Dynamics of ascidian-invaded communities over time

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Abstract Human-mediated transport of non-native species can negatively impact ecosystem services, and the scale of these impacts is predicted to increase over time. However, the manner in which invaded marine communities on artificial substrates change over time has rarely been investigated. Several marinas in Tampa Bay and Miami (Florida, USA) were surveyed to document changes in ascidian community composition of native and introduced species between 2003–2004 and 2017. Seventeen species found in 2017 were not recorded in 2003–2004; all of these 17 were rare in both Tampa Bay and Miami. Five species were found in 2003–2004 but not in 2017, with four of these five being rare. In Tampa Bay, the abundances of both native and introduced species did not change over time, though the number of native species increased while the number of introduced species was unchanged. In Miami, the number of native species increased but their abundances declined; however, introduced species number and abundances did not change. Our study showed that although populations of introduced species were dynamic, they appeared to reach carrying capacity, where overall species number and abundance remained mostly constant over time independently of recurrent introductions and the presence of native species. However, since invasion meltdown and invasion debt can happen at any time, periodic surveys and knowledge of species' biological cycles and environmental tolerance thresholds remain critical.

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Introduction

Once a non-native species is introduced to a new location, its long-term establishment will depend on the environmental conditions encountered as well as its interaction with several biotic factors, such as the presence of predators and competing species (Jones and Gomulkiewicz 2012). In addition, recurring

introductions of individuals are key in securing the necessary genetic diversity for the species' long-term survival in a new habitat (Roman and Darling 2007; Barrett and Schluter 2008). Propagule size (the absolute number of individuals arriving to a new location at a given time) and propagule number (the number of times individuals of a species have been introduced to the same location) are the two components of propagule pressure (Carlton 1996; Lockwood et al. 2005; Sinclair and Arnott 2016; Tabak et al. 2018). Several studies to date have demonstrated that high propagule pressure is expected in main entry points (Lockwood et al. 2009; Woodford et al. 2013), such as ports with sustained international traffic. On the other hand, recurrent introductions of species into smaller or isolated harbors and marinas will depend on stochastic events driven by local or regional activities such as fishing and recreational activities (Wasson et al. 2001; Darbyson et al. 2009; Davidson et al. 2010; Goldstien et al. 2010; López-Legentil et al. 2015). Likewise, environmental stochasticity such as unusual weather events (e.g., hurricanes, floods) can drastically reduce propagule size at any given time, though the importance of such events is minimized under recurrent introduction scenarios (Simberloff 2009). Finally, as the climate changes and becomes more favorable to certain non-natives, an introduced population maintained solely by constant propagule pressure will require fewer individuals for long-term establishment (Thomsen et al. 2006). Thus, over time, the establishment of new non-native species is expected, as are changes in the relative abundance of previously established ones (Lockwood et al. 2009). Non-native ascidians (Phylum Chordata, Class Ascidiacea) are conspicuous components of fouling communities and prominent examples of widely introduced species (Lambert and Lambert 2003; Lambert 2007; López-Legentil et al. 2015), causing important economic losses (Pannell and Coutts 2007; Locke et al. 2009; Aldred and Clare 2014), altering benthic community structure (Lambert and Lambert 2003; Bullard et al. 2007; Lambert 2009; Aldred and Clare 2014), and disrupting the link between pelagic and benthic communities (Lengyel et al. 2009; Mercer et al. 2009). However, few studies to date have attempted to determine the effect of propagule pressure in the long-term establishment of non-native ascidian communities around the world (Pérez-Portela et al. 2012; Goldstien et al 2013; Pineda et al. 2016). In 2003

and 2004, G. and C. Lambert accompanied members of the Smithsonian marine invasions laboratory to monitor ascidian diversity and abundance in Tampa Bay and Miami, (unpublished). Seven artificial sites located around the entrance of Tampa Bay were monitored, with Port Tampa Bay (not surveyed) located further within the Bay. Port Tampa Bay is the largest port in Florida and is one of the busiest seaports in the United States, serving container and tank ships as well as several cruise lines (Porttb 2021). Similarly, the Miami area is characterized by having one of the largest and busiest seaports in the United States, with regular arrivals and departures of international cargo and container ships as well as large numbers of recreational boats (The Port of Miami, Florida, US). The Port of Miami is also the largest passenger/cruise port in the world (The World's Busiest Cruise Ports). Both major ports are surrounded by smaller harbors and marinas, housing hundreds of fishing as well as recreational boats (Florida, United States Marinas). The intense international maritime traffic characterizing both ports, as well as their proximity to the well-connected network of smaller and easily sampled marinas, make the Miami and Tampa areas ideal to study the effects of recurrent introductions over time.

In 2017, we revisited almost all of the harbors and marinas visited in 2003–2004. We pooled the data of the original surveys (2003–2004) with data obtained in 2017 to compare ascidian diversity and abundances between both regions (Miami and Tampa Bay) and time periods (2003–2004 and 2017). We sought to determine (1) whether there was any noticeable shift in the composition of the ascidian communities between these two time periods, and (2) whether these changes occurred for both surveyed regions. As a result of recurrent introductions and increased propagule pressure, we expected to observe an increase in both the number of introduced species in 2017 (for the two regions) and the abundance of established species in 2003–2004 that were still present in 2017.

Materials and methods

Surveys

Table 1 lists all of the marinas surveyed in 2003–2004 and 2017, and Fig. 1 shows their physical locations. The first set of surveys (hereafter referred to as the

Table 1 Marinas visited in the 2003, 2004 and 2017 surveys

Marina name	City	Coordinates	2003–2004 survey	2017 survey
Tampa Bay				
Bradenton Beach Marina	Bradenton Beach	27° 28′ 04.7″ N 82° 41′ 46.4″ W	May 18, 2003	July 26, 2017
Clearwater Beach Marina	Clearwater	27° 58′ 37.1″ N 82° 49′ 32.6″ W	May 17, 2003	July 28, 2017
Land's End Marina	Apollo Beach	27°46′52.8″N 82° 25′ 22.8″ W	May 18, 2003	July 25, 2017
Leverock's Marina	Perico Island	27° 29′ 46.3″ N 82° 40′ 38.3″ W	May 18, 2003	NA
St. Petersburg Marina	St. Petersburg	27° 46′ 21.2″ N 82° 37′ 40.0″ W	May 17, 2003	July 27, 2017
Tampa Bay ranging tower	St. Petersburg	27° 42′ 35.2″ N 82° 35′ 00.9″ W	May 19, 2003	NA
Vinoy Renaissance Marina	St. Petersburg	27° 46′ 40.6″ N 82° 37′ 43.4″ W	May 17, 2003	July 28, 2017
Miami				
Bill Bird Marina	Miami	25° 54′ 24.5′ N 80° 07′ 26.3″ W	August 12, 2004	August 1, 2017
Crandon Park Marina	Key Biscayne	25° 43′ 28.1″ N 80° 09′ 17.7″ W	NA	July 30, 2017
Dinner Key Marina	Miami	25° 43′ 37.0″ N 80° 14′ 04.5″ W	NA	August 2, 2017
Homestead Marina	Homestead	25° 27′ 45.2″ N 80° 20′ 15.1″ W	August 13, 2004	July 31, 2017
Matheson Hammock Marina	Miami	25° 40′ 51.4″ N 80° 15′ 37.0″ W	August 14, 2004	August 3, 2017
Miami Beach Marina	Miami Beach	25° 46′ 18.6″ N 80° 08′ 22.3″ W	NA	August 1, 2017
Pelican Harbor Marina	Miami	25° 50′ 58.5″ N 80° 10′ 02.2″ W	August 12, 2004	July 30, 2017
Port of Miami	Miami	25° 46′ 39.9″ N 80° 10′ 18.4″ W	August 11, 2004	NA
Rickenbacker Marina	Miami	25° 44′ 43.7″ N 80° 10′ 23.5″ W	August 11, 2004	August 3, 2017
Sea Isle Marina	Miami	25° 47′ 28.4″ N 80° 11′ 01.2″ W	NA	August 1, 2017
Sunset Harbor Yacht Club	Miami Beach	25° 47′ 43.6″ N 80° 08′ 38.7″ W	August 11, 2004	NA

2003–2004 surveys) was conducted from May 17 to 19, 2003 (Tampa Bay), and from August 10 to 16, 2004 (Miami). The 2003–2004 surveys included analysis of numerous retrieved settlement panels that had been submerged, at a depth of about one meter from floating docks (and directly from the Port of Miami Municipal Pier), for the previous three months by the Invasions Lab of the Smithsonian Environmental Research Center (Edgewater, MD). The adjacent floating dock surfaces and submerged ropes and bumpers attached to the docks were also surveyed at those times. The settlement plates were 14×14 cm, 0.5 cm thick, grey high-impact polyvinyl chloride (PVC), which were lightly sanded on one side to optimize attachment and were attached to bricks with the roughened settlement surface facing down (see Lambert 2019 for a complete description of plate analysis). Since plates were submerged for only three months, most of the settled ascidians were juveniles or small colonies not yet reproductive; thus, many could not be identified to the species level. However, since ascidian tadpoles are short-lived and non-feeding, they commonly settle close to their progenitors (Lambert 2005); thus, recruitment to the panels

was considered primarily local. Adult ascidians for both solitary and colonial species were collected from nearby dock surfaces and ropes hanging at depths $=$ or >1 m from the water surface. The single exception was one site (Ranging Tower) in the 2003 Tampa Bay survey, where a wall, buoys, and a ranging tower out in Tampa Bay were sampled by snorkeling; these surfaces were not resampled in 2017. No settlement plates were deployed at Clearwater Beach Marina; only the docks and associated ropes and artificial structures were sampled. Each survey took between 30 min and 2 h, depending on the number of ropes and size of the marina. The salinity and temperature were measured at Miami sites only (Online Resource Table 1).

In 2017, we re-surveyed all of the marinas from the 2003–2004 surveys, except for Leverock's Marina in Tampa Bay, which no longer exists. This second set of surveys (hereafter referred to as the 2017 surveys), was conducted from July 25 to 28, 2017 (Tampa Bay), and July 30 to August 3, 2017 (Miami). No settlement plates were deployed; we surveyed submerged dock surfaces that were accessible at arm's length, as well as buoys and ropes. We examined the undersides and
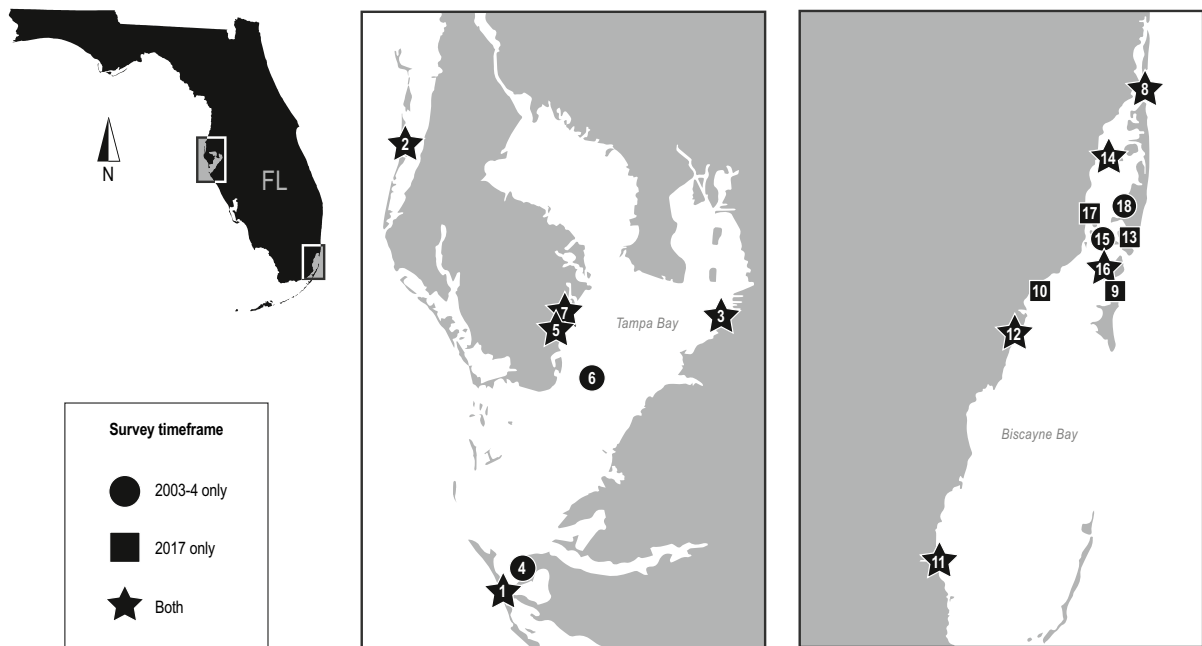
Fig. 1 Geographic locations of each survey site. 1: Bradenton Beach Marina (BBM), 2: Clearwater Beach Marina (CBM), 3: Land's End Marina (LEM), 4: Leverock's Marina, 5: St. Petersburg Marina (PMM), 6: Tampa Bay Ranging Tower, 7: Vinoy Renaissance Marina (VRM), 8: Bill Bird Marina (HPM), 9: Crandon Park Marina (CPM), 10: Dinner Key Marina (DKM), 11: Homestead Marina (HM), 12: Matheson Hammock Marina (MHM), 13: Miami Beach Marina (MBM), 14: Pelican Harbor Marina (PHM), 15: Port of Miami, 16: Rickenbacker Marina (RM), 17: Sea Isle Marina (SIM), 18: Sunset Harbor Yacht Club

sides of the docks, frequently pulling animals off the surfaces to examine them. In large marinas, we visited at least two docks in each geographic section of the marina; in small marinas, we visited every slip for every dock. In marinas where we could not reach the submerged dock surfaces, we examined every rope and boat bumper in the marina, regardless of the marina size. Each survey took between 30 min and 2 h, depending on marina size. The salinity and temperature were measured at both Tampa Bay and Miami sites (Online Resource Table 1). Clearwater Beach Marina (CBM) is located on the Gulf of Mexico rather than in Tampa Bay itself and had a salinity value of 33 ppt at the time of sampling, which is higher than the Tampa Bay sites.

At the end of each survey, estimated abundance of each species was determined based on the number of individuals observed: (1) rare: one or a few total specimens observed; (2) common: species frequently observed, a few per square meter; and (3) abundant: species occurring in great numbers, many per square meter. In 2017, a value of 4 was sometimes assigned

in the raw data for the two most abundant species: *Diplosoma listerianum* and *Styela plicata*. However, these values of 4 were converted to values of 3 in all analyses because the 2003–2004 surveys did not have any values of 4. Given the length of time spent surveying each marina, these abundances are conservative. Species were further classified as native, introduced, or cryptogenic (unknown origin), following Chapman and Carlton (1991) and Carlton (1996, 2009).

Species identifications

2003–2004

At each marina, the retrieved settlement plates were placed in seawater-filled individual Ziploc bags and stored in coolers before being transported to the laboratory for careful examination with a dissecting microscope and a recording of all species present. Representative specimens were then preserved in ethanol for permanent storage at the Smithsonian's

Maryland facility. During the field surveys of the docks and ropes, including the bricks to which the plates were attached, immediately identifiable ascidians were recorded, and, after tentative identification and estimate of abundance, additional samples were placed into Ziploc bags of seawater with menthol crystals for relaxation and later examination under a microscope at the end of each day. Some specimens were then preserved in 10% seawater formalin and examined in the lab the next day for species identification before permanent storage at the Smithsonian's Maryland facility. Identifications were made to species level when possible; immature specimens from settlement plates were sometimes identifiable only to genus level, though they were usually tentatively identified to species level based on the adult populations on the associated docks and accessory surfaces. References used for identification included Berrill (1932), Van Name (1945), Plough (1978), Monniot C. (1972a, b; 1983a, b, c), Monniot F. (1972; 1983a, b, c), and Fox and Ruppert (1985).

2017

At least two specimens of each species were photographed alive and then preserved as follows. One whole specimen with the tunic for solitary ascidians or a piece of a colony for colonial ascidians, was relaxed in seawater using menthol crystals for at least 1 h (colonial ascidians) or until the animal did not react to touch stimuli (solitary ascidians). These relaxed ascidians were then preserved in 10% seawater formalin. A second specimen was preserved in 95% ethanol. Field identifications were subsequently verified by examining morphological characters and/or barcoding of the mitochondrial cytochrome oxidase c subunit 1 (mtCO1) gene. Morphological identifications were accomplished using standard taxonomic references for the region: Berrill (1932), Bonnet and Rocha (2011), Lambert (2005), Monniot C. (1972a, b; 1983a, b, c, 2002), Monniot F. (1972; 1983a, b, c, 2018), Monniot and Monniot (1997), Plough (1978), Plough and Jones (1939), Rocha et al. (2012), and Van Name (1945).

In addition to morphological observations, species were identified by sequencing the mtCO1 gene. DNA was extracted using the Nucleospin Tissue Kit (Macherey Nagel) or the DNeasy® Blood and Tissue Kit (QIAGEN) following manufacturer's protocols.

PCR amplification was performed using OneTaq DNA Polymerase (New England Biolabs), Phusion High-Fidelity DNA Polymerase (New England Biolabs), or MyTaq HS DNA Polymerase (Bioline). OneTaq reactions comprised the following reagents: 25 µl total reaction volume with 16.375 µl of nuclease-free water (New England Biolabs), 5 µl of 5X buffer (New England Biolabs), 0.5 µl of 10 mM dNTPs, 0.5 µl of 10 µM primer of each primer, 2 µl of OneTaq and 2 µl of DNA template. Phusion reactions were as follows: 20 µl total reaction volume with 10.8 µl of nuclease-free water (New England Biolabs), 4 µl HF buffer (New England Biolabs), 0.4 µl of 10 mM dNTPs, 0.6 µl of 100% DMSO, 0.2 µl of Phusion and 2 µl of DNA template. PCR reactions using both OneTaq and Phusion DNA polymerases were conducted on a Bulldog Bio® GenePro. PCR reactions using the MyTaq DNA polymerase consisted of 1 µL of each primer (10 µM), 10.5 µL of PCR water, 12 µL of MyTaq HS MIX (2×), and 0.5 µL of DNA for a total volume of 25 µL on an Eppendorf® Mastercycler nexus X2.

Each DNA sample was amplified with one of the following two PCR primer pairs: Tun_forward/Tun_reverse2 (Stefaniak et al. 2009) or LCO1490/HC02198 (Folmer et al. 1994). Tun primers were used with OneTaq and MyTaq DNA polymerases, using the protocol: 94 °C for 1 min, 60x (94 °C for 10 s, 50 °C for 30 s, 72 °C for 50 s), 72 °C for 10 min. Folmer primers were used with Phusion and MyTaq polymerases, using this protocol: 98 °C for 30 s, 35x (98 °C for 10 s, 48 °C for 30 s, 72 °C for 30 s), 72 °C for 5 min. Excess primers and dNTPs were removed from PCR products by incubation with 1 µl each of Exonuclease I (New England Biolabs) and Antarctic Phosphatase (New England Biolabs) at 37 °C for 1 h, followed by 90 °C for 10 min. Some PCR products were sequenced at the University of Kentucky's HealthCare Genomics Core Laboratory using an ABI-3730 automated sequencer (Applied Biosystems). Forward and reverse sequences obtained this way were edited and combined into a consensus sequence using Codon Code Aligner (Codon Code Corporation). The remaining PCR products were sequenced using BigDye™ terminator v.3.1 and the same primers used in the amplification step on an Applied Biosystems 3500 genetic analyzer available at UNCW Center for Marine Science. Resulting DNA sequences were aligned using the Geneious software (v. R11.1.5 Biomatters, Auckland, New Zealand). All consensus

sequences were deposited on GenBank (Accession Numbers in Table 2). All sequences obtained here were compared to other ascidian sequences available on GenBank using the megablast algorithm. If our sequence shared 98–100% identity to a GenBank sequence, we considered our sample to be the same species as the sample on GenBank. Because GenBank sequences can be mis-identified, we only used GenBank matches for DNA sequences from a verified source (e.g., published work in a peer-reviewed journal) and for which the submitting author had also identified the sample using morphological observations. Sequences with no matches on GenBank were identified using our own morphological observations.

Changes in community composition between 2003–2004 and 2017, and between Tampa Bay and Miami for each time period

Non-metric multidimensional scaling (nMDS) plots were used to visualize the composition of the communities at each time period (2003–2004 and 2017). Separate plots for abundance data and presence/absence data were created. For the abundance plots and since data were not standardized, a square root transformation was used to fit an S17 Bray–Curtis resemblance matrix. Presence/absence data were derived from the abundance data and used to create a Bray–Curtis similarity matrix. ANOSIM analyses were conducted to determine whether nMDS distances between groups were greater than distances within group samples. Additionally, SIMPER analyses for abundance data were used to determine the percent contribution of each species to the similarity and dissimilarity between the two time periods. All analyses were conducted in PRIMER v.7 (Clarke and Gorley 2015).

Species that could not be directly compared between 2003–2004 and 2017 because of identification uncertainties were excluded: *Aplidium* sp. 1, *Ascidia* sp. 1, *Ascidia* cf. *curvata*, *Clavelina* sp. 1, and *Eudistoma* sp. 1–4. *Eudistoma capsulatum,* identified in 2017, was also excluded because it could not be unambiguously assigned to one of the three *Eudistoma* spp. identified in 2003–2004. Sites only surveyed during one time period (Leverock's Marina and Tampa Bay Ranging Tower in Tampa Bay, Dinner Key and Crandon Park Marina in Miami, and the Port of Miami Municipal Pier) were excluded. Sea

Isle Marina, in the same geographic area as the Port of Miami, was sampled in 2017 and compared with data obtained in Port of Miami during the 2004 survey. Sunset Harbor Marina was sampled in the 2004 survey, but this site had no accessible floats or ropes in 2017. Instead, Miami Beach Marina, in the same geographic area, was sampled in 2017 as a comparison to Sunset Harbor Marina.

Data obtained for both Tampa Bay and Miami sites were analyzed together and included all species listed in Table 2 minus the *Ascidia* and *Eudistoma* spp. mentioned above. Tampa Bay and Miami locations were also analyzed separately, with three data sets examined for each: all species (minus the *Ascidia* and *Eudistoma* spp.), native species only, and introduced species only. For the native species data sets, one Tampa Bay site (Land's End Marina [LEM]) and two Miami sites (Homestead Marina [HM] and Matheson Hammock Marina [MHM]) were removed because no native species were found for one of the two time periods (2003–2004 or 2017). For the Miami introduced species data set, one site had to be removed (Homestead Marina [HM]) because no introduced species were found in 2017.

To analyze changes in ascidian abundance between the 2003–2004 and the 2017 surveys, we calculated the average abundance value across all sites for each species. These abundance values were numerical: rare $= 1$, common $= 2$, abundant $= 3$ to allow graphical representation of relative abundances. The translation between a qualitative and quantitative system has its limitations, since the change between 1 and 2 may be smaller in terms of number of individuals than a change between 2 and 3. We then subtracted the 2003–2004 abundance value from the 2017 abundance value. Positive numbers represent an increase in abundance and negative numbers represent a decrease in abundance. Change in abundance, for those species that had a > 0.5 average abundance value in one or both surveys, was graphed using R v. 4.0.3. A paired two-tailed t-test of the average species abundance across marinas was performed in R v. 4.0.3 to determine whether abundances in the two time periods were significantly different for each region (Tampa Bay and Miami).

In addition to visualizing differences between time periods, we also analyzed differences in community composition between Tampa Bay and Miami in 2003–2004 and in 2017. We created nMDS plots

Table 2 Ascidian species found in surveys, organized by order

Order	Species	Origin	Morphological reference	Accession Number	# of Marinas (2003–2004)	# of Marinas (2017)
Aplousobranchia	*Aplidium bermudae*	Native	(Van Name, 1902)	NA	1	0
	Aplidium stellatum	Native	(Verrill, 1871)	MW285093	0	3
	*Aplidium sp.*1	Unknown		MW858361	0	1
	Clavelina oblonga	Native	Herdman, 1880	MW285097-MW285099	4	4
	Clavelina sp. 1	Unknown		MW858362	0	2
	Didemnum perlucidum	Introduced	Monniot F, 1983	MW285100	2	2
	Didemnum psammatodes	Introduced	(Sluiter, 1895)	NA	2	2
	Diplosoma listerianum	Introduced	(Milne Edwards, 1841)	MW285101-MW285104, MW285141	13	12
	*Diplosoma sp.*1	Unknown		NA	2	0
	Distaplia bermudensis	Native	Van Name, 1902	MW286122	5	6
	Distaplia stylifera	Introduced	(Kowalevsky, 1874)	NA	0	2
	Eudistoma capsulatum	Native	(Van Name, 1902)	MW285142-MW285144, MW286123	0	3
	Eudistoma olivaceum	Native	(Van Name, 1902)	NA	1	4
	Eudistoma sp. 1	Unknown		NA	1	NA
	Eudistoma sp. 2	Unknown		NA	1	NA
	Eudistoma sp. 3	Unknown		NA	1	NA
	Eudistoma sp. 4	Unknown		MW285107	0	1
	Lissoclinum fragile	Native	(Van Name, 1902)	MW285108	5	2
	Lissoclinum verrilli	Native	(Van Name, 1902)	MW286133	0	1
	Polyclinum constellatum	Cryptogenic	Savigny, 1816	MW285147, MW858363-MW858364	7	7
Phleobobranchia	*Ascidia archaia*	Introduced	Sluiter, 1890	NA	1	0
	Ascidia curvata	Native	(Traustedt, 1882)	NA	0	2
	Ascidia cf. curvata	Native	(Traustedt, 1882)	NA	0	2
	Ascidia interrupta	Native	Heller, 1878	NA	8	0
	Ascidia sp. 1	Unknown		NA	1	0
	Ascidia sydneiensis	Introduced	Stimpson, 1855	NA	2	2
	Ecteinascidia conklini	Native	Berrill, 1932	MW858356	0	2
	Ecteinascidia styeloides	Native	(Traustedt, 1882)	MW858352-MW858355	0	7
	Ecteinascidia turbinata	Native	Herdman 1880	MW285105-MW285106	4	9
	Perophora viridis	Native	Verrill, 1871	NA	6	12
	Phallusia nigra	Cryptogenic	Savigny, 1816	MW858365	5	8
	Rhodosoma turcicum	Introduced	(Savigny, 1816)	MW286135	3	2
Stolidobranchia						
	Botrylloides niger	Native	Herdman, 1886	MW285094-MW285095, MW858360	6	6

Table 2 (continued)

Order	Species	Origin	Morphological reference	Accession Number	# of Marinas (2003–2004)	# of Marinas (2017)
	Botryllus sp. 1	Unknown		MW285096	0	2
	Eusynstyela tincta	Native	(Van Name, 1902)	NA	0	2
	Herdmania pallida	Cryptogenic	(Heller, 1878)	NA	5	8
	Microcosmus exasperatus	Introduced	Heller, 1878	MW858357	5	8
	Molgula manhattensis	Cryptogenic	(De Kay, 1853)	NA	1	0
	Molgula occidentalis	Native	Traustedt, 1883	NA	1	1
	Polyandrocarpa anguinea	Introduced	(Sluiter, 1898)	MW285109, MW858358	0	1
	Polyandrocarpa zorritensis	Introduced	(Van Name, 1931)	MW285110-MW285131	1	1
	Polycarpa spongiabilis	Native	Traustedt, 1883	NA	0	1
	Styela canopus	Cryptogenic	(Savigny, 1816)	MW285132-MW285133	9	7
	Styela plicata	Introduced	(Lesueur, 1823)	NA	10	8
	Symplegma rubra	Cryptogenic	Monniot, C 1972	MW285135-MW285136	1	6
	Symplegma sp. 1	Unknown		MW285137-MW285138	0	1
	Symplegma sp. 2	Unknown		MW285139, MW858359	0	1
	Symplegma sp. 3	Unknown		MW285134	7	11

based on abundance data, coupled with ANOSIM and SIMPER analyses as described above. All analyses were conducted in PRIMER v.7 (Clarke and Gorley 2015).

Results

Identifications

Table 2 lists all the ascidian species found in both surveys (2003–2004 and 2017), along with the introduction status of each (native, introduced, or cryptogenic), the GenBank Accession numbers for barcode sequences, the number of marinas visited, and the number of species found. In total, 48 species were observed: 20 in the Order Aplousobranchia (including 7 identified only to genus), 12 in the Phlebobranchia (one of which was identified only to genus), and 16 in the Stolidobranchia (three of which were identified only to genus). The introduction status of the 12 species identified only to genus is unknown.

Of the 36 remaining species, 19 were native, 11 were introduced, and 6 were cryptogenic. Specific information about species identifications and photographs of samples can be found in Online Resource 1.

Abundance: individual species analyses

Online Resource Table 2 contains the raw abundance data for the 2003–2004 surveys, and Online Resource Table 3 for the 2017 survey. The change in average abundance values across sites, between 2003–2004 and 2017, are shown in Fig. 2a (Tampa Bay) and b (Miami) for all species that had an absolute average abundance value across sites > 0.5 in one or both surveys. In Tampa Bay, *Botrylloides niger, Diplosoma listerianum, Ecteinascidia turbinata, Perophora viridis, Styela canopus,* and *Symplegma* sp. 3 increased in abundance, while *Distaplia bermudensis* decreased and *Styela plicata* did not change. The means of the abundance data from 2003 and 2017, averaged across sites for each species, were significantly different ($p < 0.05$). In

Fig. 2 Graphical representation of the change in average abundance across sites from the 2003–2004 surveys to the 2017 surveys. Only species that had an average abundance across sites > 0.5 in one or both surveys were included. Abundance values are 1 (rare), 2 (common), or 3 (abundant). Positive numbers represent an increase in abundance and negative numbers a decrease in abundance. The points are arranged alphabetically on the x-axis, and the horizontal spread has no biological or numerical significance. **a** Tampa Bay sites, **b** Miami sites
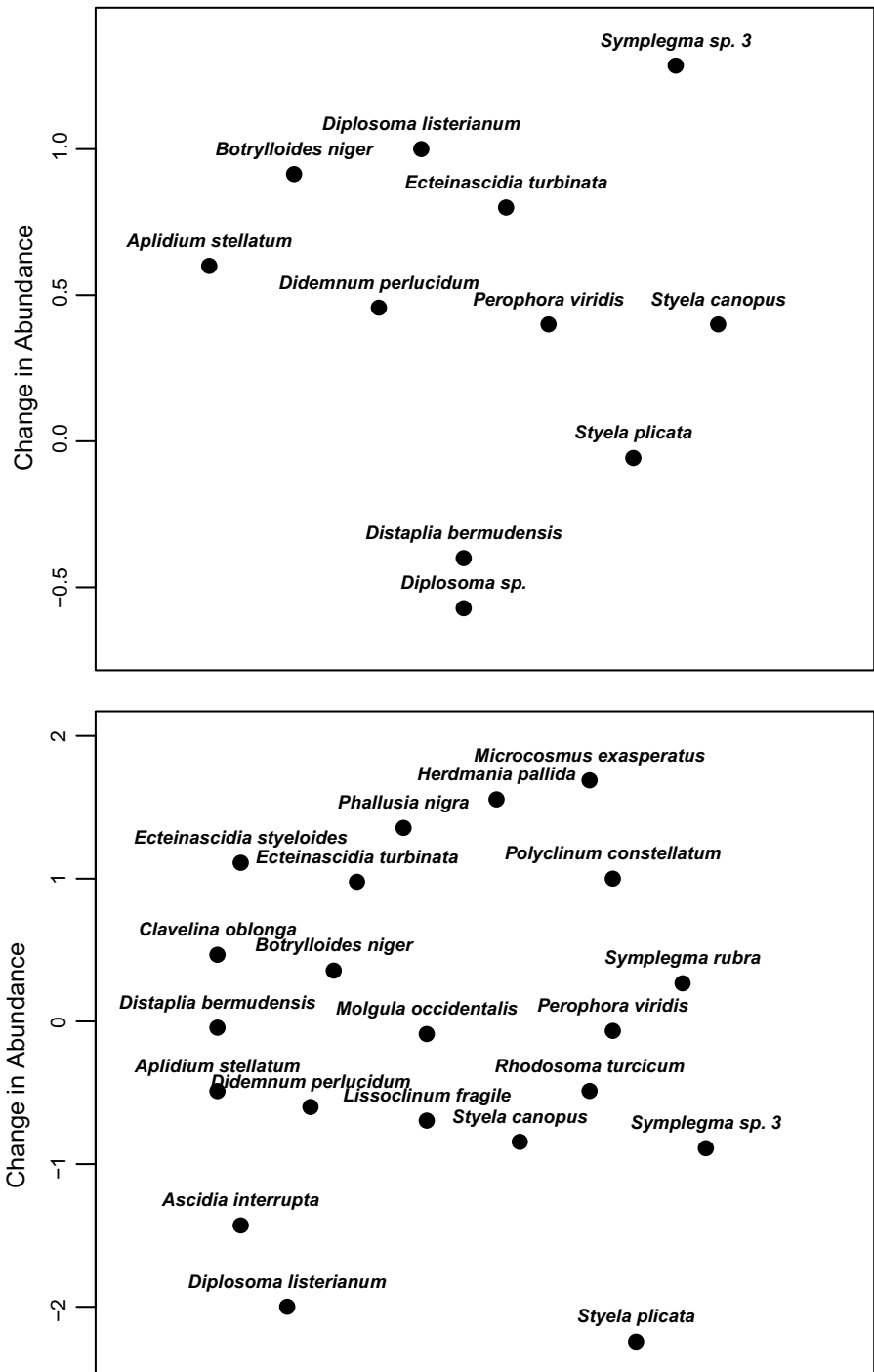
Miami, *Botrylloides niger, Clavelina oblonga, Ecteinascidia styeloides, E. turbinata, Herdmania pallida, Microcosmus exasperatus, Phallusia nigra, Polyclinum constellatum,* and *Symplegma rubra* increased in abundance, while *Aplidium stellatum,* *Didemnum perlucidum, Diplosoma listerianum, Styela canopus, S. plicata,* and *Symplegma* sp. 3 decreased; *Distaplia bermudensis, Molgula occidentalis* and *Perophora viridis* did not change. The means of the abundance data from 2004 and 2017,

averaged across sites for each species, were not significantly different ($p > 0.05$).

Abundance: community level analyses

To better visualize the relationships between the two time periods (2003–2004 and 2017), we conducted analyses on Tampa Bay (Online Resource Fig. 1) and Miami (Online Resource Fig. 2) separately. Each location was analyzed in three different ways: all species, native species only, and introduced species only. When all species were analyzed together for the Tampa Bay region, the 2003 and 2017 communities formed two apparently distinct clusters, with the exception of Clearwater Beach Marina (CBM), where the 2003 and 2017 data points were close together (Online Resource Fig. 1a). ANOSIM analysis resulted in an R of 0.184 and a p value of 0.119, indicating no significant difference between the distances of the data points within and among time periods. This means that the 2003 data points were not closer to each other than they were to the 2017 data points, and vice versa. However, the number of possible permutations of the data was only 126, and ANOSIM p values are sensitive to the number of permutations. To further assess the significance of the distinction between 2003 and 2017, in light of the distinct clusters from the nMDS, we conducted a Type I SIMPROF test in Primer v.7 (Clarke and Gorley 2003). The sample statistic (π) value was 1.74, with a p-value of 0.276 based on 999 permutations, thus corroborating the lack of community differences between 2003 and 2017 in Tampa Bay. In Tampa Bay, for the all species data set, SIMPER analyses revealed an average similarity of 50% for 2003, with the two largest contributors being *Styela plicata* (45%) and *Diplosoma listerianum* (29%) (Online Resource Fig. 3a). This means that the abundances of *Styela plicata* and *Diplosoma listerianum* were the two biggest reasons for the similarity in community composition among Tampa Bay marinas. The average similarity was 58% for 2017, and the two largest contributors were also *Styela plicata* (25%) and *Diplosoma listerianum* (26%) (Online Resource Fig. 3b).

The nMDS plot for native species in Tampa Bay did not show distinct clusters (Online Resource Fig. 1b) or any significant difference between time periods (ANOSIM, R = -0.031, $p = 0.51$, 35 permutations). The SIMPER analysis revealed two species that contributed to most of the similarity observed between the 2003 sites: *Distaplia bermudensis* (56%) and *Perophora viridis* (28%) (Online Resource Fig. 3c). Two species also contributed to most of the similarity observed between the 2017 sites: *Perophora viridis* (61%) and *Botrylloides niger* (22%) (Online Resource Fig. 3d).

The nMDS plot for introduced species in Tampa Bay did not show distinct clusters (Online Resource Fig. 1c) or any significant difference between time periods (R = 0.06, $p = 0.28$, 126 permutations). The SIMPER analysis for the introduced species data set yielded similar results to those obtained with the all species data set: *Diplosoma listerianum* and *Styela plicata* were the top two contributors for the similarity observed within the 2003 data set (average similarity 75%) and within the 2017 data set (average similarity 82%) (Online Resource Fig. 3e, f). In summary, for all three Tampa Bay data sets (all species, native species only, introduced species only), no significant differences were found between time periods.

In the Miami area, the 2004 and 2017 data for the all species data set formed distinct but spatially proximate clusters, with Homestead Marina (HM) and Matheson Hammock Marina (MHM) positioned away from the other sites for both the 2004 and 2017 sampling periods (Online Resource Fig. 2a). The R value resulting from the ANOSIM analysis was 0.182, with a p value of 0.008 based on 999 permutations. These results indicated that there were significant differences in ascidian composition and abundance among sites visited in 2004 and sites visited in 2017. SIMPER analyses for sites in the Miami area resulted in an average similarity of 48% for 2004, with five species that each contributed to 10–12% of the observed similarity: *Styela canopus, Diplosoma listerianum, Ascidia interrupta, Symplegma* sp. 3, and *Polyclinum constellatum* (Online Resource Fig. 4a). The average similarity for 2017 was 48%; *Microcosmus exasperatus* contributed 19% to this similarity, and four other species contributed between 8 and 10% of the similarity: *Herdmania pallida, Botrylloides niger, Perophora viridis,* and *Ecteinascidia turbinata* (Online Resource Fig. 4b). The average dissimilarity between the two time points was 57%. *Styela canopus* and *Ascidia interrupta* each contributed 7% of the observed dissimilarity, with 13 other species each contributing between 4–5% (Online Resource Fig. 4c). The
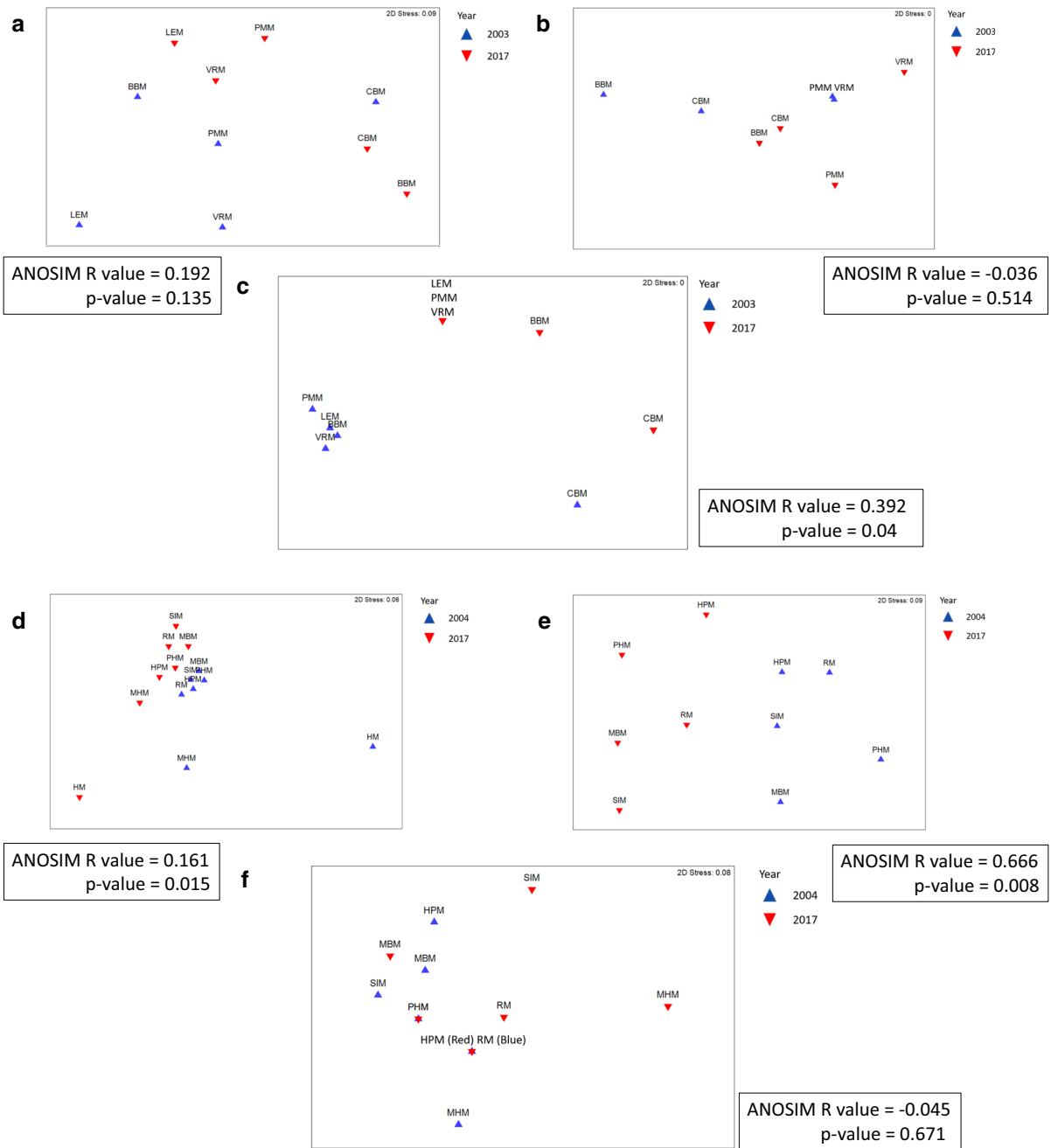
Fig. 3 **a–c** nMDS of Tampa Bay sites. Sites are Bradenton Beach Marina (BBM), Clearwater Beach Marina (CBM), Land's End Marina (LEM), St. Petersburg Marina (PMM), Vinoy Renaissance Marina (VRM). Year 1 (blue) represents 2003, Year 2 (red) represents 2017. **a** All species, **b** native species, **c** introduced species. **d–f** nMDS of Miami sites. Sites are Bill Bird Marina (HPM), Crandon Park Marina (CPM), Homestead Marina (HM), Matheson Hammock Marina (MHM), Miami Beach Marina (MBM), Pelican Harbor Marina (PHM), Rickenbacker Marina (RM), and Sea Isle Marina (SIM). Year 1 (blue) represents 2004; Year 2 (red) represents 2017. **d** All species, **e** native species, **f** introduced species

abundance of both *Ascidia interrupta* and *Styela canopus* decreased in 2017.

The nMDS plot for the native species in Miami marinas showed two distinct clusters, each corresponding to a time period (Online Resource Fig. 2b). The R value of the ANOSIM analysis was 0.642, with a p-value of 0.008 based on 126 permutations. The SIMPER analysis revealed an average similarity of 70% for 2004, with three species each contributing to 23–25% of the observed similarity: *Ascidia interrupta, Botrylloides niger,* and *Lissoclinum fragile* (Online Resource Fig. 4d). The average similarity for 2017 was 63%, with three species contributing to a majority of the similarity (>60%): *Botrylloides niger, Ecteinascidia turbinata,* and *Perophora viridis* (Online Resource Fig. 4e). SIMPER analyses for the native species data set in Miami resulted in 50% dissimilarity among sites, with *Ascidia interrupta* decreasing in abundance from 2004 to 2017 (Fig. 2b) and contributing the most to the observed dissimilarity (18%; Online Resource Fig. 4f).

In contrast to results obtained for all species and native species data sets, the nMDS plot obtained for the introduced species data set in Miami showed no difference between the two time periods (Online Resource Fig. 2c). The R value of the ANOSIM analysis was −0.048, with a *p* value of 0.63 based on 462 permutations. The SIMPER analysis showed an average similarity of 54% for 2004, with two species contributing to the majority of this similarity: *Diplosoma listerianum* and *Microcosmus exasperatus* (Online Resource Fig. 4g). The average similarity was 54% for 2017, with two species contributing to the majority of this similarity: *Diplosoma listerianum* and *Microcosmus exasperatus* (Online Resource Fig. 4 h). In summary, analysis conducted with both the all species and native species data sets for the marinas surveyed in Miami showed significant differences in species abundances between time periods. In contrast, no significant differences in abundances were retrieved within and between time periods when analyzing the introduced species data set.

Presence/absence: changes between time periods

Several species found in the 2003–2004 surveys were not observed in the 2017 survey, and vice versa (Table 2). Within the Aplousobranchia, *Aplidium bermudae* and *Diplosoma* sp. 1 were only found in 2003, while *Aplidium stellatum, Aplidium* sp. 1, *Clavelina* sp. 1, *Distaplia stylifera, Eudistoma capsulatum,* and *Lissoclinum verrilli* were only observed in 2017 (Table 2). Within the Phlebobranchia, *Ascidia archaia* was only found in 2004, *Ascidia interrupta* in 2003 and 2004, and *Ascidia curvata, Ascidia* cf. *curvata, Ecteinascidia conklini,* and *Ecteinascidia styeloides* only in 2017. Within the Stolidobranchia, *Molgula manhattensis* was only observed in 2003, and *Botryllus* sp. 1, *Eusynstyela tincta, Polyandrocarpa anguinea, Polycarpa spongiabilis,* and *Symplegma* sp. 1 and sp. 2 were only seen in 2017.

When comparing the presence/absence of species at the ten sites that were sampled in both years (Tampa Bay: Bradenton Beach, Clearwater Beach, Land's End, St. Petersburg Municipal, Vinoy Renaissance; Miami: Bill Bird, Homestead, Matheson Hammock, Pelican Harbor, Rickenbacker), the system appears even more dynamic than when comparing the survey years as a whole. Out of 38 species total at these 10 sites, 25 species were gained between sampling times at one or more sites, 16 species were lost between sampling times at one or more sites, and 18 species were constantly present at one or more sites. The numbers of gained, lost, and constantly present species add up to more than 38 species because a single species might be, for example, lost at one site and constantly present at another. Five species had all three patterns present across sites (Online Resource Table 4). For example, *Lissoclinum fragile* was gained at Bradenton Beach, was lost at Bill Bird and Pelican Harbor, and was constantly present at Rickenbacker. Species richness decreased at one of the sites (Bill Bird) and increased at the other nine sites. nMDS plots and ANOSIM analyses for presence/absence data sets for Tampa all species, native species, and introduced species are available in Fig. 3a–c; data sets for Miami all species, native species, and introduced species are in Fig. 3d–f.

Differences in ascidian communities between sites and time periods

In 2003, there were 14 total species in Tampa: 6 native, 4 introduced, 3 cryptogenic, and 1 unknown. In contrast, in 2004, there were 28 species in Miami: 9 native, 8 introduced, 5 cryptogenic, and 6 unknown. In 2017, there were 20 species in Tampa: 10 native, 5 introduced, 3 cryptogenic, and 2 unknown; and 35

species in Miami: 16 native, 8 introduced, 5 crypto-genic, and 6 unknown. Species richness values are graphed in Online Resource Fig. 5. nMDS plots comparing Tampa Bay versus Miami ascidian communities for all species, native species, and introduced species for each time period (2003–2004 and 2017) are shown in Online Resource Fig. 6. The nMDS plots showed clustering of Tampa sites together and Miami sites together in all cases. Similarly, the ANOSIM results showed significant differentiation between ascidian communities in Tampa and in Miami ($p < 0.05$), independent of their introduction status. For introduced species and all species, the SIMPER analysis revealed that *Styela plicata* was the largest contributor to the differences between ascidian communities in both 2003–2004 and 2017. For native species, *Eusynstyela tincta* was the largest contributor to the differences between these communities in both 2003–2004 and 2017.

Discussion

Artificial substrates in Florida surveyed in 2003–2004 were revisited in 2017. We predicted an increase in both the number of introduced species from 2003–2004 to 2017 (for the two regions) and the abundance of established non-native species in 2003–2004 that were still present in 2017 as a result of recurrent introductions and increased propagule pressure. Instead, we found that, while ascidian species richness increased similarly from 2003–2004 to 2017 for both regions (Miami: +7, Tampa Bay: +6), this change was driven by the addition of native species, as introduced species did not change over time in either Miami or Tampa Bay. When individual species were examined, abundance over time of species present in 2003–3004 was highly variable among species and sites, with various species increasing, decreasing, or not changing in abundance in 2017. All seventeen species found in 2017 but not recorded in 2003–2004 were rare in both Tampa Bay and Miami. In contrast, five species that were found in 2003–2004 were not observed in 2017, with four of these five being rare. While many studies on the effects of introduced ascidians often focus on the ability of these species to dominate the benthic community (e.g., Osman and Whitlatch 2007; Vance et al. 2008; Sommer et al. 2010, Kramer and Rocha 2011), these

findings highlight the importance of rare species on ascidian community composition and temporal community dynamics.

Our data show that community composition is highly diverse, not only among sites within years but also within sites between years, especially for rare species (Online Resource Tables 2 and 3). Comparisons of individual sites visited during both time periods showed that even species that were present during both survey years were not always found at the same sites between years. Therefore, our data suggested that species recorded in only one survey year may have moved into or out of our sampling sites from unsampled nearby habitats around Tampa Bay and Miami. Nine of the 17 species found only in 2017 had been previously observed in Florida, either on artificial structures or in natural habitats: *Aplidium stellatum* (Van Name 1945), *Ascidia curvata* (Hartmeyer 1908; Janiak et al. 2018)*, Botryllus* sp. 1 (Janiak et al. 2018), *Distaplia stylifera* (Van Name 1945), *Ecteinascidia* sp. (Plough and Jones 1937), *Eudistoma capsulatum* (Van Name 1945), *Eusynstyela tincta* (Van Name 1945), *Lissoclinum verrilli* (Van Name 1945, as *Echinoclinum verrilli*), and *Polycarpa spongiabilis* (Plough and Jones 1937). Two of the 17 species, *Ecteinascidia styeloides* and *Polyandrocarpa anguinea,* were previously found in the Caribbean and could have expanded their ranges northward since 2003–2004 or could have been recently introduced to Florida (*E. styeloides*: Goodbody and Cole 2006, Rocha et al. 2012, Palomino-Alvarez et al. 2019; *Polyandrocarpa anguinea:* Rocha et al. 2005, Carman et al. 2011, Heyns et al. 2016, Villalobos et al. 2017). The remaining six species of the 17 found only in 2017 were not unambiguously identified (*Aplidium* sp. 1, *Ascidia* cf. *curvata*, *Clavelina* sp. 1, *Eudistoma* sp. 4, *Symplegma* sp. 1, and *Symplegma* sp. 2); thus, their origin cannot be hypothesized.

Reestablishment of slightly different communities following environmental and anthropogenic disturbances could explain the temporal diversity found in this study. For instance, low surface salinity events following storms can cause mortality in ascidian floating dock populations (Lambert and Lambert 2003), freeing new space. Between 2004 and 2017, Hurricanes Katrina and Wilma (both in 2005) and tropical storms Fay (2008), Isaac (2012), and Andrea (2013) brought heavy rainfall to Miami (Events Index, Miami—South Florida 2022). From 2003 to

2017, Tampa Bay experienced heavy rainfall from Hurricane Frances (2004) and an unnamed storm in 2006 (Daily Historical Rainfall 2022; NWS Tampa Bay Significant Weather Events 2022). Additionally, routine dock cleaning and repair, as well as dock replacement after hurricanes, would also provide new space for settlement. In 2005, Hurricanes Katrina and Wilma damaged coastal infrastructure in Miami (Miami, Florida's history with tropical systems 2022). Because some ascidians are more likely to settle on bare space than on existing organisms (Lambert 1968; Osman and Whitlatch 1995; Lindeyer and Gittenberger 2011), the presence of available space could facilitate the recruitment of certain species of ascidians into a new site. Which species will be able to recruit depends on local or regional sources of propagules. Studies on succession in marine sessile epifauna have demonstrated that these communities were highly dynamic, with the initial species composition reflecting which species were settling at the time the space became available (Osman 1977; Sutherland and Karlson 1977; Mook 1981; Greene et al. 1983; Rajagopal et al. 1997; Lindeyer and Gittenberger 2011). In ascidians, data from recruitment and settlement plate studies have shown that recruitment varies over the course of a year for different species (e.g., Stachowicz et al. 2002; Altman and Whitlatch 2007; Nydam and Stachowicz 2007). Conversely, some ascidians never settle on bare surfaces, arriving later when communities are better established (Svane and Young 1989).

We do not have data on whether the recruitment timing of species in invaded marine communities varies in Tampa Bay and Miami; however, the variation we observed between common and rare ascidian species—most species found only in one of the two survey years were rare—also supports external disturbances as a potential mechanism for the interannual diversity in ascidian presence/absence in both Tampa and Miami. Common species present at a site before an external disturbance are likely to persist because a few individuals of their larger population are likely to survive the event and repopulate the site (Lambert and Lambert 2003). Rare species are more likely to experience total local population loss and free some space that a few migrants from another site and species could occupy. Relatedly, repeated introductions of the same species over time or increased propagule pressure may result in the arrival of a new genotype that is better adapted to the new environment

(Simberloff and Van Holle 1999; Lockwood et al. 2005). Thus, a species that was rare in the past or unable to establish long-term populations can suddenly colonize a new habitat and even expand beyond its entry port (Clark and Johnston 2009).

In Tampa Bay, results indicated that the abundances of the entire ascidian community, and native and introduced species separately, were relatively stable over time. Similarly, the abundance of introduced species over time was also stable in Miami. However, Miami marinas supported a higher species richness than Tampa Bay marinas, both in 2003–2004 and in 2017. Although we were expecting to observe an increase of introduced species number and abundances as a result of propagule number, our results showed little to no increase in introduced species richness for either region in over a decade. Thus, most introduced ascidian populations in these areas may have reached carrying capacity (sensu Dhondt 1988). In particular, for introduced species in artificial substrates, population size will ultimately be limited by environmental factors such as food and space availability, independently of recurrent introductions, reproduction rates, and the presence of competitor species.

In contrast to the pattern found for introduced species, the abundance of native species decreased over time in Miami. The decline of native ascidian species has been attributed to competitive interactions with introduced ascidian species in artificial habitats (Dijkstra et al. 2007; Gittenberger and Moons 2011). Thus, the presence of introduced species can be a major factor in defining the whole community structure (Stachowicz et al. 2002; Blum et al. 2007). In addition, large abundances of introduced ascidian species were shown to facilitate population growth of native predators, which could in turn impact the abundance of native species (Dijkstra et al. 2013). Native predatory fish species reduced cover of ascidians in artificial habitats in Indian River Lagoon, FL, although the specific fish species have not been identified (Janiak and Branson 2021). In Broward County, FL, < 20 miles from our northernmost Miami site, the most common predatory fish in a marina were juvenile *Lutjanus griseus*, *Anisotremus virginicus*, *Lutjanus synagris*, *Abudefduf saxatilis*, and *Lutjanus apodus* (Patranella et al. 2017). While there is no information in the literature about whether these species eat ascidians, invertebrates represent a substantial portion

of their diets as juveniles (Rooker 1995; Franks and VanderKooy 2000; Grossman et al. 2006; Hammerschlag et al. 2010; Sazima et al. 2010). Flatworms, gastropod molluscs, and urchins are also known to eat ascidians (see Lambert 2005 for a review).

Finally, pollution—specifically, the duration of sewage spills—was correlated with a decline in native ascidian species abundance and richness in the Mediterranean coast of Israel (Gewing et al. 2017). In Miami, sewer overflows have resulted in frequent contamination of the coastal waters (FIU Institute of Environment, 2020). These overflows resulted in the Environmental Protection Agency ordering a Consent Decree for Miami-Dade County to improve wastewater collection and treatment (FIU Institute of Environment, 2020). Ten heavy metals, ranked in order of concentration (iron, zinc, manganese, lead, nickel, copper, mercury, cadmium, selenium, arsenic) were detected in seagrass beds in the Port of Miami (Smith 2018). Copper in particular has been shown to negatively affect native ascidian species (Agell et al. 2004; Piola and Johnston 2008; Crooks et al. 2011; Pineda et al. 2012; Osborne and Poynton 2019).

Although ascidian communities in both Tampa Bay and Miami were stable overall, further decreases in native species diversity and abundances, and increased propagule pressure of non-native species, could eventually lead to an invasive process (Simberloff and Von Holle 1999). In fact, it has been demonstrated that the eventual arrival of a new non-native species that closely interacts with a long-established one could lead to what is known as an invasion meltdown (Simberloff and Von Holle 1999) and an increase of the magnitude of their impact (Braga et al. 2018). Additionally, within the existing pool of currently introduced species, a few are likely to become invasive (rapidly growing and spreading) in the future, a phenomenon called "invasion debt" (Essl et al. 2011). Thus, periodic surveys and a better understanding of the biology of all introduced species as well as their environmental tolerance thresholds remain paramount to prevent further damage to natural ecosystems. Variation in community composition over time, at local as well as regional geographic scales, emphasizes the importance of surveying multiple sites both among and within regions, rather than relying on a few sentinel sites. In addition, local conditions (e.g., propagule pressure, atmospheric and anthropogenic disturbances) are likely to play an important role in driving ascidian community dynamics.

Data availability The PRIMER7 input files and raw survey data are available from the corresponding author. All other data generated or analyzed during this study are included in this published article (and its Online Resource information files).

Declarations

Conflict of interest All authors declare no competing interests.

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