis I intended to contribute to the knowledge of sexual propagation of corals focussing on the brooding coral species *Stylophora pistillata*. Brooding coral species release fully developed larvae that can settle within a few hours after release.

I first investigated the timing and number of larvae released per individual *S. pistillata* to determine if, like broadcast spawning corals, there is synchronisation between coral reproduction and environmental variables such as temperature, solar radiation, and tidal range. Previous studies on the same species produced conflicting results as some studies claimed circalunar planulation behaviour, but others did not find it. We found that the timing of planulae release occurred before as well as after full moon, with some coral individuals showing a circa 35-day cycle and others a circa 25-day cycle. We suggest that this behaviour is more accurately described as circatrigintan and that the exact timing of planulation peaks is difficult to predict. Unlike broadcast spawning species the reproductive season of *S. pistillata* is very long and lasts for many months. The optimal timing for the collection of planula larvae is therefore less restricted for *S. pistillata* than for broadcasting species, though planula-yield on a daily basis per coral head is also lower.

The settlement of planula larvae and the type of substrate on which they settle play a major role in effective mass production of corals. The transition from planula to settler is therefore a crucial process. High success rates (low mortality rates) in capture, settlement and growth make proposed restoration methods more cost-effective. Our investigation of the settlement process revealed that the buoyant planulae of *S. pistillata* not only settled readily in a closed container, but that there was an initial preference for settling in the top of the container resulting in higher settlement rates on downward facing surfaces. We also showed that settling coral on a separate substratum allowed for easy removal and transfer of the colony. Using this method, it is possible to create transplantable units containing only a single, or if desired, multiple colonies. Less than 2% of the colonies dislodged from the transplantable unit during shipping from the lab (*ex situ*) to the mid-water coral nursery (*in situ*). Controlling the number of colonies present on a transplantable unit reduced intraspecific competition normally experienced by coral species that settle in aggregations. The capability to split a single aggregated settlement, consisting of multiple individuals, into separate individual colonies can be used to increase the number of transplantable units e.g. one aggregated coral colony consisting of 10 individuals on one transplantation unit, or 10 individual coral colonies on 10 separate transplantation units. This method can be useful when the number of planulae or the settlement success are limiting factors. The method has high survival rates and directly increased yield and reduced the need for *ex situ* manual labour.

Coral spats were reared on a mid-water nursery in the Red Sea, near Eilat, Israel, which is much less expensive than the costs associated with *ex situ* facilities. Growth and survival rates were

recorded for two different orientations, two types of nursery substrate and the presence or absence of a cage to suppress herbivory. Data was recorded for 2 years *in situ*, of which some of the coral spats spent the first 9 months in caged conditions. Horizontal, netted nursery substrate with a cage for 9 months resulted in the highest survival after 2 years *in situ* (80.8%). Caged conditions had the greatest contribution to the survival of the young colonies, followed by horizontal orientation. Colonies grown inside the mid-water nursery grew twice as fast as previously described natal colonies.

Combining the previously described methods we created the next step to capture and settle planulae in enclosed conditions and transport the containers (Coral Settlement Box), with settlements, directly to a mid-water nursery, effectively skipping all *ex situ* steps previously performed. The corals experienced relatively low survival rates using this method, but we also proposed improvements that could potentially increase the yield from its current estimated 5% to 40% or higher. This would be similar to the methods we developed using an *ex situ* step to mass-produce coral colonies (60% settlement rates, 80% survival the first one and a half months *ex situ* and 80-90% survival from 1 to 24 months *in situ*). The use of Coral Settlement Boxes for mass production of brooding corals from larvae requires little labour and is inexpensive, simple, and can be upscaled to larger quantities.

In conclusion, although the new methods advanced in this thesis can still be further improved, my results show that sexual propagation of brooding corals is a feasible strategy to mass produce coral colonies that can be used in coral reef restoration.

Samenvatting

Innovatieve methoden voor de massaproductie van broedend koraal via seksuele voortplanting

Momenteel worden verschillende methodieken ontwikkeld om koraalriffen, die zijn aangetast door klimaatverandering en andere antropogene invloeden, te herstellen. Met dit proefschrift draag ik bij aan deze ontwikkeling door het beschrijven van nieuwe methodieken om koralen te kweken via seksuele voortplanting. Eén van de grootste obstakels bij de productie van koraalsoorten via seksuele voortplanting is de hoge kosten die dat met zich meebrengt. Het kost namelijk veel tijd en geld om de larven van koralen te vangen, deze op te laten groeien onder semi-artificiële omstandigheden, en uiteindelijk het jongvolwassen koraal te transplanteren naar het koraalrif. Om deze redenen wordt koraalproductie via seksuele voortplanting vooral toegepast voor wetenschappelijke doeleinden. Het onderzoek naar de productie van koraal via seksuele voortplanting heeft zich tot nu toe voornamelijk gericht op kuitschietende koraalsoorten. Kuitschietende koralen planten zich voort door massale verspreiding van hun gameten in het zeewater gedurende korte perioden op (semi-)voorspelbare tijden. In dit proefschrift hebben we een andere strategie gevolgd door de massaproductie van broedende koralen te onderzoeken. Broedende koralen onderscheiden zich van kuitschietende koraalsoorten door zich voort te planten via het “uitbroeden” van de bevruchte eitjes tot larven. Broedende koraalsoorten laten hun larven pas vrij in het zeewater als deze vrijwel volledig zijn ontwikkeld en deze larven kunnen zich daarna binnen enkele uren vestigen om een nieuwe koraalkolonie te beginnen. Als model voor broedende koralen is in dit onderzoek gekozen voor *Stylophora pistillata*.

Ik heb eerst onderzoek gedaan naar de timing en het aantal larven dat wordt vrijgelaten door individuele S. *pistillata* kolonies. Hierbij heb ik ook gekeken of er, net als bij de kuitschietende koralen, synchronisatie plaatsvindt tussen koraalreproductie en omgevingsvariabelen zoals bijvoorbeeld de stand van de maan, temperatuur, zonnestraling en getijverschil. Eerdere studies bij dezelfde soort leverden tegenstrijdige resultaten op, aangezien sommige studies wel een correlatie vonden tussen het vrijlaten van larven en de stand van de maan, en anderen niet. Ik ontdekte dat het vrijkomen van de larven zowel voor als na volle maan plaatsvond, waarbij sommige koraalindividuen een cyclus van rond de 35 dagen hebben en anderen een cyclus van rond de 25 dagen. Omdat er niet voldoende bewijs is dat de maan direct invloed heeft op de timing, stel ik voor om deze cyclus te omschrijven als “circatrigintan”, dat wil zeggen een cyclus met een periode van ongeveer 30 dagen. Tevens blijkt uit mijn bevindingen dat de exacte timing waarop de meeste larven worden vrijgegeven nogal onvoorspelbaar kan zijn. In tegenstelling tot kuitschietende koraalsoorten is het voortplantingsseizoen van *S. pistillata* erg lang (maanden, in plaats van enkele dagen per jaar). De optimale tijd voor de collectie van larven is daardoor in mindere mate een beperkende factor voor het kweken van broedend koraal zoals *S. pistillata* dan voor de kuitschietende koraalsoorten. Echter, op dagelijkse basis, produceren broedende koralen veel minder larven per koraalhoofd in vergelijking met de massale productie van gameten door kuitschietende koralen.

Het type substraat en de manier waarop de larven zich daaraan bevestigen spelen een belangrijke rol bij de overleving van koraal. De overgang van larve naar koraal is daarom een cruciaal proces. De hier voorgestelde methoden voor het vangen, vestigen en kweken van *S. pistillata* hebben een hoog slagingspercentages (getypeerd door lage sterftecijfers). Uit de resultaten bleek dat de larven van *S. pistillata* niet alleen gemakkelijk in een gesloten container konden worden opgevangen, maar dat de larven aanvankelijk een voorkeur hadden om zich helemaal bovenin de container te vestigen. Naar alle waarschijnlijkheid werd dit veroorzaakt door het positieve drijfvermogen van de larven, wat leidde tot hogere vestigingspercentages op oppervlakken die naar beneden gericht waren. Door de larven zich te laten vestigen op een apart flexibel substraat,

dat bevestigd was aan de container, konden de gevestigde koraalkolonies gemakkelijk worden los gemaakt nadat deze een calcium skelet hadden gevormd. Hierdoor waren deze kolonies ook gemakkelijk over te plaatsen naar een andere plek. Met deze methode is het mogelijk om transplanteerbare eenheden te creëren die slechts één of, indien gewenst, meerdere kolonies bevatten. Minder dan 2% van de koraalkolonies ging verloren tijdens transport van het laboratorium naar een kraamkamer in de zee. Door het aantal kolonies op een transplanteerbare eenheid te beperken, verminderde de intraspecifieke concurrentie die gewoonlijk plaatsvindt bij koraalsoorten die zich in aggregaties vestigen op substraat. Door aggregaties van gevestigde koraal op te splitsen in meerdere losse individuen kan de productie van transplanteerbare eenheden verder worden vergroot. Dit soort technieken kunnen van belang zijn voor het creëren van een groter aantal transplanteerbare eenheden als de aanvoer van larven of de succesvolle vestiging van aggregerende koraalsoorten een beperkende factor is. Deze methode heeft een hoog overlevingspercentage, verhoogt direct de opbrengst en vermindert de hoeveelheid arbeid die anders nodig zou zijn voor langere kweektijden.

De jonge koralen werden gekweekt in een koraalkraamkamer in de Rode Zee, vlakbij de stad Eilat in Israël. De koraalkraamkamer is essentieel voor koraal productie omdat het langdurig kweken in zee vele malen goedkoper is dan het langdurig kweken in een laboratorium. Ik onderzocht de groei- en overlevingskans van jonge koralen (1,5 maand oud) in de koraalkraamkamer. Hierbij is gekeken naar het effect van oriëntatie, vestigingssubstraat en, gedurende de eerste 9 maanden, de aan- of afwezigheid van een beschermende kooi. De koralen werden 2 jaar lang in de koraalkraamkamer gevolgd. De horizontale oriëntatie van koralen in een beschermende kooi gaf het beste resultaat na 2 jaar (80,8% overleving). De aanwezigheid van een kooi had de grootste invloed op het overleven van de jonge kolonies in de koraalkweekkamer. Het opgroeien in een horizontale oriëntatie was de tweede meest invloedrijke factor. Kolonies in de koraalkweekkamer groeiden twee keer zo snel in vergelijking met eerder gepubliceerde gegevens over de groei van *S. pistillata* kolonies in het wild.

De combinatie van bovenstaande bevindingen leidde tot een nieuwe methode om koraallarven, direct na het vrijkomen in het water, in een gesloten en beschermde omgeving (container) op te vangen en te laten vestigen zonder deze uit zee te halen. Deze containers (Coral Settlement Boxes, CSB) met neergestreken koraal werden na het vangen van de larven rechtstreeks naar de koraalkweekkamer gebracht. Door de larven direct in een container op te vangen en de mogelijkheid te geven om zich in de container te vestigen konden alle gebruikelijke kweekstappen in het laboratorium effectief worden overgeslagen. Deze nieuwe methode produceerde een relatief lage opbrengst van koraal. We stellen dan ook verbeteringen voor die de opbrengst zouden kunnen verhogen van de huidige geschatte 5% tot 40% of meer. Met die verbeteringen zouden volgens onze inschatting de overlevingskansen van de nieuwe methode (CSB) vergelijkbaar zijn met de overlevingskansen van onze eerder beschreven methodes: 1) het vestingen van de larven in een container in een laboratorium (60% overleving) en *ex situ* kweek tot 1,5 maand oud (80% overleving). 2) het horizontaal met een kooi voor 24 maanden laten opgroeien in een koraalkweekkamer (80-90% overleving). Het gebruik van de CSB voor de massaproductie van broedend koraal vereist weinig arbeid, is goedkoop, eenvoudig en kan gemakkelijk worden opgeschaald. Deze nieuwe methode (CSB) biedt ruime mogelijkheden om verder ontwikkeld te worden. In het totaal laten de resultaten in dit proefschrift zien dat het produceren van broedend koraal via seksuele voortplanting een geschikte mogelijkheid is die kan worden ingezet voor het herstel van koraalriffen.

Author contributions

Chapter 2: BL and BR conceived the project and designed the experiment. BL carried out the field work. BL analysed the data with inputs of BR and JH. BL, BR and JH wrote the manuscript. All authors gave final approval for publication.

Chapter 3: BL and BR conceived the project and designed the experiment. BL carried out the field work. BL analysed the data with inputs of BR. BL and BR wrote the manuscript. Both authors gave final approval for publication.

Chapter 4: BL and BR conceived the project and designed the experiment. BL carried out the field work. BL analysed the data with inputs of BR. BL and BR wrote the manuscript. Both authors gave final approval for publication.

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