HOST - MICROBIOTA INTERACTIONS IN AN ESTUARINE METAZOAN UNDER CHANGING CONDITIONS

Faculty of Mathematics and Natural Sciences

Doctor of Philosophy in Natural Sciences

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SUMMARY

First life on earth arose about 4.1 billion years ago and likely was of prokaryotic nature. Therefore, multicellular eukaryotic organisms evolved in a World dominated by Bacteria and Archaea and these three domains of life co-evolved in close association, forming complex interactions. All multicellular organisms are colonized by microbes and live in a mutualistic relationship with them. These evidence, combined with constantly growing scientific knowledge over the "microbial World", brought to the switch of the conception of microbes as just pathogens, towards a much wider view of them as also provider of benefits *e.g.,* development and homeostasis of their multicellular hosts, biotechnologies, bioremediation, pharmacy and medicine, etc.

Multicellular model organisms and their associated microorganisms are being employed as subject of studies aimed at disentangling the mechanisms of such associations and investigating their potential to foster rapid species evolution and adaptation to environmental changes.

In this thesis work, was investigated the emerging marine model organism *Nematostella vectensis*, an anthozoan cnidarian and its associated microbiota composition. *N. vectensis* is a widespread, euryhaline and eurytherm animal, capable of surviving under highly variable conditions and harbours a diverse and dynamic microbial community. These plasticity from both the host and the associated microbiota sides, gave us the hint that this symbiosis constitutes a highly specific and essential tool for the whole metaorganism maintenance under different conditions.

In long-term experiments we first investigated the associated microbiota composition from different natural *N. vectensis* populations under both field and lab conditions and from genetically distinct animals under different thermal regimes. Contextually, we acclimated genetically identical *N. vectensis* polyps at three different thermal regimes, to exclude differences derived from the host genotype. We demonstrated that *N. vectensis* is able to diversify its associated microbiota according with its genotype and provenance geographic location, field vs laboratory conditions, and culture temperature, and that animals acclimated to higher temperatures acquire resistance to heat stress. Moreover, these differences in thermal resistance could be transferred to non-acclimated animals through microbiota transplantation alone, and to the next generation together with the microbiota vertical transmission. On the base of this last

evidence, we investigated the specificity of this host-microbiota association among the different host' s developmental stages and between the two sexual lines. This last study confirmed the consistency of the microbiota association between animal's generations, the differential transmission of symbionts from maternal and paternal polyps trough the gametes to the offspring and an active and specific shaping of the microbiota composition by the host during development and from the surrounding environment.

Together these results provide an overview and substantially set out the intimacy, specificity and consistency of the association between *N. vectensis* and its microbial symbionts. They indicate that host-driven microbiota plasticity contributes to the metaorganism thermal acclimation and that its transmission to the next generation and its transfer to unacclimated individuals may represent a rapid mechanism for adaptation.

INDEX

LIST OF ABBREVIATIONS

AB	antibiotics
ASV	amplicon sequence variant
AT	acclimation temperature
DT	developmental temperature
F1	“first filial generation”
LDA	linear discriminant analysis
NM	*Nematostella* medium
OTU	operational taxonomic unit
Vs	“versus”
Woa	weeks of acclimation

Materials

Organisms

Investigated organism	*Nematostella vectensis* (taxonomy ID: 45351)	
Prey organism	*Artemia salina*	INVE

Antibiotics

Ampicillin	Sigma
Neomycin sulfate	Roth
Rifampicin	Roth
Spectinomycin	Roth
Streptomycin sulfate	Roth

Chemicals

Agarose	Roth
Dimethylsulfoxide (DMSO)	Roth
DNA Loading Dye (10x)	Thermo Scientific

dNTPs (10 mM) Fermentas
EDTA Sigma
Ethanol Roth
GeneRuler™ DNA Ladder Mix Thermo Scientific
HCl (37%) Merck
Sea salt (Red Sea Salt®) Red Sea
TRIzol Sigma-Aldrich
β-mercaptoethanol Sigma-Aldrich

Media and buffers

Artemia Medium 31.8 g sea salt. ad 1 L Millipore H2O
Marine broth Plates 1 L Marine broth Medium, 15 g Agar-Agar
Nematostella Medium 16 g sea salt, ad 1 L Millipore H2O

Kits

AllPrep DNA/RNA/miRNA Universal QIAGEN
DNeasy® Blood & Tissue Kit QIAGEN

Enzymes

DNase Thermo Scientific and QIAGEN
GoTaq® DNA Polymerase Promega
Proteinase K QIAGEN
Ribolock™ RNase Inhibitor Thermo Scientific
RNase QIAGEN

Oligonucleotides

Name	Sequence (5' -> 3')	Tm [°C]	company
V2_27F	AGAGTTTGATCCTGGCTCAG	57.3	biomers.net
V2_338R	TGCTGCCTCCCGTAGGAGT	61.0	biomers.net

V2_1492R GGHTACCTTGTTACGACTT 53.1 biomers.net

Devices

1205 MP Weighing scale Sartorius
Centrifuge 5415 D Eppendorf
Centrifuge 5417 R (Cooling Centrifuge) Eppendorf
Certomat Incubator B. Braun
Refrigerated incubator AL654 Aqualytic
Gel-Doc™ XR+ Bio-Rad
Kern 770 Weighing scale Kern
LaminAir® HB 2448 Clean bench Heraeus instruments
Milli-Q Academic System Millipore
Mini Spin Eppendorf
NanoDrop® 3300 Thermo Scientific
pH-Meter pH 211 Hanna Instruments
Qubit 3.0 Fluorometer Thermo Scientific
Real-Time Cycler 7300 Applied Biosystems
Separation system B1A Owl Separation Systems
Separation system B2 Owl Separation Systems
Separation system D3 peqLab
THq Homogenizer Omni

Web resources

Compagen http://www.compagen.org/
GBIF https://www.gbif.org/
LEfSe https://huttenhower.sph.harvard.edu/galaxy/
NCBI http://www.ncbi.nlm.nih.gov/
RDP https://rdp.cme.msu.edu/

Softwares

GraphPad Prism 7
Primer v7
JASP 0.16.4
QGIS
QIIME 1.9
QIIME 2

This work was supported by the Human Frontier Science Program (HFSP - Young Investigators' Grant RGY0079/2016) and the Deutsche Forschungsgemeinschaft (DFG - CRC grant 1182) “Origin and Function of Metaorganisms” (project B1). The Sequencing was supported by the DFG Research Infrastructure NGS_CC (project 407495230) as part of the Next Generation Sequencing Competence Network (project 423957469). NGS was carried out at the Competence Centre for Genomic Analysis in Kiel (CRC1182, project Z3).

INTRODUCTION

The metaorganism concept

Since Wallin published his book "Symbiontism and the Origin of Species" in 1927 (Macklin, 1927), there has been a growing recognition by the scientific community and the society that multicellular organisms live and evolve in close relationship and interdependence with microbes. After him, Lynn Margulis (Margulis, 1993) consolidated the idea that symbiosis has been an important factor in evolution, although, at that time, the focus was on eukaryotes-eukaryotes symbioses. Nowadays we are very much aware of the importance and ubiquity of associations between prokaryotes and archaea with animals and plants. There are no germ-free multicellular organisms in nature. Epithelia in contact with the environment are colonized by microbial communities, and all multicellular organisms must be considered an association of the macroscopic host in synergistic interdependence with bacteria, archaea, fungi, and numerous other microbial and eukaryotic species (Bosch & Miller, 2016; McLoughlin et al., 2016). These associations that can be analyzed, measured, and sequenced, are referred to as “holobiont” or “metaorganism” (Bosch & Miller, 2016). The term “metaorganism” was first used (G. Bell, 1998) to refer to organisms which are between two levels of organization. Currently, the term is increasingly used to refer to the totality of any multicellular organism derived from millennia of coevolution with microbiota, and even humans have been reviewed as “metaorganisms” as a result of a close symbiotic relationship with the intestinal microbiota (Turnbaugh et al., 2007). Various scientists (Gordon et al., 2013) proposed to use for the functional entity formed by a macrobe and its associated symbiotic microbes and viruses the term “holobiont.” Both terms, “metaorganism” and “holobiont” refer to an association of organisms which occupies an ecological niche, adapts, and may even be the organizational level at which natural selection acts. If adaptation occurs by swapping microbial constituents or by reshuffling the relative proportions of current bionts is still matter of investigation. The fact that all multicellular organisms coexist with bacteria (Knowlton & Rohwer, 2003) and references therein) suggests that these symbiosis are indispensable. How organisms tolerate microbes and how stable these partnerships are achieved represents a big matter of studies. Like all partnerships, it seems to exist a trade-off between conflict and cooperation.

Symbioses in nature are more appropriately represented as a spectrum of interactions ranging from symbiotic to outright parasitic (Bosch & Miller, 2016). Microbial communities display a variety of selection scenarios that reveal constant selection pressures, but also often comprise a degree of complexity that can only be captured by frequency- and density- dependent selection pressures (Coleman & Chisholm, 2010; Zeng et al., 2017).

Prokaryotes enable eukaryotes to acquire and metabolize otherwise unavailable substances, but also shape their development and behavior. Interactions between the members of the holobiont, *i.e.*, viruses, prokaryotes, eukaryotic symbionts, and host cells, have probably been critical to enabling the key transitions in organisms' evolution. However, the reciprocal is also true, eukaryotes have dramatically transformed the physical environment that is available for microbial colonization by providing niches that do not exist elsewhere and by exercising enormous selective forces on microbial populations. The health of animals, including humans, appears to be fundamentally multiorganismal. Any disturbance within the complex community has drastic consequences for the wellbeing of all the members (adapted from (Bosch & Miller, 2016).

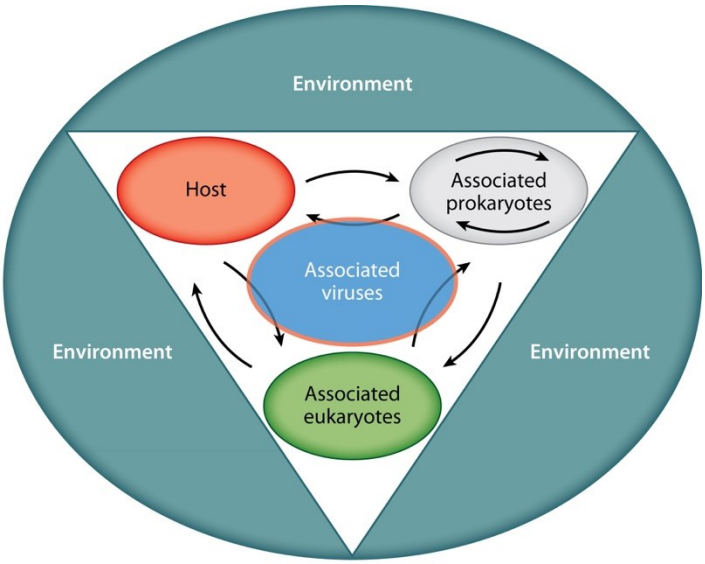

Figure 1. Multicellular organisms are metaorganisms composed of the macroscopic host and synergistically interdependent bacteria, archaea, viruses, and numerous other microbial and eukaryotic species including fungi and algal symbionts. From (Bosch, 2013).

Acclimation in the marine environment

The Intergovernmental Panel on Climate Change (IPCC) predicted an increase of the sea surface temperature up to +2 °C by 2060 in the best SSP (Shared Socioeconomic Pathway) scenario (IPCC, 2022 (H.-O. Pörtner et al., 2022). The oceans constitute an important sink for atmospheric CO_2, thereby strongly influencing the Earth's climate, and have absorbed around 40% of global anthropogenic CO_2 emissions (Foo & Byrne, 2016; H.-O. Pörtner et al., 2022). In seawater, dissolved CO_2 forms carbonic acid and causes a decrease in carbonate ion concentration coupled with an increase in bicarbonate ion concentration and a consequent release of hydrogen ions thus causing pH lowering (*i.e.,* "ocean acidification"). In this context, marine organisms are synergistically exposed to multiple anthropogenic stressors, *e.g.,* hypercapnia, the increase in organism partial pressure of CO_2,; the decrease in calcium carbonate saturation; decreased salinity; hypoxia; nutrient enrichment; among others (Przeslawski et al., 2015). Environmental stressors, are a significant evolutionary force that influences the shape of marine communities through selection (Hoffmann & Sgró, 2011). The adaptive capacity of marine species involves several mechanisms, *i.e.,* acclimation, shifts in distribution, microevolution/adaptation, and the adaptation capacity of species is greatly influenced by their stress tolerance, dispersal ability, the latitudinal range the species inhabit and the potential for genetic exchange (Bernhardt & Leslie, 2013). The shift in distribution might be unfeasible for species that have reduced migration capacity or are sessile, while adaptation occurs over many generations and relies on genetic change in response to environmental selection (Hoffmann & Sgró, 2011). Acclimatization, on the other hand, allows species adjustment to a changing environment within an individual's lifetime. It involves phenotypic plasticity, wherein changes in physiology, as a result of environmental conditions, can generate a range of phenotypes from a single genotype (Bay & Palumbi, 2015; Edwards, 2020; Foo & Byrne, 2016). In the short term, acclimation may allow adjustment to changing conditions in some species and provide the time required for genetic adaptation to occur (Chevin et al., 2013). Acclimated individuals may generate preadapted offspring that exhibit increased fitness under the new environmental conditions; a phenomenon known as "transgenerational acclimation" (A. M. Bell & Hellmann, 2019; Clark et al., 2019; Donelson et al., 2018; JM Donelson et al., 2012; Munday, 2014; Veilleux et al., 2015). This effect can take place thanks to

different mechanisms: transgenerational plasticity (*e.g.,* upregulation of immune and stress related genes) (A. M. Bell & Hellmann, 2019; Clark et al., 2019; Donelson et al., 2018), differential selection for favorable alleles in a population (Salinas & Munch, 2012), 'gamete imprinting', consisting of protective factors enriched in the eggs from acclimated adults (*e.g.,* heat shock proteins, antioxidant enzymes) (Hamdoun et al., 2007), epigenetic inheritance (*e.g.,* DNA methylation and histones modification), that alter gene expression without changing the DNA sequence (A Klosin, 2016; Boyko & Kovalchuk, 2011; Fallet et al., 2020; Ho & Burggren, 2010; Manuscript, 2012), and the vertical transmission of beneficial symbionts (Björk et al., 2019; Hirose & Fukuda, 2006; JL Padilla-Gamiño, 2012; Quigley et al., 2017). Since the early stages are often the most vulnerable to extreme environmental conditions, these plastic responses across generations may facilitate populations' persistence (Foo & Byrne, 2016). Usually, species adapted to unstable environments and fluctuating conditions show higher plasticity and acclimation capacity compared to those adapted to more stable habitats (Foo & Byrne, 2016; Sanford & Kelly, 2011). Temperature is a key factor determining the biogeography and distribution of marine species (H. O. Pörtner, 2002; Somero, 2010; Sunday et al., 2014; Tomanek, 2010) and individuals from warmer climates are more thermotolerant than those from the cooler regions of the range (Dixon et al., 2015; B. J. Dunphy et al., 2013; Gaitán-Espitia et al., 2017; KE Ulstrup, 2006; Peck, 2016; Pereira et al., 2017; Visser, 2008). Despite this thermotolerance capacity, many marine species appear to be already operating at the edge of their upper thermal limit, and further acclimatization to warming may be narrow (Sunday et al., 2014). The "tolerance–plasticity trade-off hypothesis" predicts that individuals already adapted to high temperatures are more vulnerable to climate change because they cannot evolve both high tolerance and plasticity (Barley et al., 2021; H. O. Pörtner et al., 2006; van Heerwaarden & Kellermann, 2020).

Marine metaorganisms

In the context of multicellular species rapid acclimation, symbiotic microorganisms have now been identified as active contributors (Bang et al., 2018). In aquatic environments, observed differences in bacterial communities retrieved from multicellular hosts versus those from the surrounding water, indicate that host-bacteria associations are non-random but subjected to selective mechanisms (S Sunagawa,

2010; Sweet et al., 2010). Maintaining an appropriate microbiota composition is of pivotal importance for the host to preserve its health, and associated microbial communities alterations are usually observed in diseased states and in under adverse environmental conditions (D. Bourne et al., 2008; Garren et al., 2016; Jones et al., 2004; R. Littman et al., 2011). Some studies report that host-associated bacterial communities depend mainly on environmental factors and geographical location (Hester et al., 2016; O. O. Lee et al., 2012; R. A. Littman, Willis, Pfeffer, et al., 2009; Pantos et al., 2015), while others indicate that bacterial assemblages are host species-specific (Adair & Douglas, 2017; Apprill et al., 2012; Bosch, 2012; Chu et al., 2016; Hernandez-Agreda et al., 2017; Hester et al., 2016; Ziegler et al., 2019). It is a common idea that water animals' associated microbiotas consist of three components: (1) a small conserved core, (2) a larger group of associates specific to a geographic site, depth, and local environmental characteristics and (3) a variable/stochastic community (Hernandez-Agreda et al., 2017; Kellogg et al., 2017; van de Water et al., 2017, 2018). High microbiome flexibility presumably supports holobiont adaptation to unstable environments with the risk of losing important associates/functions or acquiring pathogens (Bennett & Moran, 2015). Hence, redundancy of important microbial functional groups is often observed and considered a maintaining factor of metaorganism structure and function during environmental changes (Bang et al., 2018).Conversely, in more stable environments, low microbiome flexibility helps to maintain stable and robust relationships with conserved microbial functions at the cost of a higher susceptibility to rapid environmental change. (Voolstra & Ziegler, 2020; Ziegler et al., 2019). Host-genotype specificities have recently been described also for sponge (Glasl et al., 2018), plant, crustacean, and human microbiomes (Berg, 2009; Cahana & Iraqi, 2020; Sullam et al., 2018).

The model organism *N. vectensis*

When searching for general concepts, simple animal models may help to study mechanisms and identify key players or mediators. Cnidaria is the oldest eumetazoan phylum and forms the sister group to all bilaterians and are separated from Porifera and Placozoa by possessing real tissues (**Figure 2)**. Unlike Bilateria, whose tissues consist of three blastodermic layers (ecto-, meso- and endoderm), Cnidaria's tissues just consist of two (ecto- and endoderm) separated by the acellular mesogloea layer.

Differently from Bilateria, Cnidaria possess radial body symmetry rather than bilateral and a nerve net rather than a central nervous system, and, uniquely in the animal kingdom, they possess the nematocytes, a type of cells with predation, defense and adhesion functions (Kelava et al., 2015; Technau et al., 2015; Technau & Steele, 2011) and references therein). Cnidaria have preserved much of the genetic complexity of the common metazoan ancestor. For instance, most of the signaling pathways that regulate development (Technau et al., 2005) and innate immunity (D. J. Miller et al., 2007) important in bilaterian animals (including humans) (Faltine-Gonzalez & Layden, 2019; Pukhlyakova et al., 2018; N. H. Putnam et al., 2007; Ryan et al., 2007). (Al-Shaer et al., 2021) pointed out that establishing a genetically amenable, high-throughput, cnidarian model would improve our understanding of many aspects of cnidarian biology, which has been hindered by the inability to easily access, observe and culture many species within this phylum. Although corals can be harvested and kept under laboratory conditions, their natural history makes it very difficult to control spawning behavior and therefore makes it so that embryos are only available up to a few times a year (Cleves et al., 2018). In addition, there are few tools and resources available for conducting molecular, cellular or physiological research in non-model cnidarian systems (Technau & Steele, 2011).

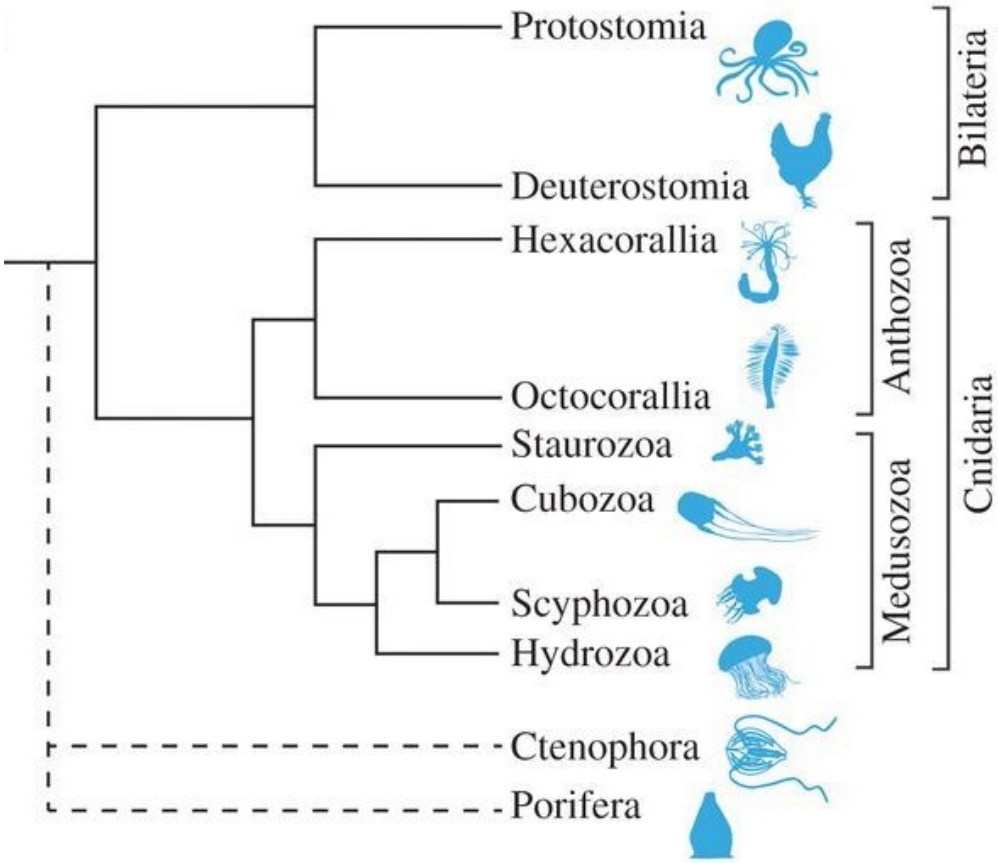

Figure 2. Phylogenetic relationships of cnidarians. As the phylogenetic position of ctenophores and sponges is still not completely resolved, their lineages are marked with a dashed line (Kelava et al., 2015).

Cnidaria are divided into two major classes, Anthozoa and Medusozoa; the starlet sea anemone *Nematostella vectensis* (Stephenson 1935), like corals, belongs to the class Anthozoa, that unlike the Medusozoa, lack a free-swimming medusa stage in the life cycle. *N. vectensis* body is divided into three parts: i) the *capitulum* or head with the central oral opening surrounded by 16 tentacles; ii) the *scapus* or column with the pharynx and mesenteries; and iii) the *physa* or foot (Stefanik et al., 2013) (**Figure 3**).

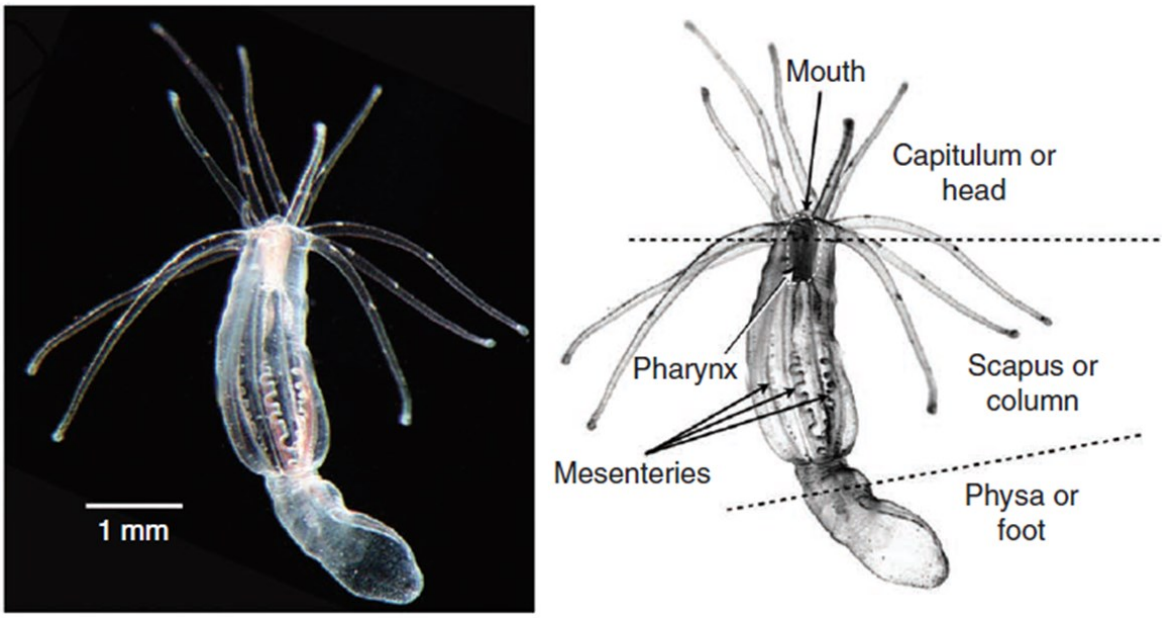

Figure 3. *N.vectensis* body divided into three major regions. The *capitulum* (head) with mouth and tentacles, the *scapus* (column) with the pharynx and mesenteries and the *physa* (foot) (Stefanik et al., 2013).

N. vectensis can reproduce both sexually and asexually. Asexual reproduction occurs by transverse fission via either physal pinching or polarity reversal. Physal pinching consists of constriction and pinching off the foot. In the next days, this isolated portion of the body regenerates all missing organs and structures into a fully functional polyp. The polarity reversal consists of a new head formation at the aboral site of the animal. This results in a sea anemone with two heads but no *physa*. The animal then divides in its midsection resulting in two individuals that will regenerate the missing aboral portions (Darling et al., 2005). *N. vectensis* is dioecious (Reitzel et al., 2007), gametes develop in the mesenteries and the fertilization is external, the oocytes are released in bundles of hundreds embedded into a gelatinous matrix. After 2-3 days, free-swimming, ciliated planula larvae leave the oocytes mass and, after 5-10 days, settle and develop into primary polyps that possess four tentacles and begin to predate. After two months, they possess their full set of 16 tentacles and after 3-6 months reach sexual maturity (Hand & Uhlinger, 1992, 1994) (**Figure 4**).

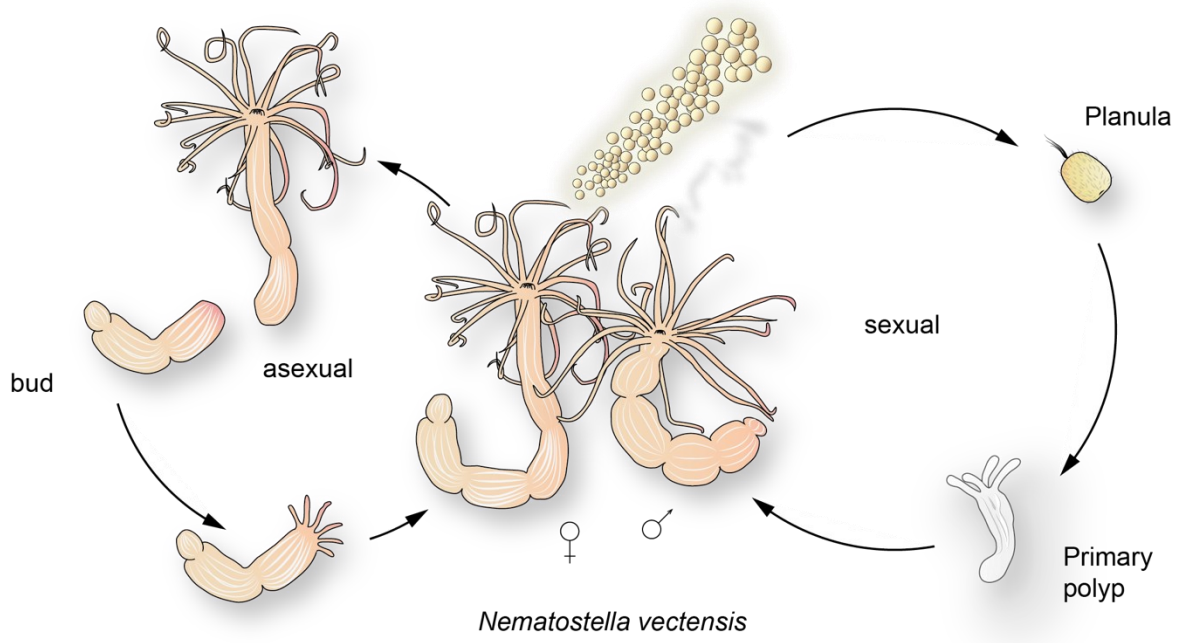

Figure 4. Life cycle of *N. vectensis*, with both sexual and asexual reproduction. From (Kelava et al., 2015).

N. vectensis is a sedentary predator that resides exclusively in estuaries and brackish water environments, where it lives borrowed in sediments (Hand & Uhlinger, 1992, 1994). It is a widespread species that has been found in both Pacific and Atlantic coasts of the US and of the UK (**Figure 5**). In their natural habitats, wild populations of *N. vectensis* experience high variations of salinity, temperature and pollutants (Darling et al., 2004, 2005; Pearson et al., 2002; Reitzel, Chu, et al., 2013; Stefanik et al., 2013). This species is considered to have overall pretty limited dispersal abilities (Darling et al., 2004), gene flow between subpopulations is easily restricted by physical barriers, resulting in conspicuous genetic structuring of the metapopulation (Darling et al., 2004; Reitzel et al., 2010). Completely or largely clonal populations exist all through the distribution range of *N. vectensis* (Darling et al., 2004; Pearson et al., 2002; Reitzel, Darling, et al., 2008), however microsatellite and SNP markers indicated an extensive intraspecific genetic diversity and genetic structuring between populations (Darling et al., 2009; Reitzel, Herrera, et al., 2013). In 2013, Reitzel and colleagues (Reitzel, Chu, et al., 2013) showed that *N. vectensis* adults and developmental stages, have pretty high tolerance thresholds to high temperature but also that this species is living very close to its physiological limit in the field.

Furthermore, that study showed that temperature has a significant negative effect on both growth and regeneration rates, with a significant negative relationship with animals' latitude of origin. Despite that, individuals from higher latitudes did not exhibit higher growth rates at cooler temperatures. These results showed evidence for local adaptation to higher temperatures in populations living at lower latitudes that would be physiologically compromised by future warming. In a study from Mortzfeld et al. (Mortzfeld et al., 2016) was shown that *N. vectensis* individuals originated from different wild populations harbor distinct associated microbiota following a north-south gradient and that temperature is the environmental factor that mainly drives these differences.

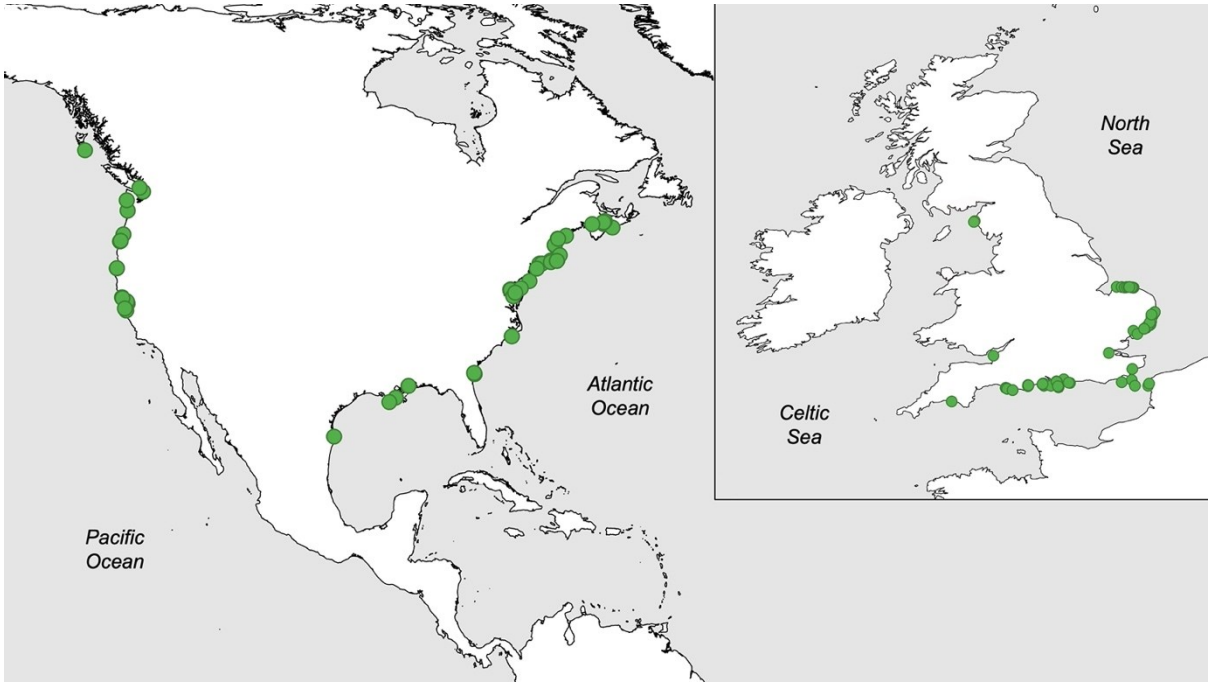

Figure 5. Current distribution area of *N. vectensis* showing the observed occurrences (green dots) available on the GBIF database (updated to March 2023).

No eukaryotic symbionts are known for this species. In addition to these eco-physiological characteristics, *N. vectensis* is more amenable to genetic approaches than the related coral species (Ikmi et al., 2014; Layden et al., 2013). Literature about this animal is extensive and continuously evolving. An annotated genome (N. H. Putnam et al., 2007), a molecular toolkit, including protocols for gene suppression and transgenesis (Nakanishi & Martindale, 2018), sequencing of transcriptomes (A. Kimura et al., 2009; Oren et al., 2015; Reitzel, Sullivan, et al., 2008; Tulin et al., 2013)

and data on genome methylation (Ying et al., 2022; Zemach et al., 2010), histone modifications (Schwaiger et al., 2014) and miRNAs (Y. Moran et al., 2014) are available. Under lab conditions, all the developmental stages are procurable on a weekly basis and spawning is induced by a shift in temperature and exposure to light (Fritzenwanker & Technau, 2002). *N. vectensis* can be easily cultured in high numbers and clonally propagated to eliminate genetic confounding effects (Hand & Uhlinger, 1992). Altogether these characteristics make this sea anemone a perfect model organism to investigate the mechanisms of acclimation to diverse environmental stresses and of microbes-host interactions (Fraune et al., 2016).

An intriguing possibility is that *N. vectensis* may be employed as a good proxy to investigate fundamental molecular programs in other cnidarians. This way, hypotheses could be quickly tested in this developed model so that resources can be mobilized most efficiently in hard-to-study cnidarian species. Additionally, *N. vectensis* can be used as a cnidarian model for environmental stress tolerance and adaptation. This could broaden our understanding of how imperiled cnidarians are likely to cope with ongoing environmental change. Plus, understanding the underlying mechanisms responsible for environmental plasticity in *N. vectensis* could potentially be exploited in the conservation of other species.

AIM OF THE STUDY

Thanks to the several pioneer studies mentioned above, we had reason to hypothesize that *N. vectensis* associated microbial community is not randomly assembled but actively selected by the host and that it might play specific roles in the whole metaorganism homeostasis maintenance and even adaptation to changing conditions. Due to the increasing attention that *N. vectensis* has gained in the last years as model organism for eco-evo-devo studies, it is needed to extend the knowledge of this animal in the context of the upcoming global climate changes. The purpose of this doctoral research was to broaden and deepen the knowledge about the host-microbiota interactions within the holobiont *N. vectensis.* We aimed at investigating the different aspects of these relationships at different levels *i.e.,* among animals belonging to different natural populations, among genetically identical or biogeographically diverse animals subjected to different environmental conditions, and among different life

stages. A focus on the impact of long-term lab culturing has been also included. Specifically, we addressed the following points: i) detect which abiotic and/or biotic factors shape the associated bacterial community composition; ii) assess if the associated bacteria contribute to host acclimation to changing external conditions; iii) shed light on symbionts transmission modalities between generations and with the environment. To address these points, we took advantage of all the main characteristics of *N. vectensis* as a model organism: i) its natural populations biogeographical structuring; ii) its plasticity under changing environmental conditions; iii) its fast clonal and sexual reproduction modalities; iv) the availability of well assessed molecular tools and reference databases.

The results of this comprehensive work confirmed that *N. vectensis* microbiota has a specific composition in relation to developmental stage, sex, individual genetics, biogeography, season and culture temperature and that it plays a pivotal role in sustain the holobiont homeostasis under rapidly changing conditions.

GENOTYPE-ENVIRONMENT INTERACTIONS DETERMINE MICROBIOTA PLASTICITY IN *NEMATOSTELLA VECTENSIS*

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Abstract

Most multicellular organisms harbor microbial colonizers that provide various benefits to their hosts. Although these microbial communities may be host species- or even genotype-specific, the associated bacterial communities can respond plastically to environmental changes. In this study, we estimated the relative contribution of environment and host genotype to bacterial community composition in *Nematostella vectensis*, an estuarine cnidarian. We sampled *N. vectensis* polyps from five different populations along a north-south gradient on the Atlantic coast of the United States and Canada. In addition, we sampled three populations at three different times of the year. While half of the polyps were immediately analyzed for their bacterial composition by 16S rRNA gene sequencing, the remaining polyps were cultured under laboratory conditions for one month. Bacterial community comparison analyses revealed that laboratory maintenance reduced bacterial diversity by fourfold but maintained a population-specific bacterial colonization. Interestingly, the differences between bacterial communities correlated strongly with seasonal variations, especially with ambient water temperature. To decipher the contribution of both ambient temperature and host genotype to bacterial colonization, we generated twelve clonal lines from six different populations in order to maintain each genotype at three different temperatures for three months. The bacterial community composition of the same *N. vectensis* clone differed greatly between the three different temperatures, highlighting the contribution of ambient temperature to bacterial community composition. To a lesser extent, bacterial community composition varied between different genotypes under identical conditions, indicating the influence of host genotype. In addition, we identified a significant genotype x environment interaction determining microbiota plasticity in *N. vectensis*. From our results we can conclude that *N. vectensis*-associated bacterial communities respond plastically to changes in ambient temperature, with the association of different bacterial taxa depending in part on the host genotype. Future research will reveal how this genotype-specific microbiota plasticity affects the ability to cope with changing environmental conditions.

Introduction

Most multicellular organisms live in association with microbial symbionts (McFall-Ngai et al., 2013; Zilber-Rosenberg & Rosenberg, 2008). It has been widely demonstrated that these symbionts provide various benefits for the survival and persistence of their hosts (Peixoto et al., 2017; Reshef et al., 2006; Rosado et al., 2019). The quality and quantity of associated microbial species is characteristic for host species (Baker, 2003; Fraune & Bosch, 2007; Kvennefors et al., 2010), genotype (Cahana & Iraqi, 2020; Glasl et al., 2019), biogeography (Linnenbrink et al., 2013; Mortzfeld et al., 2016; Terraneo et al., 2019), life stage (Baldassarre et al., 2021; Damjanovic, Menéndez, Blackall, & van Oppen, 2020; Domin et al., 2018; Vijayan et al., 2019), diet (David et al., 2014; Leeming et al., 2019; Zarrinpar et al., 2014) and environmental conditions (Baldassarre et al., 2022; Mortzfeld et al., 2016; Sehnal et al., 2021; Terraneo et al., 2019). Starting from these evidences, many studies demonstrated that the host plays an active role in shaping its symbiont microbiota (Augustin et al., 2017; Franzenburg et al., 2012; Fraune & Bosch, 2007; Groussin et al., 2017; S. T. M. Lee et al., 2016). In addition to the effects of the host and the environment, the interaction between these two factors is also discussed as a potential factor influencing the plasticity of the microbiota (Oyserman et al., 2021).

N. vectensis is a small, burrowing estuarine sea anemone found in tidally restricted salt marsh pools. The distribution of this species extends over the Atlantic and Pacific coasts of North America, and the southeast coast of England (Reitzel, Darling, et al., 2008) and its range encompasses large latitudinal variation in temperature and salinity (Sheader et al., 1997). *N. vectensis*' wide environmental tolerance and broad geographic distribution (Hand & Uhlinger, 1992; Reitzel, Darling, et al., 2008), combined with the availability of a genome sequence (N. H. Putnam et al., 2007) make it an exceptional organism for exploring adaptations to variable environments. *N. vectensis* has separated sexes and it is able to reproduce both sexually through external fertilization (Darling et al., 2005; Hand & Uhlinger, 1992; Reitzel et al., 2007) and asexually through transverse fission (Reitzel, Darling, et al., 2008). Although a free-swimming larval stage is present, this species is considered to have overall pretty limited dispersal abilities (Darling et al., 2004). Seasonal population fluctuations in density may lead to frequent bottlenecks, and when gene flow between subpopulations is restricted by physical barriers, such fluctuations could result in

conspicuous genetic structuring between locations over short geographic distances.(Darling et al., 2004; Reitzel et al., 2010). Completely or largely clonal populations exist all through the distribution range of *N. vectensis* (Darling et al., 2004; Pearson et al., 2002; Reitzel, Darling, et al., 2008), however microsatellite and SNP markers indicated an extensive intraspecific genetic diversity and genetic structuring between populations in their native range along the Atlantic coast of North America (Darling et al., 2009; Reitzel, Herrera, et al., 2013).

Within a single estuary, *N. vectensis* occupies tidal streams that flush with each tide or, isolated still-water high-marsh pools, that can differ substantially in a set of ecological variables including temperature and salinity (Friedman et al., 2018; Smith & Able, 1994). Previous works showed that different *N. vectensis* genotypes from same natural pools within a single estuary, have significantly different tolerances to oxidative stress (Friedman et al., 2018) and that individuals from different field populations respond differently to same thermal conditions during lab culturing (Reitzel, Chu, et al., 2013).

An initial categorization of the *N. vectensis* microbiota has shown that individuals from different field pools of the North American Atlantic coast have significantly different microbiota and that these differences follow a north-south gradient (Mortzfeld et al., 2016). The different ecological conditions that distinguish these pools from each other and the genetic structuring of *N. vectensis* populations led us to hypothesize that the microbiota is a subject to local selection. In particular, locally adapted host genotypes may associate with symbionts that provide advantages at the specific ecological conditions of each native pool. Recently, we have shown that genetically identical animals differentiate their microbiome composition in response to a change in environmental temperature. By transplanting the adapted microbiome onto non-adapted animals, we demonstrated that the observed microbiome plasticity leads to increased tolerance of the animals to thermal stress (Baldassarre et al., 2022). However, the influence of the host genotype on the plasticity of the microbiome and thus on the ability to cope with changing environmental conditions remained unclear.

In this study we analyzed the microbiota composition of polyps from different populations directly after sampling and after one month of laboratory maintenance. We first investigated which factors among ambient temperature, salinity, season and geographic location, contribute to microbiota differentiation. The results of these analyses show that the composition of the microbiota changes with both season and

geographic location, and that these differences persist under laboratory conditions. Consistent with previous laboratory observations (Mortzfeld et al., 2016), our field data confirmed that temperature, over salinity, is correlating the most with differences in bacterial community compositions. Starting from these evidences, we investigated the influence of ambient temperature on the microbiota plasticity of twelve individual genotypes derived from six different populations. We found that after three months of laboratory culture, temperature was the factor most driving microbiota differentiation, although differences according to genotype were also detectable. In addition, we demonstrated that microbiota plasticity in relation to temperature is genotype-specific, suggesting that microbiota plasticity is also influenced by interactions between genotype and temperature.

With this study, we have taken an important step toward understanding the contribution of both local environmental conditions and host genotype in shaping the microbiota. Furthermore, we have shown that although microbial community dynamics are plastic, each genotype is associated with a microbiota that exhibits genotype-specific flexibility. These results suggest that local populations of the same species may have different abilities to adapt to environmental changes through microbiota-mediated plasticity.

Materials and methods

Animal sampling and culture

All experiments were carried out with polyps of *N. vectensis* (Stephenson 1935). Adult animals were collected from field populations of Nova Scotia (10/03/2016), Maine (11/03/2016, 02/06/2016, 11/09/2016), New Hampshire (11/03/2016, 02/06/2016, 11/09/2016), Massachusetts (12/03/2016, 03/06/2016, 13/09/2016), Maryland (long-term lab culture) and North Carolina (16/03/2016) by sieving them from loose sediments. Environmental parameters (air temperature, water temperature and salinity) were also recorded at the moment of sampling and used as metadata for further analysis (see **Table S1** for details). Half of the animals from March sampling were kept for one month in the laboratory, under constant, artificial conditions, at 20 °C, without substrate or light, in *N. vectensis* Medium (NM), which was adjusted to 16ppt salinity with Red Sea Salt and Millipore H_2O (according to (Hand & Uhlinger,

1992). Polyps were fed two times a week with first instar nauplius larvae of *Artemia salina* as prey (Ocean Nutrition Micro *Artemia* Cysts 430 - 500 gr, Coralsands, Wiesbaden, Germany) and washed once a week with media pre-incubated at 20 °C.

Animal acclimation

Independently from the sampling effort described above, individually sampled polyps from six wild populations were asexually propagated for more than one year under laboratory conditions. After that, two strains from each original population were selected for the following experiment. Three polyps (three replicates) for each of the twelve strains selected were placed separately into 6-well plates and let acclimate for three months at each of the three different acclimation temperatures (15, 20 and 25 °C). After three months, the polyps were collected, frozen in liquid N and stored at -80 °C before DNA extraction and 16S sequencing.

DNA extraction

The specimens from the field were preserved in RNAlater until DNA extraction. For the samples from the field and after one month of lab culture and for negative controls, gDNA was extracted with the AllPrep DNA/RNA Mini Kit (Qiagen), as described in the manufacturer's protocol. The animals from the experiment were washed two times with 2 ml autoclaved MQ, instantly frozen in liquid N without liquid and stored at −80 °C until extraction. The gDNA was extracted from whole animals plus a negative control with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), as described in the manufacturer's protocol. Elution was done in 50 µl and the eluate was stored at −80 °C until sequencing. DNA concentration was measured by gel electrophoresis (5 µl sample on 1.2% agarose) and by spectrophotometry through Nanodrop 3300 (Thermo Fisher Scientific).

16S rRNA sequencing

For each sample the hypervariable regions V1 and V2 of bacterial 16S rRNA genes were amplified. The forward primer (5´-**AATGATACGGCGACCACCGAGATCTACAC** XXXXXXXX TATGGTAATTGT AGAGTTTGATCCTGGCTCAG-3´) and reverse primer (5´-

CAAGCAGAAGACGGCATACGAGAT XXXXXXXX AGTCAGTCAGCC TGCTGCCTCCCGTAGGAGT -3´) contained the Illumina Adaptor (in bold) p5 (forward) and p7 (reverse). Both primers contain a unique 8 base index (index; designated as XXXXXXXX) to tag each PCR product. For the PCR, 100 ng of template DNA (measured with Qubit) were added to 25 µl PCR reactions, which were performed using Phusion Hot Start II DNA Polymerase (Finnzymes, Espoo, Finland). All dilutions were carried out using certified DNA-free PCR water (JT Baker). PCRs were conducted with the following cycling conditions (98 °C—30 s, 30 × [98 °C—9s, 55 °C—60s, 72 °C—90s], 72 °C—10 min) and checked on a 1.5% agarose gel. The concentration of the amplicons was estimated using a Gel Doc XR+ System coupled with Image Lab Software (BioRad, Hercules, CA USA) with 3 µl of O'GeneRuler 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as the internal standard for band intensity measurement. The samples of individual gels were pooled into approximately equimolar subpools as indicated by band intensity and measured with the Qubit dsDNA br Assay Kit (Life Technologies GmbH, Darmstadt, Germany). Subpools were mixed in an equimolar fashion and stored at -20 °C until sequencing. Sequencing was performed on the Illumina MiSeq platform with v3 chemistry (Rausch et al., 2016). The raw data are deposited at the Sequence Read Archive (SRA) and available under the project PRJNA757926.

Analyses of bacterial communities

The 16S rRNA gene amplicon sequence analysis was conducted through the Qiime2 2022.8 package (Bolyen et al., 2019, p. 2). Adapters trimming and sequences quality filtering was performed through Dada2 (Callahan et al., 2016). Sequences with at least 100% identity were grouped into ASVs and clustered against the Silva 138 reference sequence database. Samples with less than 5000 sequences were also removed from the dataset, being considered as outliers. For the successive analysis the number of ASVs per sample was normalized to the lowest number of reads after filtering.

Alpha-diversity represents the total number of different ASVs observed in each sample. Beta diversity matrices were generated through Qiime2 according with the different beta-diversity metrics available (Bray-Curtis, Jaccard, Weighted-Unifrac and Unweighted-Unifrac). Statistical values of clustering were calculated using the nonparametric comparing categories methods PERMANOVA and Anosim. A Mantel

test was applied to infer correlation between the different beta-diversity and environmental parameters distance matrices. The multifactorial PERMANOVA was performed through Primer 7.0.21 (https://www.primer-e.com), by testing the impact of temperature and genotype on the microbiota beta-diversity as fixed factors, since all categories of our experiment were included in the test. In order to test the different impacts between pairs of genotypes originated from the same geographic location, the genotype was nested within the location applied as random factor.

Statistical tests were performed through JASP v0.16.4 (https://jasp-stats.org). Data were subjected to descriptive analysis, and normality and variance homogeneity tests as described herein. For univariate analyses, statistical differences were tested through non-parametric Mann-Whitney U-test; for multivariate analyses, statistical significance was tested through non-parametric Kruskal-Wallis test followed by Dunn's post-hoc comparisons.

Bacterial ASVs specifically associated with each genotype and each temperature were identified through LEfSe (http://huttenhower.sph.harvard.edu/galaxy) (Segata et al., 2011). LEfSe uses the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance, with respect to the biological conditions of interest; subsequently LEfSe uses Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature. Assuming that different genotypes from the same location may naturally share a number of symbionts, we only performed pairwise comparisons between genotypes from different locations. In addition to that, presence-absence calculations were performed directly on the ASV tables in order to detect bacterial ASVs that are unique for a specific genotype or AT.

Results

Laboratory maintenance results in loss of bacterial diversity associated with *N. vectensis* polyps

Genomic DNA samples from 168 *N. vectensis* polyps were submitted for 16S rRNA gene sequencing. While 53 samples were collected from five different populations (Nova Scotia, Maine, New Hampshire, Massachusetts and North Carolina) in March 2016, the sampling in Maine, New Hampshire and Massachusetts was repeated also in June and September (31 and 34 samples respectively). In addition, we maintained 50 polyps sampled in March, for one month under laboratory conditions before we extracted gDNA. Sequencing was successful for 156 samples. A total of 25.737 different ASVs were detected, with 5.208 to 106.793 reads per sample.

Maintaining *N. vectensis* polyps for one month under laboratory conditions resulted in a major shift in the associated bacterial communities compared to the bacterial communities of polyps directly sampled from the field (**Figure 1A** and **Table 1**). The bacterial variability between polyps significantly decreases during one month of laboratory culturing (**Figure 1B and S1**) and the alpha-diversity decreases to around one quarter of that observed in field sampled *N. vectensis* polyps (**Figure 1C**).

The loss of bacterial diversity in laboratory-maintained polyps became also evident by comparing the major bacterial groups (**Figure 1D**). While Cyanobacteria, Campilobacteria and Desulfobacteria disappeared and Bacteroidota decreased in relative abundance in laboratory-maintained animals, Gammaproteobacteria, Firmicutes and Spirochaetota increased in relative abundance (**Figure 1D**).

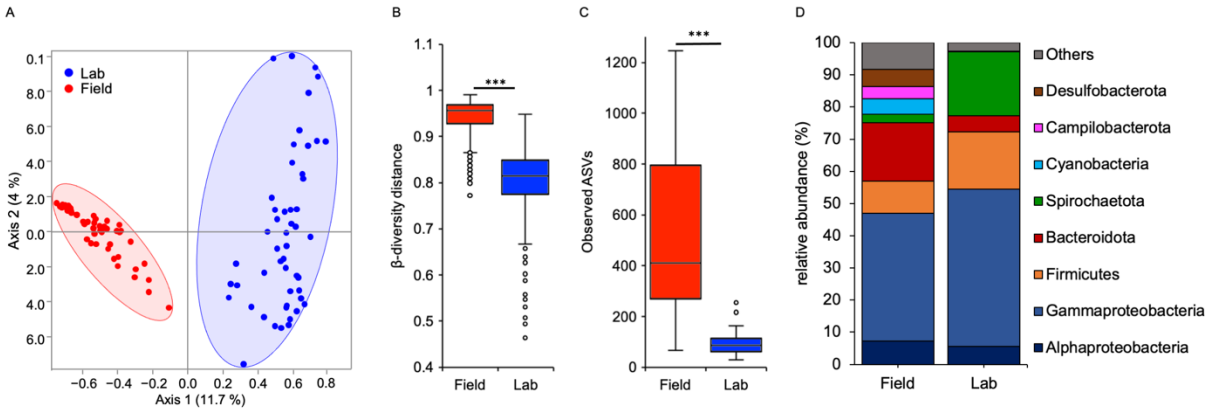

Figure 5. Laboratory maintenance reduced bacterial diversity associated with *N. vectensis* polyps. (A) PCoA (based on Jaccard metric, sampling depth = 5000) illustrating similarity of bacterial communities based on sample source; (B) beta-diversity distance box-plots of the field and lab samples; (C) alpha-diversity comparisons between field and lab samples (max rarefaction depth = 5000, num. steps = 10). Differences in B and C were tested through Mann-Whitney U-test (*** = p ≤ 0.001); (D) relative abundance of main bacterial groups among the two different samples sources.

Table 1. Statistical analysis determining the influence of animal laboratory maintenance on bacterial colonization. Statistical analyses were performed (methods PERMANOVA and ANOSIM, number of permutations = 999) on each of the pairwise comparison distance matrices generated.

		PERMANOVA		Anosim	
	beta-diversity metric	***pseudo-F***	***p-value***	***R***	***p-value***
Source	Bray-Curtis	13.129	0.001	0.488	0.001
	Jaccard	12.580	0.001	0.803	0.001
	Weighted-Unifrac	28.804	0.001	0.584	0.001
	Unweighted-Unifrac	26.693	0.001	0.950	0.001

To determine whether bacterial communities from polyps collected from different locations reveal a biogeographic signal, and to test whether this signal is preserved in polyps maintained in the laboratory, we analyzed the two data sets, field and laboratory samples, separately.

Microbial diversity in the field correlates with host biogeography and environmental factors

Analyzing the bacterial communities associated with *N. vectensis* polyps sampled in the field in March 2016, principal coordinates analysis (PCoA) revealed a clear clustering of the associated bacterial community by provenance location (**Figure 2A, B, Table 2**). Based on the different beta diversity measures, geographic location explained between 56% and 83% of the bacterial variability (**Table 2**). The beta-diversity distance between samples within the same location was significantly lower than that between the different locations, stressing the clustering of the samples sharing the same provenance (**Figure 2C**).

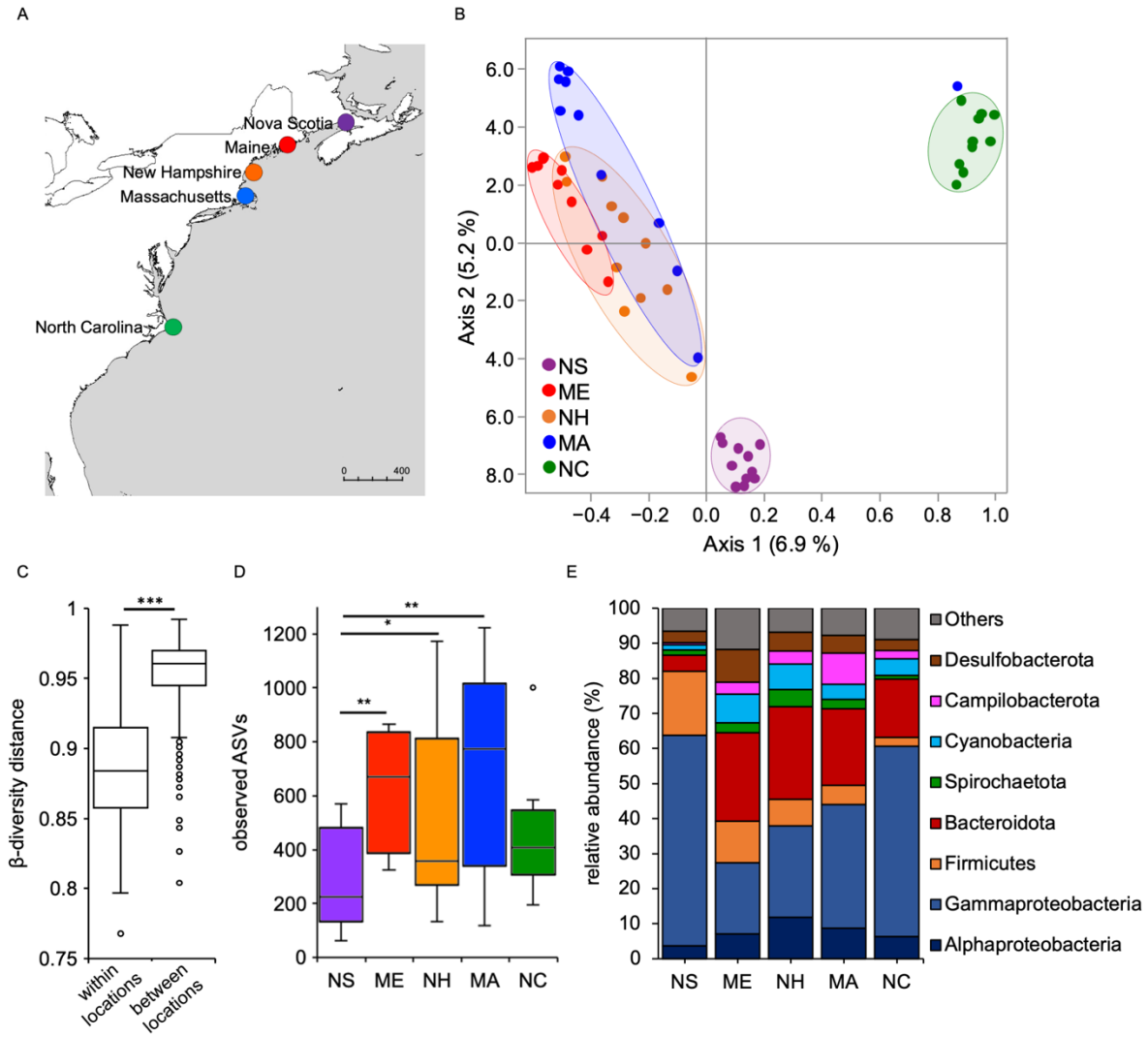

Figure 6. Natural *N. vectensis* populations are associated with specific microbiota. (A) Sampling sites map. The base layer was obtained at https://www.diva-gis.org/Data. (B) PCoA (based on Jaccard metric, sampling depth = 5000) illustrating similarity of bacterial communities based on geographic

location of the March-field samples; (C) beta-diversity distance box-plots within and between geographic locations, differences were tested through Mann-Whitney U-test (*** = p ≤ 0.001); (D) alpha-diversity comparisons between geographic locations (max rarefaction depth = 5000, num. steps = 10), differences were tested through Kruskal-Wallis test followed by Dunn's post-hoc comparisons (H = 12.63, * = p ≤ 0.05, ** = p ≤ 0.01); (E) relative abundance of main bacterial groups among different geographic locations. NS (Nova Scotia), ME (Maine), NH (New Hampshire), MA (Massachusetts), NC (North Carolina).

Table 2. Statistical analysis determining the influence of geographic location on bacterial colonization in March-field samples. Statistical analyses were performed (methods PERMANOVA and ANOSIM and) on each of the pairwise comparison distance matrices generated (Number of permutations = 999).

		PERMANOVA		**Anosim**	
	beta-diversity metric	***pseudo-F***	***p-value***	***R***	***p-value***
Geographic location	Bray-Curtis	6.549	0.001	0.800	0.001
	Jaccard	2.684	0.001	0.833	0.001
	Weighted-Unifrac	7.766	0.001	0.559	0.001
	Unweighted-Unifrac	2.831	0.001	0.583	0.001

We next investigated the influence of geographic distance, water temperature and water salinity on a continuous scale by applying Mantel tests to each of the five measures of beta-diversity (**Table 3**). Mantel tests revealed that geographic distance is the main factor impacting beta-diversity, explaining approximately 25–73% of the variation (**Table 3**). While both environmental factors, temperature and salinity also correlated significantly with bacterial diversity, water temperature explained the highest proportion (**Table 3**).

Table 3. Statistical analysis determining the influence of geographic distance, field temperature and salinity on bacterial colonization. Mantel tests were performed between the three different parameters distance matrices and the beta-diversity matrices generated. (Number of permutations = 999).

		Mantel test	
parameter	**beta-diversity metric**	**Mantel *r***	**Mantel *P***
Geographic distance	Bray-Curtis	0.594	0.001
	Jaccard	0.732	0.001
	Weighted-Unifrac	0.253	0.001
	Unweighted-Unifrac	0.400	0.001
Temperature	Bray-Curtis	0.568	0.001
	Jaccard	0.630	0.001
	Weighted-Unifrac	0.289	0.001
	Unweighted-Unifrac	0.400	0.001
Salinity	Bray-Curtis	0.235	0.001
	Jaccard	0.197	0.001
	Weighted-Unifrac	0.258	0.001
	Unweighted-Unifrac	0.155	0.006

In addition, alpha-diversity showed also a biogeographic signal. Polyps from the extreme northern and southern locations (Nova Scotia and North Carolina) had lower bacterial alpha-diversity than polyps from central locations (**Figure 2D**). By looking at the principal bacterial groups in the field samples, a north-south pattern was evident regarding the Gammaproteobacteria that increased in relative abundance moving from Maine through North Carolina, while Firmicutes and Desulfobacteria decreased in abundance moving in the same direction. The samples from Nova Scotia showed a different trend, with the Gammaproteobacteria and Firmicutes reaching the highest overall abundances while all the other groups the lowest (**Figure 2E**).

For the locations in which the samplings have been repeated at three different seasonal time points (Maine, New Hampshire and Massachusetts), we investigated the differences in the microbiota composition according to sampling month (March, June and September). A clustering of the samples with sampling time point was significant (**Figure 3A**), contributing up to 40% of the total difference (**Table 4**).

Interestingly, the samples from June clustered in between those from March and September (**Figure 3A**), and showed a, although not significant, higher alpha-diversity than the other two sampling time points, suggesting a gradual shift of associated bacteria along seasons (**Figure 3B**). The Firmicutes increased in abundance moving from March to September in all the three locations (Maine, New Hampshire and Massachusetts). Overall, the Gammaproteobacteria and Bacteroidota were more abundant in March samples, while Spirochaetota and Cyanobacteria were more abundant and Gammaproteobacteria less abundant in the samples from June, respectively (**Figure 3C**).

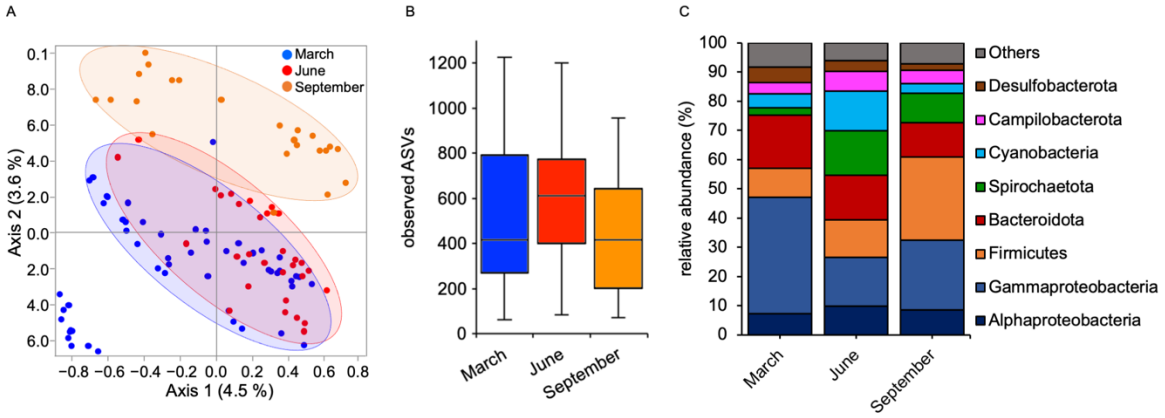

Figure 7. Natural microbiota in *N. vectensis* vary according to season. (A) PCoA (based on Jaccard metric, sampling depth = 5000) illustrating similarity of bacterial communities based on sampling month; (B) alpha-diversity comparisons between sampling months (max rarefaction depth = 5000, num. steps = 10), differences were tested through Kruskal-Wallis test (not significant); (C) relative abundance of main bacterial groups among different sampling months.

Table 4. Statistical analysis determining the influence of season on bacterial colonization. Statistical analyses were performed (methods PERMANOVA and ANOSIM, number of permutations = 999) on each of the pairwise comparison distance matrices generated.

		PERMANOVA		Anosim	
	beta-diversity metric	***pseudo-F***	***p-value***	***R***	***p-value***
Season	Bray-Curtis	7.817	0.001	0.295	0.001
	Jaccard	3.030	0.001	0.228	0.001
	Weighted-Unifrac	11.493	0.001	0.405	0.001
	Unweighted-Unifrac	3.827	0.001	0.207	0.001

N. vectensis polyps cultured in the laboratory maintain population-specific microbiota

To test whether the biogeographic signal of the bacterial communities associated with polyps is maintained under laboratory conditions, we analyzed the laboratory samples separately (**Figure 4**). A clear clustering of the samples according with the provenance location was still present and become even more evident after one month under laboratory conditions (**Figure 4A** and **B**). All the ANOVA comparisons performed and the Mantel tests were highly significant ($p < 0.001$) (**Table 5**), and showed that the provenance geographic location explained between 55 and 74% of the beta-diversity difference for the lab samples, proving that the population-specific bacterial fingerprints were maintained (**Table 5**). The beta-diversity distance between samples originating from the same location was significantly lower than that between the different locations, stressing the clustering of the samples sharing the same provenance (**Figure 4B**). For the lab samples, the alpha-diversity was also the highest in the samples from the intermediate locations (**Figure 4C**). Animals from the extreme locations (Nova Scotia and North Carolina) where colonized by the highest abundances of Firmicutes and Gammaproteobacteria respectively, while those from the central latitudes were associated mainly with greater abundances of Bacteroidota and Spirochaetota (**Figure 4D**).

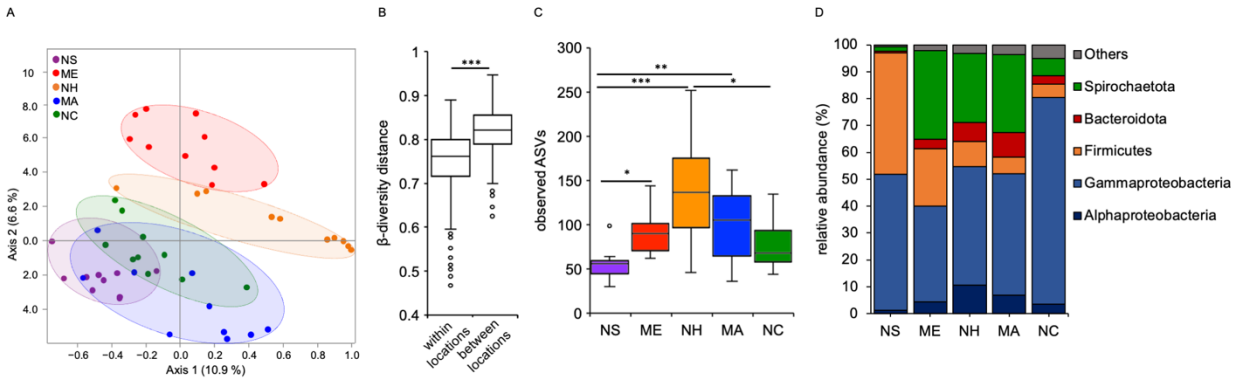

Figure 8. Population-specific microbiota are maintained under laboratory conditions. (A) PCoA (based on Jaccard metric, sampling depth = 5000) illustrating similarity of bacterial communities based on geographic population; (B) beta-diversity distance box-plots of the lab samples within and between geographic locations, differences were tested through Mann-Whitney U-test (*** = p ≤ 0.001); (C) alpha-diversity comparisons between geographic locations (max rarefaction depth = 5000, num. steps = 10); (D) relative abundance of main bacterial groups among different geographic locations. Differences were tested through Kruskal-Wallis test followed by Dunn's post-hoc comparisons (H = 18.35, * = p ≤ 0.05, ** = p ≤ 0.01, *** = p ≤ 0.001). NS (Nova Scotia), ME (Maine), NH (New Hampshire), MA (Massachusetts), NC (North Carolina).

Table 5. Statistical analysis determining the influence of geographic distance and geographic location on bacterial colonization in laboratory-maintained populations. Statistical analyses were performed (methods PERMANOVA and ANOSIM) on each of the pairwise comparison distance matrices generated according with provenance geographic location. Mantel test was performed between the geographic location distance matrix and the different beta-diversity matrices. (Number of permutations = 999).

		PERMANOVA		Anosim		Mantel test	
	beta-diversity metric	***pseudo-F***	***p-value***	***R***	***p-value***	**Mantel *r***	**Mantel *P***
Geographic location	Bray-Curtis	10.653	0.001	0.736	0.001	0.608	0.001
	Jaccard	2.986	0.001	0.604	0.001	0.352	0.001
	Weighted-Unifrac	8.902	0.001	0.551	0.001	0.433	0.001
	Unweighted-Unifrac	3.753	0.001	0.599	0.001	0.384	0.001

Under different temperatures, *N. vectensis* maintains genotype-specific microbiota

The variation of bacterial communities associated with *N. vectensis* polyps in the field correlated mostly with ambient water temperature (**Table 3**). Based on these findings, we aimed to measure experimentally the contribution of temperature and host

genotype and their interaction on the microbiota composition. We selected in total twelve genotypes originating from six different geographic locations (two genotypes/location) (**Figure 5A**). To be able to maintain each genotype at different ambient temperatures, we clonally propagated the polyps to reach at least nine clones/genotype. Subsequently, we maintained each genotype at three different temperatures (15, 20 and 25 °C, n=3) for three months (**Figure 5A**). Nine polyps out of 108 didn´t survive the treatment. Interestingly, culturing at high temperature (25 °C) resulted in higher mortality in animals from Nova Scotia, New Hampshire, and Massachusetts, while animals from Maine had the highest mortality at low temperatures (15 and 20 °C) (**Figure S2**).

After three months of culturing at different temperatures, gDNA from 99 polyps were submitted for 16S rRNA gene sequencing. A total of 985 different ASVs were detected, with the number of reads per sample ranging between a maximum of 65.402 and a minimum of 15.850. After setting the minimum number of reads/sample at 15.800, 92 samples remained for the successive analyses.

PCoA revealed that ambient temperature explained most of the detected bacterial diversity associated with the polyps (between 15% and 59% diversity explained) (**Figure 5B, Table 6)**, while no significant differences in beta-diversity distances were evident between the three different temperatures (**Figure S3A**). While principal component 1 (PC1) mostly separates samples according to the ambient temperature (**Figure 5B**), PC2 mostly explains variations within the different genotypes (**Figure 5C**). The ANOSIM results indicated that host genotype contributed between 13% and 22% to the total bacterial diversity observed (**Table 6**). The alpha-diversity slightly increased, although not significantly, from the 15 °C samples through the 25 °C ones (**Figure S3B**), no clear pattern from the host genotypes on the alpha-diversity analysis was evident (**Figure S4**).

Interestingly, comparison of the beta diversity distances of the different genotypes (**Figure 5D**) revealed that they differ significantly (Kruskal Wallis $p < 0.001$) in terms of microbiota flexibility (**Table S2**). These results suggest that each genotype is endowed with a microbiota that exhibits genotype-specific flexibility. In particular, we identified genotypes whose microbiota exhibit low flexibility (*e.g.*, MA1 and MD2), in contrast to genotypes whose microbiota exhibit high flexibility (*e.g.*, NS3 and NH3).

In order to detect genotype-specific bacterial adjustments to temperature variation, we performed a multifactorial PERMANOVA, by testing the influence of the genotypes

within each provenance location separately. The results revealed that genotype x temperature interactions significantly influenced microbial plasticity despite the possible genotype similarities within the same location (**Table 7**). Plotting the average PC2 eigenvalues of each genotype at the three different ambient temperatures **(Figure 5E)** indicated that the microbial plasticity differed between the twelve different genotypes. Interestingly, the adjustments in bacterial diversity within the twelve genotypes can be divided into two main patterns (**Figure S6A and B**). Together, these results suggest different metaorganism strategies to cope with environmental changes.

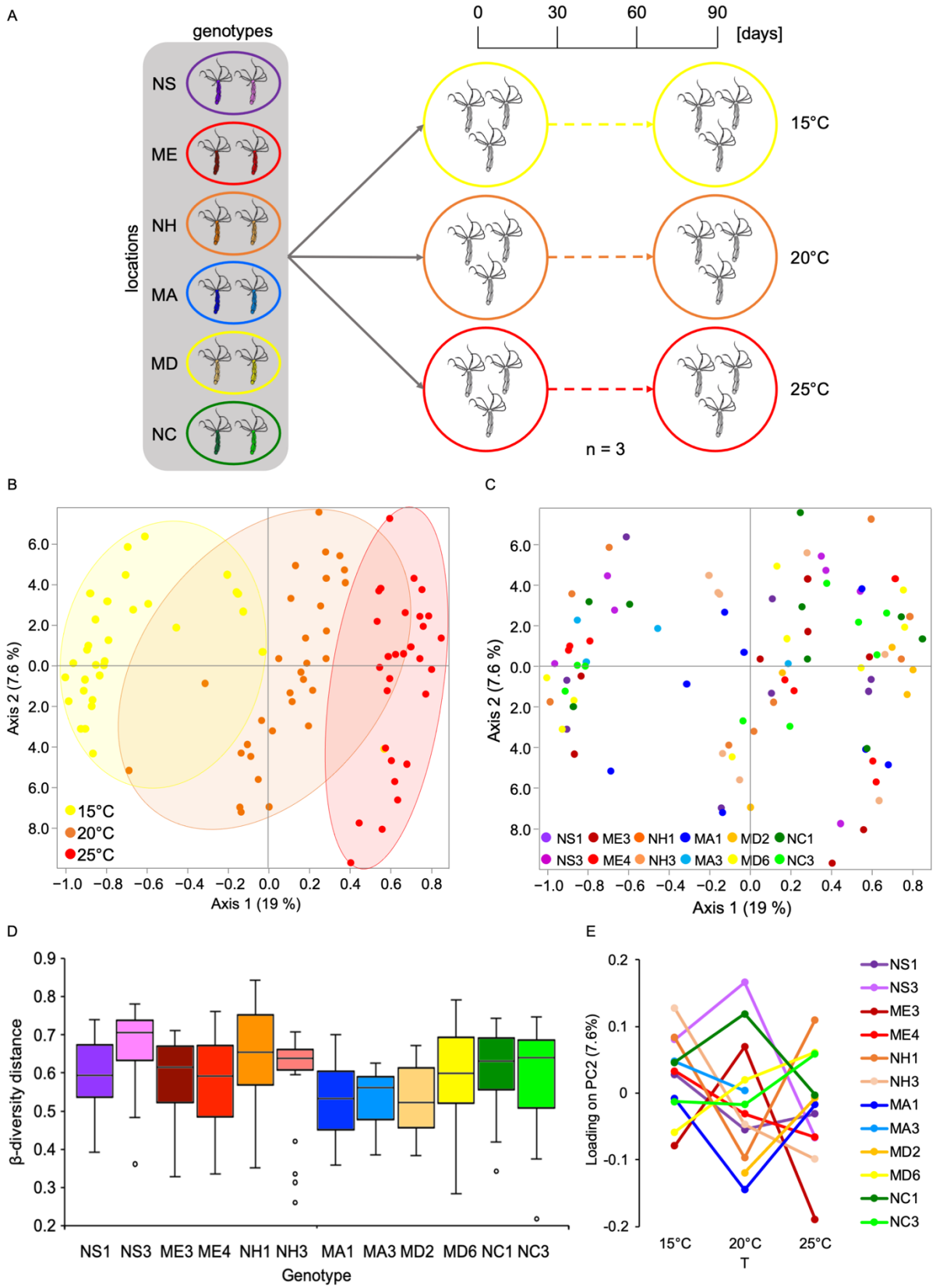

Figure 9. Influence of host genotype and temperature on bacterial colonization. (A) Experimental design, 2 genotypes for each geographic location were kept in 3 replicates at 3 different temperatures for 3 months; (B) PcoA (based on Jaccard metric, sampling depth = 15800) illustrating similarity of bacterial communities based on ambient temperature; (C) PCoA (based on Jaccard metric, sampling

depth = 15800) illustrating similarity of bacterial communities based on host genotype; (D) beta-diversity distance box-plots between different genotypes (Jaccard metric, sampling depth = 15800), differences were tested through Kruskal-Wallis test (H = 38.91, p = < 0.001); for clarity the Dunn's post-hoc comparisons are reported in Table S2. (E) Reaction norms plotting average principal component 2 eigenvalues for each of the twelve genotypes at each temperature. NS (Nova Scotia), ME (Maine), NH (New Hampshire), MA (Massachusetts), MD (Maryland), NC (North Carolina), numbers near the location abbreviations indicate the different genotypes.

Table 6. Statistical analysis determining the influence of host genotype and temperature on bacterial colonization in experimental animals. Statistical analyses were performed (methods PERMANOVA and ANOSIM, number of permutations = 999) on each of the pairwise comparison distance matrices generated.

		PERMANOVA		**Anosim**	
parameter	**beta-diversity metric**	***pseudo-F***	***p-value***	***R***	***p-value***
Temperature	Bray-Curtis	12.991	0.001	0.497	0.001
	Jaccard	11.027	0.001	0.591	0.001
	Weighted-Unifrac	5.630	0.001	0.154	0.001
	Unweighted-Unifrac	8.438	0.001	0.376	0.001
Genotype	Bray-Curtis	2.372	0.001	0.219	0.001
	Jaccard	1.773	0.001	0.132	0.001
	Weighted-Unifrac	3.041	0.001	0.225	0.001
	Unweighted-Unifrac	1.869	0.001	0.155	0.001

Table 7. Statistical analysis determining the influence of host genotype x temperature interaction on bacterial colonization in experimental animals. Multifactorial PERMANOVA test was performed on each of the beta-diversity distance matrices generated. (Number of unrestricted permutations = 9999; type I (sequential) sums of squares; temperature and genotype as fixed factors, genotype nested within location as random factor).

		PERMANOVA	
parameter	**beta-diversity metric**	***pseudo-F***	***P value***
Temperature * Genotype	Bray-Curtis	2.260	0.0001
	Jaccard	1.823	0.0001
	Weighted-Unifrac	2.342	0.0001
	Unweighted-Unifrac	1.775	0.0001
Temperature	Bray-Curtis	10.373	0.0001
	Jaccard	6.038	0.0001
	Weighted-Unifrac	4.903	0.0002
	Unweighted-Unifrac	5.453	0.0001
Genotype	Bray-Curtis	1.738	0.0011
	Jaccard	1.921	0.0001
	Weighted-Unifrac	1.620	0.0183
	Unweighted-Unifrac	1.875	0.0001

In a further step, we aimed to detect indicator taxa specifically associated with ambient temperature and genotypes **(Figure 6 and Table S3 and S4)**. Through LEfSe we were able to detect indicator ASVs that are overrepresented in each sample category in comparison with all the others. We observed that extreme ambient temperatures showed higher numbers of unique associated ASVs (**Figure 6A**). Interestingly, calculating the relative abundance of indicator ASVs (**Figure 6B and D**) revealed that around 36% and 29% of bacterial abundance at 15 °C and 25 °C respectively, were represented by temperature specific ASVs. In contrast genotype-specific ASVs represented on average 5% of the bacterial total abundance, while the two genotypes isolated from MD (the only long-term lab culture) didn't show any genotype-specific ASV (**Figure 6D**). Interestingly, genotypes isolated from the same location show similarities in terms of specific ASVs and their relative abundances, and notably NS1 and NS3 share three out of their four genotype-specific ASVs (**Figure 6C and D**).

These results suggest that genotypes from the same locations might be close relatives.

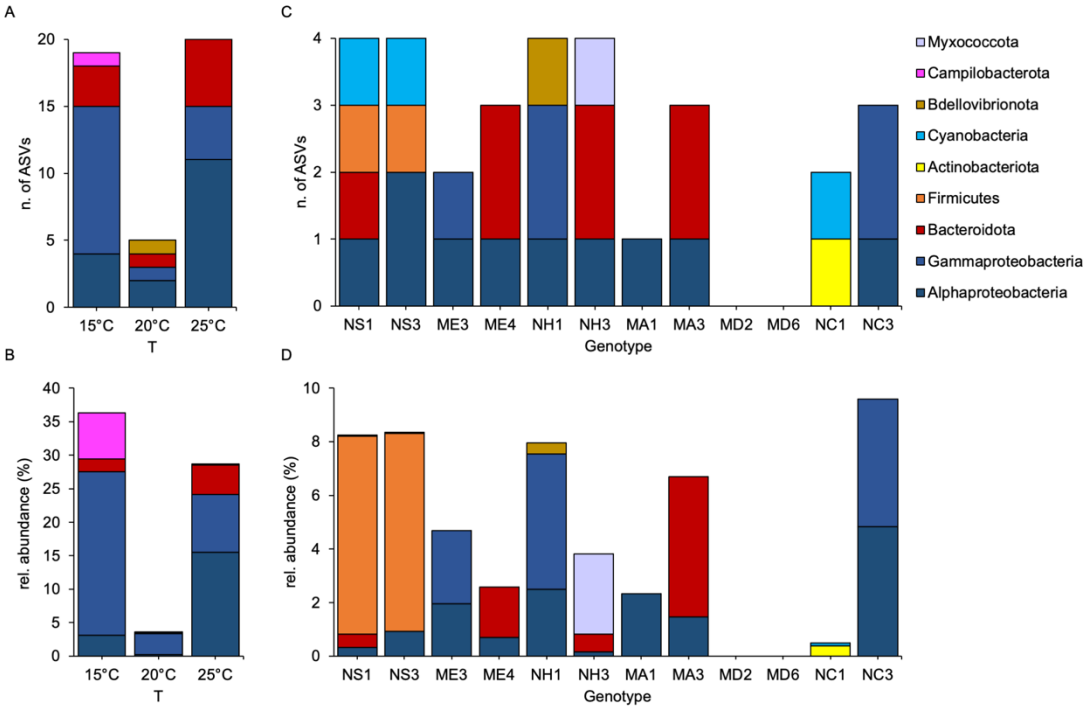

Figure 10. Bacterial ASVs representative of host genotype and acclimation temperature. Number of bacterial ASVs overrepresented at each temperature (A and B) and in each genotype (C and D) compared to the others, divided by major groups. Absolute ASV number (A and C), relative ASVs abundances on the total number of reads (B and D).

Discussion

Environmental factors can explain most but not all variability of *N. vectensis* associated microbiota

To estimate the contributions of both environmental factors and genotype to the bacterial diversity associated with *N. vectensis*, we started with a huge sampling effort to collect individuals of *N. vectensis* from multiple populations of the US Atlantic coast along a north south gradient of more than 1500 km and correlated the microbial composition data to the environmental factors temperature and salinity. In addition, we sampled individuals from three populations also in three different seasons. Our results showed that temperature and salinity, although explaining a similar percentage of the observed variability, could not explain all of the observed bacterial variation. In addition, we showed that the associated microbial community changes gradually along a temporal pattern during the year. Previous studies in corals have also shown that associated bacterial communities change depending on the season (Cai et al., 2018; Li et al., 2014; Rubio-Portillo et al., 2016; Sharp et al., 2017), *e.g.*, due to changes in dissolved oxygen concentrations and rainfall (Li et al., 2014). In addition, seasonal changes in host physiology associated with winter quiescence, may drive microbiota diversity (Sharp et al., 2017). Besides these cues, natural seasonal fluctuations in bacterial communities can also impact the availability of certain symbiotic species (La Rivière et al., 2013).

Maintenance in the laboratory reduces bacterial diversity but preserves population-specific bacterial signatures

After sampling polyps from the wild, we additionally kept individuals of *N. vectensis* from each population under constant laboratory conditions for one month and compared these samples to those sampled directly from the field in terms of microbial diversity. In accordance with what was previously found from studies on lab-mice (Bowerman et al., 2021), insects (Ibarra-Juarez et al., 2018; Morrow et al., 2015; Staubach et al., 2013) and corals (Damjanovic, Blackall, et al., 2020; Dungan et al., 2021) laboratory-reared *N. vectensis* individuals host a significantly lower bacterial diversity than in the wild. Interestingly, the homogenous lab environment did not

eliminate the original differences in bacterial colonization observed in the animals directly sampled from the field. Surprisingly, the population specific signature became even more evident in the laboratory-maintained animals. These results indicate that the bacterial diversity loss mainly affects bacteria that are not responsible for the population-specific signature. Therefore, bacteria that are lost under laboratory condition most likely are loosely associated environmental bacteria, food bacteria or might stem from taxa that are only transiently associated with the host (Ainsworth et al., 2015; Dungan et al., 2020). In future studies, the amount of bacterial sequences derived from dead bacteria or eDNA could be reduced by sequencing bacterial RNA instead of DNA. However, bacteria that are persisting during laboratory maintenance most likely represent bacteria that are functionally associated with *N. vectensis* (Baldassarre et al., 2022) and might have co-evolved with its host (Ainsworth et al., 2015; Hernandez-Agreda et al., 2017; Schmitt et al., 2012).

Genotype x environment interactions shape microbiota plasticity of *N. vectensis*

For several animal and plant species it has been observed that associated microbial community dissimilarities increase with geographical distance (C. M. Dunphy et al., 2019). Host selection, environmental filtering, microbial dispersal limitation and microbial species interactions have all been suggested as key drivers of host-microbial composition in space and time (Costello et al., 2012). Also a previous study in *N. vectensis* evidenced that individuals from different populations harbor distinct microbiota (Mortzfeld et al., 2016).

In order to disentangle the contribution of the host, the environment, and their interaction on the microbiota composition in *N. vectensis*, we selected twelve genotypes from six different field populations and kept clones of each genotype for three months under different temperatures. We found bacterial taxa that are associated with both specific genotypes and specific temperature conditions. These results suggest that both intrinsic and extrinsic factors shape the host-associated microbiota, although environmental conditions appear to have a stronger influence. In contrast to previous observations in corals (Brener-Raffalli et al., 2018; Glasl et al., 2019), where host genotype had a greater impact on microbiota composition than environmental conditions, in our study we observed that environmental conditions (in this case, temperature), tend to even the microbiota of different genotypes. Similar

results were shown in fire coral clones, where both host genotype and reef habitat contributed to bacterial community variabilities (Dubé et al., 2021). Genomic function predictions suggested that environmentally determined taxa lead to functional restructuring of the microbial metabolic network, whereas bacteria determined by host genotype are functionally redundant (Dubé et al., 2021). As previously suggested (Damjanovic et al., 2019), these observations confirm that both environmental and host factors are drivers of associated microbial community composition and that different genotype x environment combinations can create unique microhabitats suitable for different microbial species with different functions.

One mechanism by which host selection can occur is through innate immunity, e. g. the secretion of antibiotic compounds via the mucus layer that target non-beneficial or pathogenic microbes (Augustin et al., 2017; Franzenburg et al., 2012; Fraune & Bosch, 2007; Ritchie, 2006). Our results suggest that *N. vectensis* also plays an active role in shaping its symbiotic microbiota in response to environmental variability and that these mechanisms depend on genotypic differences and local adaptation.

Microbial plasticity is linked to animal adaptation

Differences in prokaryotic community composition in different environments have been documented in many other marine invertebrates and are considered to reflect local acclimation (A Hernandez-Agreda et al., 2016; Glasl et al., 2019; Goldsmith et al., 2018; van Oppen et al., 2018). We have recently shown that the restructuring of microbial communities due to temperature acclimation is an important mechanism of host plasticity and adaptation in *N. vectensis* (Baldassarre et al., 2022). The higher thermal tolerance of animals acclimated to high temperature could be transferred to non-acclimated animals through microbiota transplantation (Baldassarre et al., 2022). In our study, high temperature conditions were particularly challenging for some genotypes native to north habitats, where they experience colder climate. Whether this is the result of local adaptation of the host to colder temperatures or the symbiotic microbiota, needs to be clarified. We also observed that the bacterial species richness increases in intermediate latitudes, seasons and temperature, while it decreases at the extremes, suggesting a dynamic and continuous remodeling of the microbiota composition along environmental conditions gradients.

Evidence from reciprocal transplantation experiments in corals followed by short-term heat stress suggests also that coral-associated bacterial communities are linked to variation in host heat tolerance (Ziegler et al., 2017) and that associated bacterial community structure responds to environmental change in a host species-specific manner (Ziegler et al., 2019). Here we show that not only do different species exhibit different microbial flexibility, but genotypes can also differ in the flexibility of their microbiota.

We hypothesize that host organisms may evolve faster than on their own due to plastic changes in their microbiota. Rapidly dividing microbes are predicted to undergo adaptive evolution within weeks to months. Adaptation of the microbiota can occur via changes in absolute abundances of specific members, acquisition of novel genes, mutation and/or horizontal gene transfer (Baldassarre et al., 2021; Bang et al., 2018; Bay & Palumbi, 2015; Edwards, 2020; van Oppen et al., 2018). Here, we provide evidence for genotype-specific microbial plasticity and flexibility, leading to genotype-specific restructuring of the microbial network in response to environmental stimuli. Together these results may indicate that the genotype-specific bacterial colonization reflects local adaptation. Future studies will reveal whether lower plasticity and flexibility of the microbiome is associated with lower adaptability to changing environmental conditions and which host factors determine the plasticity and flexibility of the microbiome. In particular, genotypes adapted to highly variable environments might favor flexibility over fidelity regarding the associated microbiota composition; conversely, under more stable conditions less dynamic and more strict association might be advantageous (Voolstra & Ziegler, 2020).

MICROBIOTA MEDIATED PLASTICITY PROMOTES THERMAL ADAPTATION IN THE SEA ANEMONE *NEMATOSTELLA VECTENSIS*

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Abstract

At the current rate of climate change, it is unlikely that multicellular organisms will be able to adapt to changing environmental conditions through genetic recombination and natural selection alone. Thus, it is critical to understand alternative mechanisms that allow organisms to cope with rapid environmental changes. Here, we use the sea anemone *Nematostella vectensis*, which has evolved the capability of surviving in a wide range of temperatures and salinities, as a model to investigate the microbiota as a source of rapid adaptation. We long-term acclimate polyps of *N. vectensis* to low, medium, and high temperatures, to test the impact of microbiota-mediated plasticity on animal acclimation. Using the same animal clonal line, propagated from a single polyp, allows us to eliminate the effects of the host genotype. The higher thermal tolerance of animals acclimated to high temperature can be transferred to non-acclimated animals through microbiota transplantation. The offspring fitness is highest from F0 females acclimated to high temperature and specific members of the acclimated microbiota are transmitted to the next generation. These results indicate that microbiota plasticity can contribute to animal thermal acclimation and its transmission to the next generation may represent a rapid mechanism for thermal adaptation.

Introduction

Changes in the climate are proceeding worldwide at a rate never registered before and temperatures will rise dramatically in the coming decades. Species able to migrate could move toward new favorable areas, but those that have limited dispersal capacities or are sessile will have only two options: adaptation or extinction. Since the Modern Synthesis (Huxley, 1942), the balance between mutation and natural selection has been considered the main source of phenotypic novelty and thus of the ability of populations to adapt to new environmental conditions. However, in some organisms acclimation to environmental change can occur within one generation.

Changes in gene expression have long been considered the most important explanation for this ability (Bay & Palumbi, 2015; SR Palumbi et al., 2014), although genetics alone cannot fully explain an organism's phenotype. Unlike the genes and regulatory regions of the genome, the microbial composition can be rapidly modified by environmental cues, and may thus represent a mechanism for rapid acclimation and adaptation of individuals to a changing environment (Bang et al., 2018; Fraune et al., 2016; Kolodny & Schulenburg, 2020; Reshef et al., 2006). Recently, the microbiota-mediated transgenerational acclimatization concept was proposed (N. S. Webster & Reusch, 2017), suggesting that changes in microbiota assemblages may be passed on through generations to confer long-lasting fitness advantages to changing environments by individuals and populations.

To be able to separate host genetic contribution from the microbial contributions to thermal acclimation, we here resort to the model system *Nematostella vectensis* (Totton, 1935). *N. vectensis*, an anthozoan cnidarian, is a sedentary predator that resides exclusively in estuaries and brackish water environments, where it lives burrowed in sediments (Hand & Uhlinger, 1994). It is a widespread species that has been found in both the Pacific and Atlantic coasts of the US and of the UK. In their natural habitats, wild populations of *N. vectensis* experience high variations of salinity, temperature and pollutants (Darling et al., 2004, 2005; Hand & Uhlinger, 1992; Pearson et al., 2002; Reitzel et al., 2008; Stefanik et al., 2013). Under laboratory conditions, all the developmental stages are procurable weekly and spawning is induced by a shift in temperature and exposure to light (Fritzenwanker & Technau, 2002). *N. vectensis* can be easily cultured in high numbers (Hand & Uhlinger, 1992) and clonally propagated to eliminate genetic confounding effects. A detailed analysis

of its microbiota revealed that *N. vectensis* harbors a specific microbiota whose composition changes in response to different environmental conditions and among geographic locations (Mortzfeld et al., 2016). Recently, it was shown that female and male polyps transmit different bacterial species to the offspring and that additional symbionts are acquired from the environment during development (Baldassarre et al., 2021). Furthermore, a protocol based on antibiotic treatment was established to generate germ-free animals that allow controlled recolonization experiments to be conducted (Domin et al., 2018). Altogether, these characteristics make the sea anemone *N. vectensis* a uniquely informative marine model organism to investigate the effects of bacterial plasticity on thermal acclimation (Fraune et al., 2016).

Here, we use a clonal lineage of *N. vectensis* to characterize the physiological and microbial plasticity of the holobiont under different long-term thermal acclimation regimes while eliminating the variability due to different host genotypes. Using microbial transplantations to non-acclimated polyps, we prove the ability of acclimated microbes to confer resistance to thermal stress. We further show that polyps acclimated to high temperatures pass on higher fitness to the next generation.

Altogether, we provide strong evidence that microbiota-mediated plasticity contributes to the adaptability of *N. vectensis* to high temperature and that the transmission of acclimated microbiota represents a mechanism for rapid adaptation.

Methods

Animal culture

All experiments were carried out with polyps of *N. vectensis (*Stephenson 1935). The adult animals of the laboratory culture were F1 offspring of CH2XCH6 individuals collected from the Rhode River in Maryland, USA (Fritzenwanker & Technau, 2002; Hand & Uhlinger, 1992). They were kept under constant, artificial conditions without substrate or light in plastic boxes filled with ca. 1L. *Nematostella* Medium (NM), which was adjusted to 16‰ salinity with Red Sea Salt and Millipore H_2O. Polyps were fed 2 times a week with first instar nauplius larvae of *Artemia salina* as prey (Ocean Nutrition Micro *Artemia* Cysts 430 - 500 gr, Coralsands, Wiesbaden, Germany) and washed once a week with media pre-incubated at the relative culture temperatures. No individual animals were used for more than one experiment of this study. The animals that survived/were left from each experiment were immediately sacrificed.

Animal acclimation

A single female polyp from the standard laboratory culture conditions (16 ‰, 20 °C) was isolated and propagated via clonal reproduction. When a total of 150 new clones was reached, they were split into 15 different boxes with 10 animals each. The boxes were moved into 3 different incubators (5 boxes each) set at three different acclimation temperatures (ATs) (15, 20, and 25 °C). The animals were kept under a constant culture regime as described above. When the total of 50 polyps per box was reached, it was maintained constant by manually removing the new clones formed. Every week the number of new clones, dead, and spontaneous spawning events were recorded.

Dry weights

Ten animals from each AT were rinsed quickly in pure ethanol and placed singularly in 1.5 ml tubes, previously weighed on an analytical scale. The animals were left dry at 80 °C in a ventilated incubator for 4 hours. After complete evaporation of fluids, the animals with the tubes were weighed on the same analytical scale and the dry weight was calculated.

Generation of axenic polyps

To reduce the total bacterial load (axenic state), animals belonging to the same clonal line were treated with an antibiotic cocktail of ampicillin, neomycin, rifampicin, spectinomycin, and streptomycin (50 µg/ml each) in filtered (on 0.2 µm filter membrane), autoclaved NM (modified from (Fraune et al., 2015)). The polyps were incubated in the antibiotic cocktail for two weeks in 50 ml Falcon tubes (10 animals each). The medium and the antibiotics were changed every day and the tubes 3 times per week. After the treatment, the polyps were incubated for 1 week in sterile NM without antibiotics to let them recover before the recolonization. After the 2 weeks of antibiotic treatment, the axenic state was checked by homogenizing single polyps with an electric tissue grinder (Omni THq Homogenizer) into 1 ml sterile NM and by plating 100 µl of the homogenate on marine broth plates, successively incubated for 1 week at 20 °C. In addition, we performed a PCR with primers specific for the V1-V2 region of the bacterial 16S rRNA gene (27F and 338R). No CFUs on the plates and a weaker signal in the PCR electrophoretic gel compared with wild-type controls were considered evidence of bacterial reduction and an axenic state of the animals.

Heat stress experiment (HS)

Adult polyps for each AT were placed singularly in 6-well plates and incubated at 40 °C for 6 hours (adapted from (Reitzel et al., 2013)). The day after, the number of survivors was recorded and the mortality rate was calculated.

Bacterial transplantation

For this experiment, the protocol for conventionalized *Hydra* polyps was modified (Fraune et al., 2015). For each acclimated culture (n = 5), 6 axenic adult polyps were recolonized with the supernatant of 1 adult polyp, singularly homogenized (as described above) in 2 ml sterile NM. The solution was centrifuged for 5 seconds at 1 g to sediment the coarsest tissue debris. One ml of supernatant was added into single Falcon tubes, containing 6 axenic animals each and filled with 50 ml sterile NM. One additional animal from each acclimated culture was collected for DNA extraction and 16S sequencing (donors). The tubes with the recolonized animals were placed at 20 °C and after 24 hours, the medium was exchanged to remove tissue debris and non-

associated bacteria. One month after recolonization, 3 recolonized animals of each replicate were tested for heat stress tolerance as described above (n = 15) and 3 recolonized polyps of each replicate were sampled for DNA extraction and 16S sequencing.

Induction of spawning

Acclimated animals separated singularly in 6-well plates, were induced for sexual reproduction via light exposure for 10 h and temperature shift to 20 °C for the animals acclimated at 15 °C (Fritzenwanker & Technau, 2002). The animals acclimated at 20 and 25 °C were shifted to 25 °C. At each fertilization event, sperms from a single induced male were pipetted directly onto each oocyte pack. Fertilization was performed within 3 hours after spawning. The developing animals were then cultured for 1 month under different developmental temperatures (DTs) (15, 20 or 25 °C).

Offspring survival test

Two female polyps from each of the acclimated cultures (n=5) and one male polyp from the standard culture conditions were induced separately for spawning. After spawning, the adult polyps were removed and the oocyte packs fertilized as described above. Fertilization was confirmed by observation under a binocular of the oocytes' first cleavages. After fertilization, each oocyte pack was split with a scalpel into 3 parts that were transferred into 3 distinct Petri dishes. The 3 oocyte-pack sub-portions were placed into 3 different incubators, set at 15, 20 and 25 °C respectively, and let develop for 1 month. Right after fertilization and after 1 month of development, pictures of the oocytes and the juvenile polyps were acquired for successive counting. Ratios between the initial number of oocytes and the surviving juvenile polyps were calculated and the survival rate was estimated.

Bacteria vertical transmission test

One female polyp from each of the acclimated cultures (n = 5) and one male polyp from the standard conditions were induced separately for spawning as described above. Immediately after spawning the parental polyps were collected, frozen in liquid nitrogen and stored at -80 °C for successive DNA extraction. Oocyte packs were

fertilized, split into 3 parts each and let develop for 1 month at the 3 different DTs, as described for the offspring survival test. After 1 month of development, the juvenile polyps were collected, frozen in liquid nitrogen and stored at -80 °C. DNA was extracted from both the adults and the offspring as described herein.

DNA extraction

DNA was extracted from adult polyps starving for 3 days before sacrifice and from never-fed juveniles. The recolonized animals were not fed for the whole duration of the antibiotic treatment and the transplantation (7 weeks in total). Animals were washed two times with 2 ml autoclaved MQ, instantly frozen in liquid nitrogen without liquid, and stored at −80 °C until extraction. The gDNA was extracted from whole animals with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany), as described in the manufacturer's protocol. Elution was done in 50 µl and the eluate was stored at −80 °C until sequencing. DNA concentration was measured by gel electrophoresis (5 µl sample on 1.2 % agarose) and by spectrophotometry through Nanodrop 3300 (Thermo Fisher Scientific).

RNA extraction

Adult animals were starved for 3 days before sacrifice. Polyps were washed two times with 2 ml autoclaved MQ, instantly frozen in liquid nitrogen without liquid and stored at −80 °C until extraction. Total RNA was extracted from the body column only, with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany), as described in the manufacturer's protocol. RNA elution was done in 20 µl of RNAse-free water and the eluates were stored at −80 °C until sequencing. RNA concentration was measured through electrophoresis by loading 1 µl of each sample on 1 % agarose gel and by spectrophotometry through Nanodrop 3300 (Thermo Fisher Scientific).

16S RNA sequencing and analysis

For each DNA sample, the hypervariable regions V1 and V2 of bacterial 16S rRNA genes were amplified. The forward primer (5´-**AATGATACGGCGACCACCGAGATCTACAC** XXXXXXXX TATGGTAATTGT AGAGTTTGATCCTGGCTCAG-3´) and reverse primer (5´-

CAAGCAGAAGACGGCATACGAGAT XXXXXXXX AGTCAGTCAGCC TGCTGCCTCCCGTAGGAGT -3´) contained the Illumina Adaptor (in bold) p5 (forward) and p7 (reverse) (Fadrosh et al., 2014). Both primers contain a unique 8 base index (index; designated as XXXXXXXX) to tag each PCR product. For the PCR, 100 ng of template DNA (measured with Qubit) were added to 25 µl PCR reactions, which were performed using Phusion Hot Start II DNA Polymerase (Finnzymes, Espoo, Finland). All dilutions were carried out using certified DNA-free PCR water (JT Baker). PCRs were conducted with the following cycling conditions (98 °C—30 s, 30 × [98 °C—9s, 55 °C—60s, 72 °C—90s], 72 °C—10 min) and checked on a 1.5 % agarose gel. The concentration of the amplicons was estimated using a Gel Doc TM XR+ System coupled with Image Lab TM Software (BioRad, Hercules, CA USA) with 3 µl of O'GeneRulerTM 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as the internal standard for band intensity measurement. The samples of individual gels were pooled into approximately equimolar sub-pools as indicated by band intensity and measured with the Qubit dsDNA br Assay Kit (Life Technologies GmbH, Darmstadt, Germany). Subpools were mixed in an equimolar fashion and stored at -20 °C until sequencing. Sequencing was performed on the Illumina MiSeq platform with v3 chemistry (2 × 300 cycle kit) (Rausch et al., 2016). The raw data are deposited at the Sequence Read Archive (SRA) and available under the project ID PRJNA742683.

The 16S rRNA gene amplicon sequence analysis was conducted through the QIIME 1.9.0 package (Caporaso et al., 2010). Sequences with at least 97% identity were grouped into OTUs and clustered against the QIIME reference sequence collection; any reads, which did not hit the references, were clustered *de novo*. OTUs with less than 50 reads were removed from the dataset to avoid false positives which rely on the overall error rate of the sequencing method (Faith et al., 2013). Samples with less than 3600 sequences were also removed from the dataset, being considered as outliers. For the successive analysis, the number of OTUs per sample was normalized to that of the sample with the lowest number of reads after filtering.

Alpha-diversity was calculated using the Chao1 metric implemented in QIIME. Data were subjected to descriptive analysis, and normality and homogeneity tests as described herein. When normality, homogeneity and absence of significant outliers assumptions were met, statistical significance was tested through one-way ANOVA. When at least one of the assumptions was violated, the non-parametric Kruskal-Wallis

test was performed instead. When a significant difference between treatments was detected, post-hoc comparisons were performed to infer its direction and size effect. Tukey's post-hoc comparisons were applied after ANOVA, while Dunn's post-hoc after Kruskal-Wallis.

Beta-diversity was calculated in QIIME according to the different beta-diversity metrics available (Binary-Pearson, Bray-Curtis, Pearson, Weighted-Unifrac and Unweighted-Unifrac). Statistical values of clustering were calculated using the nonparametric comparing categories methods Adonis and Anosim.

Bacterial groups associated with specific conditions were identified by LEfSe (http://huttenhower.sph.harvard.edu/galaxy) (Segata et al., 2011). LEfSe uses the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance, concerning the biological conditions of interest; subsequently, LEfSe uses Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature. In addition to that, presence-absence calculations were performed directly on the OTU tables to detect bacterial OTUs that are shared between donor and recipient and between F0 and F1 bacterial communities. Statistical tests were performed through JASP v0.16 (https://jasp-stats.org).

Transcriptome analyses

The analysis was performed on five animals from each AT in two repeated sequencing runs. mRNA sequencing with previous poly-A selection was performed for 15 libraries on the Illumina HiSeq 4000 platform, with 75 bp and 150 bp paired-end sequencing respectively. The quality of raw reads was assessed using FastQC v0.11.7 (Andrews, 2014). Trimmomatic v.0.38 (Bolger et al., 2014) was then applied to remove adaptors and low-quality bases whose quality scores were less than 20. Reads shorter than 50 bp were removed, and only paired-end reads after trimming were retained. Reads were mapped to the Ensembl metazoa *Nematostella vectensis* genome *(*release 40) using the splice-aware aligner hisat2 v2.1.0 (Kim et al., 2015) with rna-strandness RF option and default parameters (**Table S7**).

RNA-seq data was used to improve the predicted *N. vectensis* gene model downloaded from Ensembl Metazoa database release 40. Using mapped reads from each temperature condition as input, StringTie v2.0 (Pertea et al., 2015) and Scallop

v0.10.4 (Shao & Kingsford, 2017) were applied to perform genome-guided transcriptome assemblies. The assembled transcripts were subsequently compared and merged using TACO (Niknafs et al., 2016). This produced 42,488 genes with 81,163 transcripts, among which 21,245 genes had significant matches (blastx with parameter e-value 1^{e-5}) with proteins in the SwissProt database. Assembled genes were compared with the Ensembl gene model using gffCompare v0.11.2 (Pertea & Pertea, 2020), from which genes with lower blastx e-value were selected. Ensembl genes without matching assembled genes were retained, and assembled genes without matching Ensembl genes but with significant matching SwissProt proteins were added to the gene model. The final gene model included 20,376 Ensembl genes, 4,400 improved genes and 2,751 novel assembled genes (**Suppl. Data 4**). The gene model statistics and the completeness of gene models were assessed using BUSCO v5.2.2 (Manni et al., 2021) on the Metazoa dataset (**Table S8, S9**). Total counts of read fragments aligned to the annotated gene regions were derived using the FeatureCounts program (Subread-2.0.0) (Liao et al., 2014) with default parameters. Genes whose combined counts from all samples were lower than 5 counts per million (cpm) mapped reads were excluded from the analyses. Differential expression analyses were performed in parallel using DESeq2 (v1.28.1) BioConductor package (Love et al., 2014), and limma (voom v3.44.3) package (Law et al., 2014). Differentially expressed genes (DEGs, **Table S10**) were determined based on False Discovery Rate (FDR, Benjamini-Hochberg adjusted p-value ≤ 0.05). Gene ontology annotation was derived from the best-matching SwissProt proteins. Enriched GO-terms in DEGs were identified by the topGO (v2.40.0) BioConductor package (**Suppl. Data 1**).

Results

Long-term acclimation at high temperature increases heat resistance in *Nematostella vectensis*

Before starting the acclimation experiment, we propagated a single female polyp to 150 clones and split these clones into 15 different cultures with 10 clonal animals each, to ensure the same genotype in all acclimation regimes. We further propagated these animals to 50 animals per culture and constantly maintained this number throughout the experiment. Subsequently, we acclimated these independent cultures at low (15 °C), medium (20 °C) and high temperature (25 °C) (5 cultures each) for 3 years (161 weeks) (**Figure 1**).

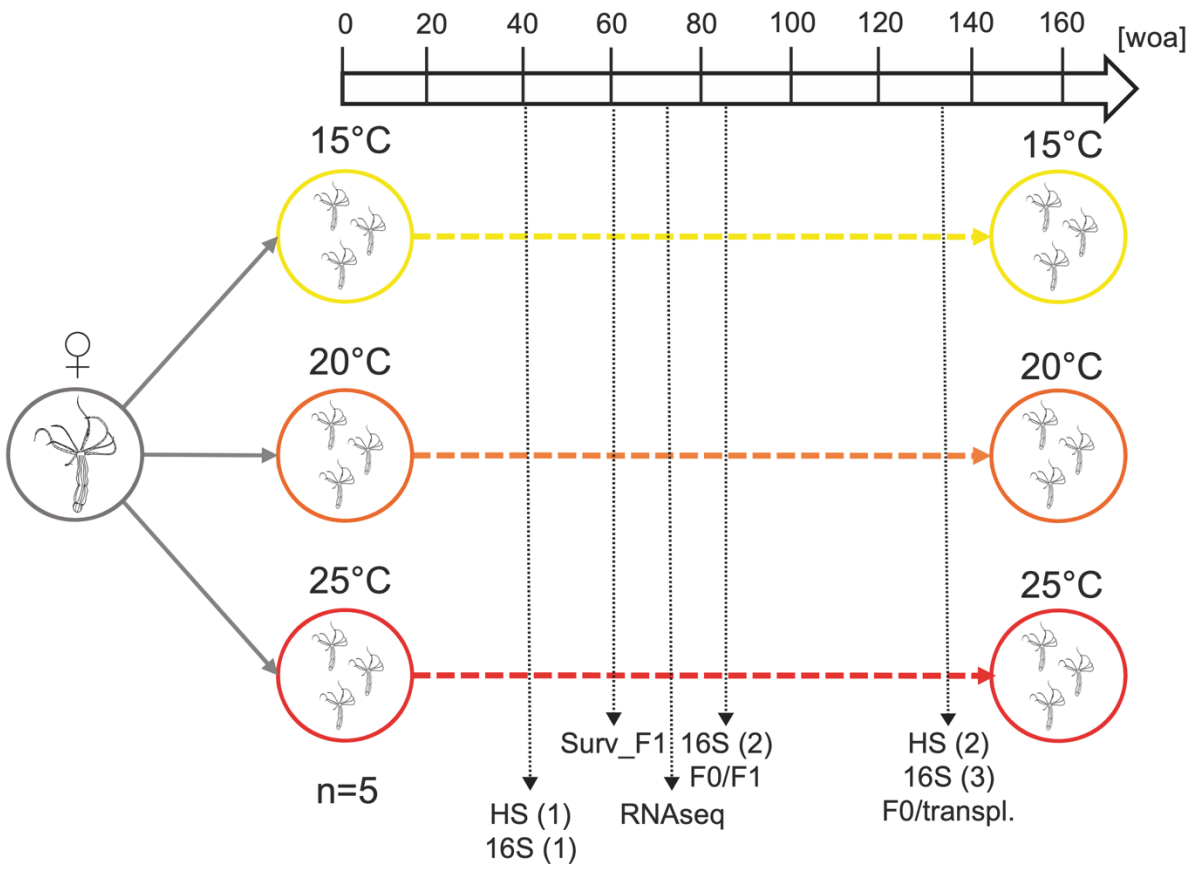

Figure 11. Experimental setup. A single female polyp from the standard culture conditions (16‰, 20 °C) was isolated and propagated via clonal reproduction. When a total of 150 clones was reached, they were split into 15 different culture boxes of 10 animals each. The boxes were put at three different acclimation temperatures (ATs) (15, 20, and 25 °C, n = 5) and the number of animals/box was kept equal to 50. Heat stress experiments (HS) (6 h, 40 °C) were performed at 40 (n = 10) and 132 (n = 5)

weeks of acclimation (woa). Sexual reproduction was induced at 60 and 84 woa for the juvenile survival test (Surv_F1, n =10) and the vertical transmission experiment (F0/F1, n = 5). At 40, 84 and 132 woa samples were collected for 16S sequencing (16S, n = 5); at 76 woa sampling for RNA sequencing was performed (n =5).

After 40 weeks of acclimation (woa), we tested, for the first time, the heat tolerance of acclimated polyps as a proxy for acclimation. We individually incubated polyps of each acclimated culture in 10 replicates for 6 hours at 40 °C and recorded their mortality (**Figure 2A**). Already after 40 woa, significant differences in the mortality rates of clonal animals were detectable. While all animals acclimated to low temperature died after the heat stress, animals acclimated at 20 °C showed a significant higher survival rate of 70 %. Animals acclimated at 25 °C showed a survival rate of 30 %, although this was not significantly different from the survival rate at 15 °C (**Figure 2A**). We repeated the measurement of heat tolerance 2 years later (132 woa). Interestingly, we observed a drastic increase in fitness in animals acclimated at high temperature, while the animals acclimated at 15 °C and 20 °C showed 100 % mortality (**Figure 2A**).

We also monitored the mortality rate in the acclimated cultures throughout 161 weeks (**Figure 2B**). While the mortality in cultures acclimated at 15 °C and 20 °C was below 0.5 polyps per week, the mortality rate was significantly reduced in cultures acclimated at 25 °C. An additional phenotypic difference between the acclimated animals was the clonal growth, as animals acclimated at 25 °C propagated asexually nearly seven times more than animals acclimated at 15 °C (**Figure 2C**). This may explain the differences in body size, where animals acclimated at 15 °C were more than three times bigger than the animals acclimated at 20 and 25 °C (**Figure 2D**). The different ATs also affected the fecundity of the animals: the polyps acclimated at the high ATs showed a significantly higher number of spontaneous spawning events recorded along the whole course of the experiment, compared with the 15 °C acclimated animals that never spawned if not artificially induced (**Figure S1**).

These results indicate that *N. vectensis* possesses remarkable plasticity at long-term temperature acclimation realized through differences in thermal tolerance, body size, asexual propagation, and fecundity. In the following, we analyzed the associated microbiota and host transcriptomic responses as a source of thermal acclimation in *N. vectensis*.

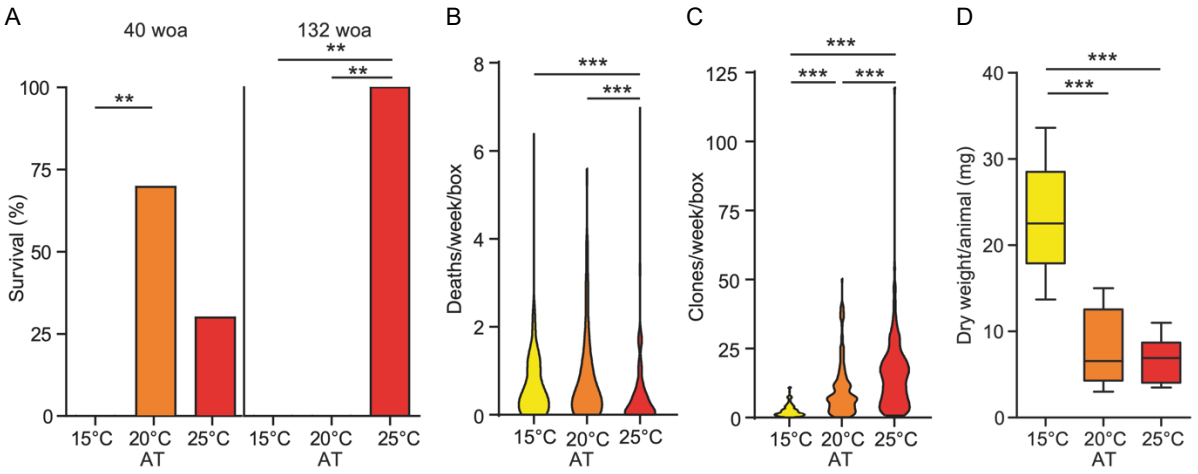

Figure 12. Phenotypic plasticity in response to thermal acclimation. (A) Survival of acclimated polyps after heat stress (40 °C, 6 h). Statistical analyses were performed by a Fisher's exact test (n = 10 (40 woa), **p = 0.0031), n = 5 (132 woa, **p = 0.0079). (B) Average of dead polyps per week per box throughout the whole acclimation period (n = 5, 161 woa) (Kruskal-Wallis test followed by Dunn's post-hoc comparisons, H = 24.09, ***p < 0.001). (C) Average of clones generated per week per 50 animals throughout the whole acclimation period, n = 5, Kruskal-Wallis test followed by Dunn's post-hoc comparisons, H = 191.6, ***p < 0.001. (D) Dry weights of acclimated polyps at 161 woa, n = 10, Kruskal-Wallis test followed by Dunn's post-hoc comparisons, H = 19.01, ***p < 0.001. Box plots are presenting center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

Thermal acclimation leads to dynamic, but consistent changes in the microbiota

To monitor the dynamic changes in the associated microbiota of acclimated animals, we sampled single polyps from each of the 15 clonal cultures at 40, 84 and 132 woa and compared their associated microbiota by 16S rRNA sequencing (**Figure 1**). To determine the impact of AT and sampling time point on the assemblage of the bacterial community, we performed principal coordinates analysis (PCoA) (**Figure 3A and B**). While principal component 1 (PC1) mostly separates samples according to the AT (**Figure 3A**), PC2 correlates with the different sampling time points (**Figure 3-b**). Using five different beta-diversity metrics, we found that bacterial colonization is significantly influenced by both AT and woa (**Table 1**).

Assigning the different microbial communities by sampling time points revealed a shared clustering after 84 and 132 woa (**Figure 3B**), suggesting a stabilization within the microbial communities after around 2 years of acclimation. In contrast, assigning the samples by AT revealed a clear clustering of the microbial communities (**Figure 3A**) with the bacterial communities acclimated at 20 °C clustering between the two

extremes (15 °C and 25 °C). This indicates that the three different ATs induced differentiation of three distinct microbial communities since the beginning of the acclimation process and that this differentiation is more pronounced between the extreme ATs. While most bacterial groups maintain a stable association with *N. vectensis (***Figure 3C**), bacteria that contribute to the differentiation at the end of the acclimation process are Alphaproteobacteria, which significantly increase at high temperatures (one-way ANOVA, $F = 17.27$, $p = 0.0004$) (**Table S1)**, and Epsilonproteobacteria, which significantly increase at low temperature (one-way ANOVA, $F = 25.96$, $p < 0.0001$) (**Figure 3C, Table S2**).

Using the Binary-Pearson distance matrix, we calculated the distances between samples within all three acclimation regimes (**Figure 3D**) and sampling time points (**Figure 3E**). Continuous acclimation under the different temperature regimes revealed no differences in the within-treatment distances (**Figure 3D**), indicating similar microbial plasticity at all three ATs. In contrast, Binary-Pearson distances of the different sampling time points significantly increased between 40 and 84 woa (**Figure 3E**) and stabilized between 84 and 132 woa. The alpha-diversities of bacteria associated with acclimated polyps were significantly higher at 20 °C and 25 °C, compared to those associated with polyps acclimated at 15 °C (**Figure 3F**). As for the β-diversity, the α-diversity was significantly increasing within the first 84 woa and stabilized between 84 and 132 woa (**Figure 3G**).

Altogether, these results show that the microbiota of *N. vectensis* reacts plastically to environmental changes. The microbial composition changes stabilize within two years of acclimation indicating a new homeostatic bacterial colonization status.

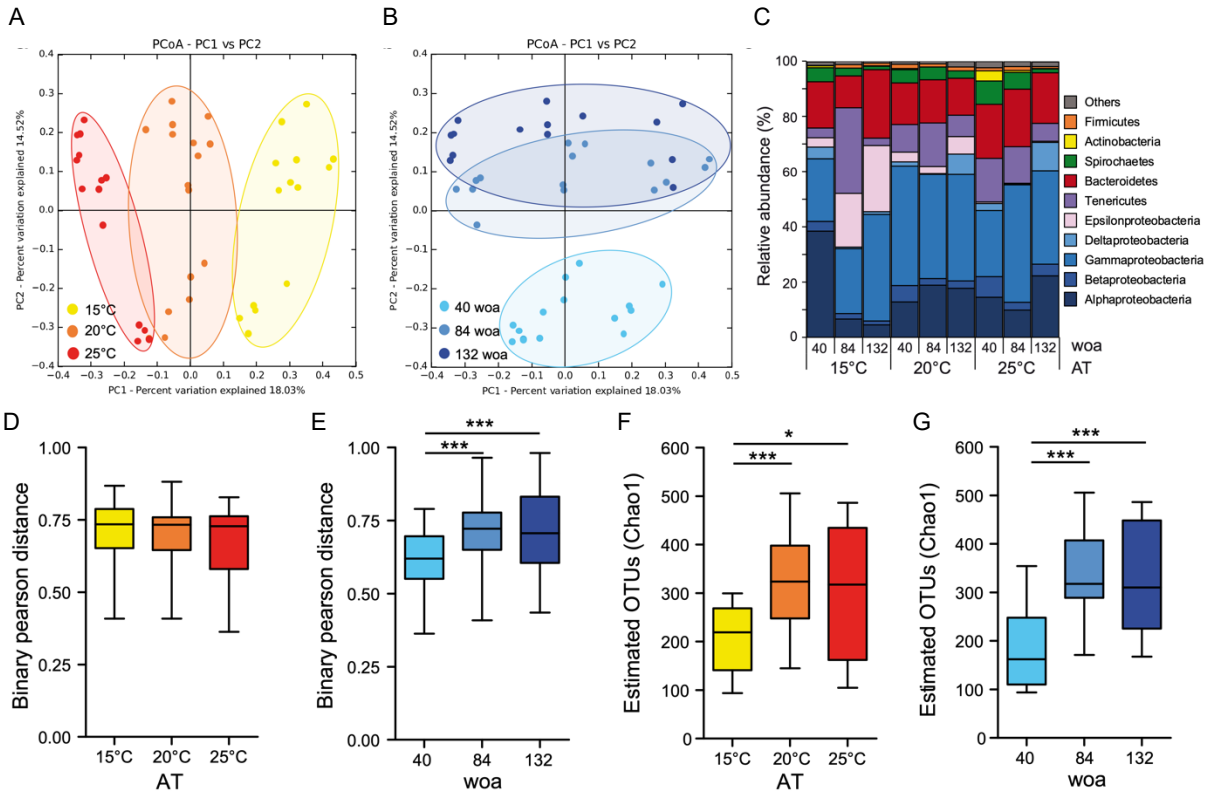

Figure 13. Bacterial community changes in response to thermal acclimation. (A) PCoA (based on the binary-Pearson metric, sampling depth = 3600) illustrating similarity of bacterial communities based on acclimation temperature (AT); (B) PCoA (based on Binary-Pearson metric, sampling depth = 3600) illustrating similarity of bacterial communities based on weeks of acclimation (woa). In (A) and (B) the same data are plotted, but with different color codes. (C) Relative abundances of principal bacterial groups, the abundances were summarized under the relative higher taxonomic categories (classes and phyla) and reported as percentages of the total. (D and E) beta-diversity distances within each AT (D) and within woa (E), statistical analyses were performed using a non-parametric Kruskal-Wallis test followed by Dunn´s post hoc comparisons (e) H = 43.66; ***p < 0.001. (F) Alpha-diversity (Chao1) comparison by AT (max rarefaction depth = 3600), statistical analyses were performed using a non-parametric Kruskal-Wallis test followed by Dunn´s post hoc comparisons (H = 9.801; *p = 0.011, ***p < 0.001). (G) Alpha-diversity (Chao1) comparison by woa (max rarefaction depth = 3600), statistical analyses were performed by using one-way ANOVA followed by Tukey's post hoc comparisons (F = 12.036; ***p < 0.001). Box plots are presenting center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

Table 1. Statistical analysis determining the influence of AT and woa on bacterial colonization. (number of permutations =999).

		Adonis		Anosim	
parameter	**beta-diversity metric**	***R2***	***P-value***	***R***	***P-value***
AT	Binary-Pearson	0.208	0.001	0.544	0.001
	Bray-Curtis	0.219	0.001	0.466	0.001
	Pearson	0.256	0.001	0.360	0.001
	Weighted-Unifrac	0.147	0.001	0.238	0.001
	Unweighted-Unifrac	0.193	0.001	0.521	0.001
woa	Binary-Pearson	0.230	0.001	0.608	0.001
	Bray-Curtis	0.199	0.001	0.372	0.001
	Pearson	0.217	0.001	0.277	0.001
	Weighted-Unifrac	0.149	0.001	0.173	0.001
	Unweighted-Unifrac	0.192	0.001	0.498	0.001

Thermal acclimation induces a robust tuning of host transcriptomic profiles

To evaluate the contribution of host transcriptional changes to the observed increased thermal tolerance in animals acclimated at high temperature, we analyzed gene expression profiles of *N. vectensis* after 75 woa (**Figure 1**). We sampled from each replicate culture one animal, extracted its mRNA and sequenced it by Illumina HiSeq 4000. The constant acclimation at 15, 20 and 25 °C induced a robust tuning of the host transcriptomic profiles (**Figure 4A**).

From pairwise comparisons, we determined the differentially expressed (DE) genes (**Figure 4B**) in all acclimated animals. While the comparison of transcriptomic profiles from polyps acclimated at 15 and 25 °C revealed the highest number of DE genes, the comparison of 20 and 25 °C acclimated animals revealed the lowest number of DE genes. In all three comparisons, we observed a similar fraction of up- and down-regulated DE genes (**Figure 4B**).

To retrieve molecular processes and signaling pathways enriched at the different ATs, we performed a gene ontology (GO) enrichment analysis and concentrated on GO categories significantly enriched in the comparison between 15 and 25 °C acclimated polyps (**Figure 4C, Suppl. Data 1**). Animals acclimated to high temperature significantly increased expression in genes involved in innate immunity, gene

regulation, epithelial cells proliferation, steroid biosynthesis, and metabolism (**Figure 4C, Suppl. Data 1**). While genes associated with enriched GO categories show opposite expression levels at 15 °C and 25 °C, an intermediate expression level was evident in the animals acclimated at 20 °C (**Figure 4C**). The animals acclimated to low temperature showed upregulation of genes associated with viral processes, which seems to be compatible with their general lower viability.

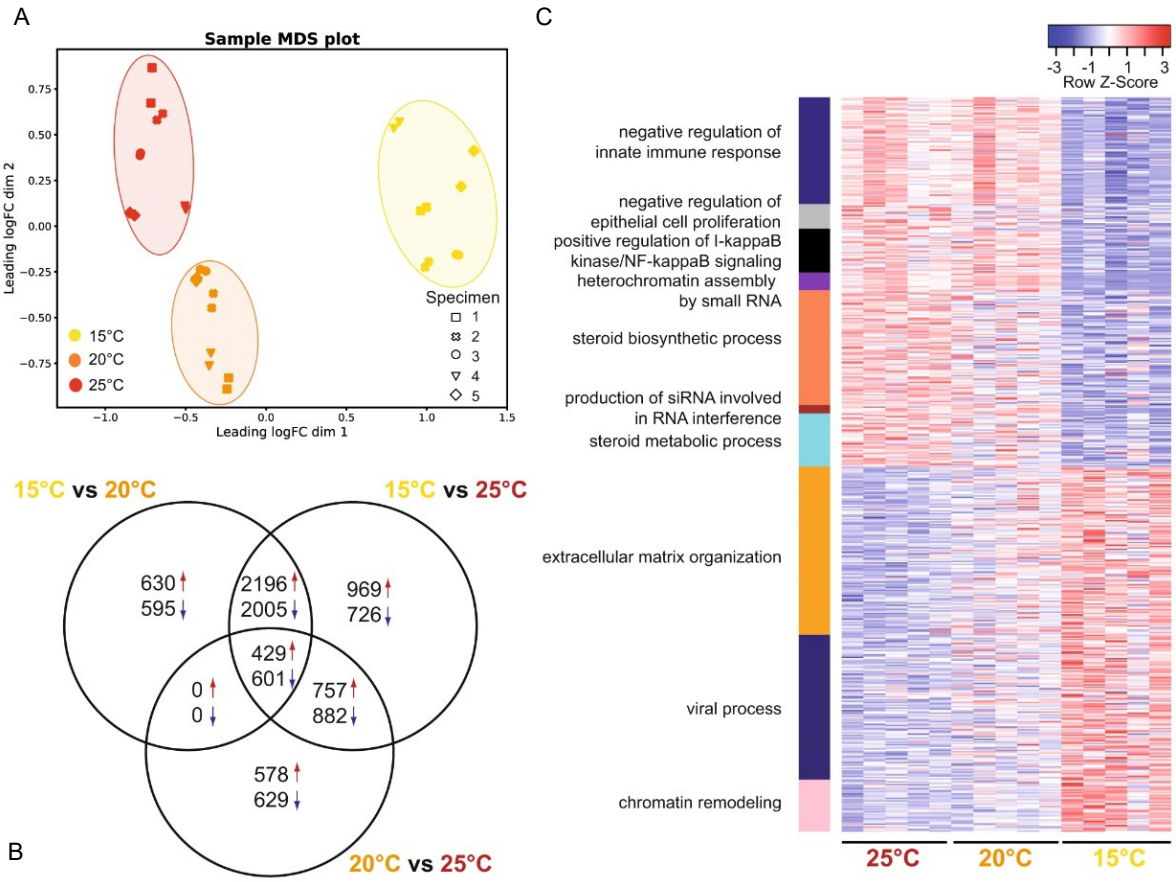

Figure 14. Host transcriptome changes after thermal acclimation. (A) MDS plot showing clustering of the transcriptome profiles according to the AT (samples were sequenced in technical replicates, indicated by the different symbols); (B) Venn diagram showing the numbers of differentially expressed genes from pairwise comparisons between the three ATs; (C) heat-map of differentially expressed genes in significantly enriched GO term categories from the comparison between 15 and 25 °C acclimated polyps.

Transplantation of acclimated microbiota induces differences in heat tolerance

To separate the effects of transcriptomic from bacterial adjustments on the thermal tolerance of acclimated polyps, we performed microbial transplantation experiments.

We generated axenic non-acclimated animals (recipients) and recolonized these animals with the microbiota of long-term acclimated polyps (donors) from the same clonal line. We maintained recipient animals for one month at 20 °C to allow the adjustment of stable colonization.

The 16S rRNA gene sequencing of donor and recipient animals and subsequent PCoA of the recipient polyps revealed that they grouped according to the AT of the donor microbiota 1 month after transplantation (**Figure 5A, Table 2**). To evaluate the rate of vertical bacterial transmission we included the donor samples into the PCoA (**Figure S2, Table S3**). While principal component 1 explained the bacterial variation due to transplantation, principal component 3 explained the bacterial variation due to the difference in acclimation temperature in donor polyps (**Figure S2 A-C**). In addition, presence-absence analysis based on the OTU read table revealed that recipient polyps received a high proportion of differentially abundant bacteria from the corresponding donor polyps during transplantation (**Suppl. Data 2**). Nevertheless, not all bacteria could be transplanted with the same efficiency. Recipient polyps showed a reduction of bacterial alpha-diversity by approximately 30% compared to the donor polyps (**Figure S2D**). While most bacterial classes were present in similar proportions in donor and recipient polyps, Epsilonproteobacteria did not appear to be transplantable (**Figure S2E**).

Subsequently, we tested the recipient animals for their heat tolerance as previously performed for the acclimated animals. The recipient animals showed clear differences in mortality after heat stress depending on the microbial source used for transplantation. A significant gradient in survival was evident from the animals transplanted with the 15 °C-acclimated microbiota (33 %) to those transplanted with the 25 °C-acclimated microbiota (80 %) (**Figure 5B**). The animals transplanted with the 20 °C-acclimated microbiota showed an intermediate survival (60 %).

These results indicate that the high thermal tolerance of animals acclimated to high temperature can be transferred to non-acclimated animals by microbiota transplantation alone. Therefore, we conclude that microbiota-mediated plasticity provides a rapid mechanism for a metaorganism to cope with environmental changes. Through LEfSe analysis, we were able to detect bacterial OTUs differentially represented between the polyps acclimated at 15 and 25 °C, and in the corresponding transplanted animals (**Table S4**). These bacteria belong to the families Phycisphaeraceae, Flavobacteriaceae, Emcibacteraceae, Rhodobacteraceae,

Methylophilaceae, Francisellaceae, Oceanospirillaceae, and Vibrionaceae, which are known to include various commensals, symbionts, and pathogens of marine organisms. Therefore, the OTUs overrepresented in the 25 °C microbiota may constitute good candidates for providing thermal resistance to their host.

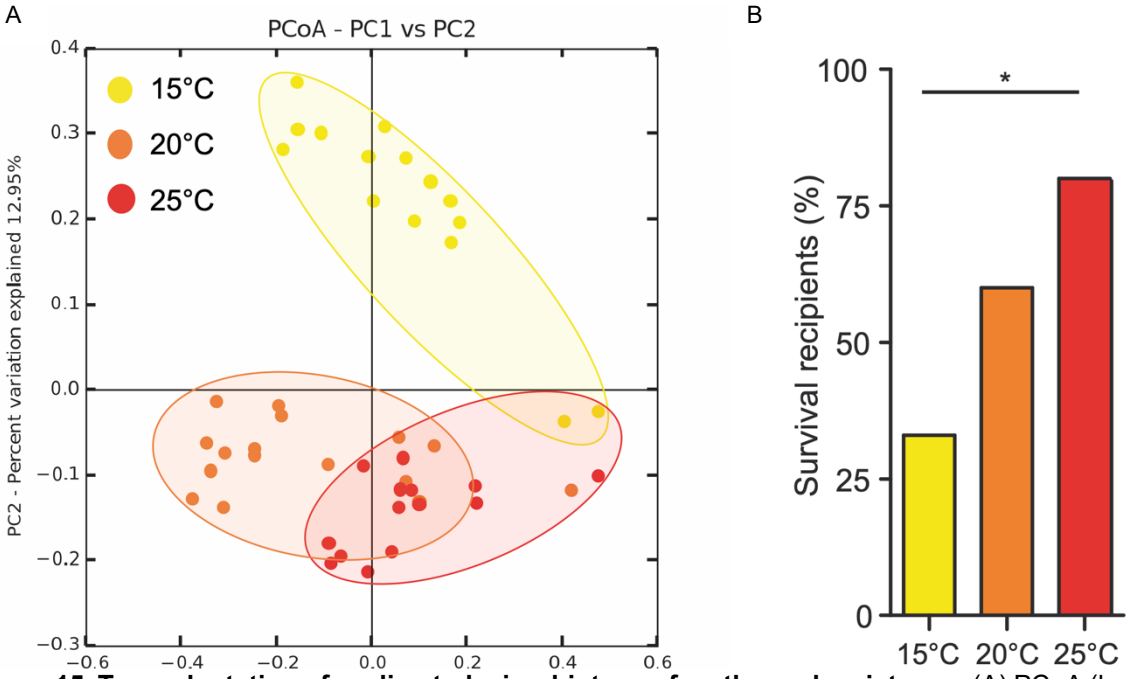

Figure 15. Transplantation of acclimated microbiota confers thermal resistance. (A) PCoA (based on the binary-Pearson metric, sampling depth = 3600) illustrating similarity of recipient bacterial communities based on AT of donor microbiota; (B) heat stress (40 °C, 6 h) survival of recipient polyps. Statistical analyses were performed by pairwise Fisher's exact test (n = 15, *p = 0.025). Box plots are presenting center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range.

Table 2. Statistical analysis determining the influence of donor polyps' AT on recipient microbiota (number of permutations = 999).

		Adonis		Anosim	
parameter	**P-value**	***R2***	***P-value***	***R***	***P-value***
	Binary-Pearson	0.199	0.001	0.486	0.001
	Bray-Curtis	0.183	0.001	0.346	0.001
Donors' AT	Pearson	0.165	0.001	0.194	0.001
	Weighted-Unifrac	0.161	0.001	0.272	0.001
	Unweighted-Unifrac	0.184	0.001	0.416	0.001

Acclimated microbiota and higher fitness are transmitted to the next generation

In the next step, we tested if the higher temperature resistance of F0 animals acclimated to high temperature is transmitted to the offspring (F1 generation). Therefore, two female polyps from each long-term acclimated culture and one non-acclimated male polyp were induced separately for spawning. All oocyte-packs were fertilized with the sperm of the same male polyp, split into 3 parts, counted and let develop for 1 month at the 3 different developing temperatures (DTs) in a full factorial design (**Figure 6A**).

After one month of development, the survived juvenile F1 polyps were counted and corresponding survival rates were calculated (**Figure 6B**). The offspring from the polyps acclimated at 25 °C showed a significantly higher overall survival rate compared to the offspring from polyps acclimated at medium and low temperature. In contrast, the offspring of polyps acclimated at 15 °C showed the lowest survival rate at 25 °C DT (**Figure 6B**). In a second step, the F0 and the juvenile F1 polyps were subjected to 16S rRNA sequencing to evaluate the transmission of acclimated microbes to the next generation. PCoA of F1 animals revealed a significant clustering according to both F1 DT and F0 AT (**Figure 6C and D, Table 3**). While, on average, around 50 % of bacterial variation can be explained by the DT of the juvenile polyps, around 20 % of the bacterial colonization in juveniles can be explained by the acclimation temperature of the F0 polyps (**Table 3**). The comparison of F0 and F1 samples in PCoA revealed a clustering between F0 and F1 samples along PC1 (**Figure S3A**), indicating that differential bacterial communities colonize juvenile and adult polyps, as described in earlier studies (Baldassarre et al., 2021; Mortzfeld et al., 2016). In contrast, PC2 separated samples according to AT in both the F0 and the F1 samples (**Figure S3A, Table S5**), indicating the successful transplantation of parts of the acclimated bacterial communities.

Successful transmission is also indicated by the concordance of bacterial groups present in the F0 and F1 animals (**Figure S3B**). For the identification of individual OTUs potentially transmitted from F0 to F1 animals, we performed a presence/absence analysis (**Suppl. Data 3**) revealing the differential transmission of bacterial species from F0 female polyps acclimated at different temperatures. Using LEfSe analysis, we found that bacterial OTUs were overrepresented in polyps acclimated at 15, 20 and 25 °C as well as in the corresponding F1 animals (**Table S6**).

While F0 female polyps acclimated at 15 °C mainly transmitted Bacteroidetes and Alphaproteobacteria, polyps acclimated at 25 °C transmitted mainly Gammaproteobacteria to their offspring (**Table S6**).

These results demonstrate that acclimation at high temperature of the F0 generation improved the overall fitness of its offspring. The fact that also specific members of the acclimated microbiota are transmitted and persisted in the F1 generation suggests that vertically transmitted acclimated bacteria can be adaptive to high temperature.

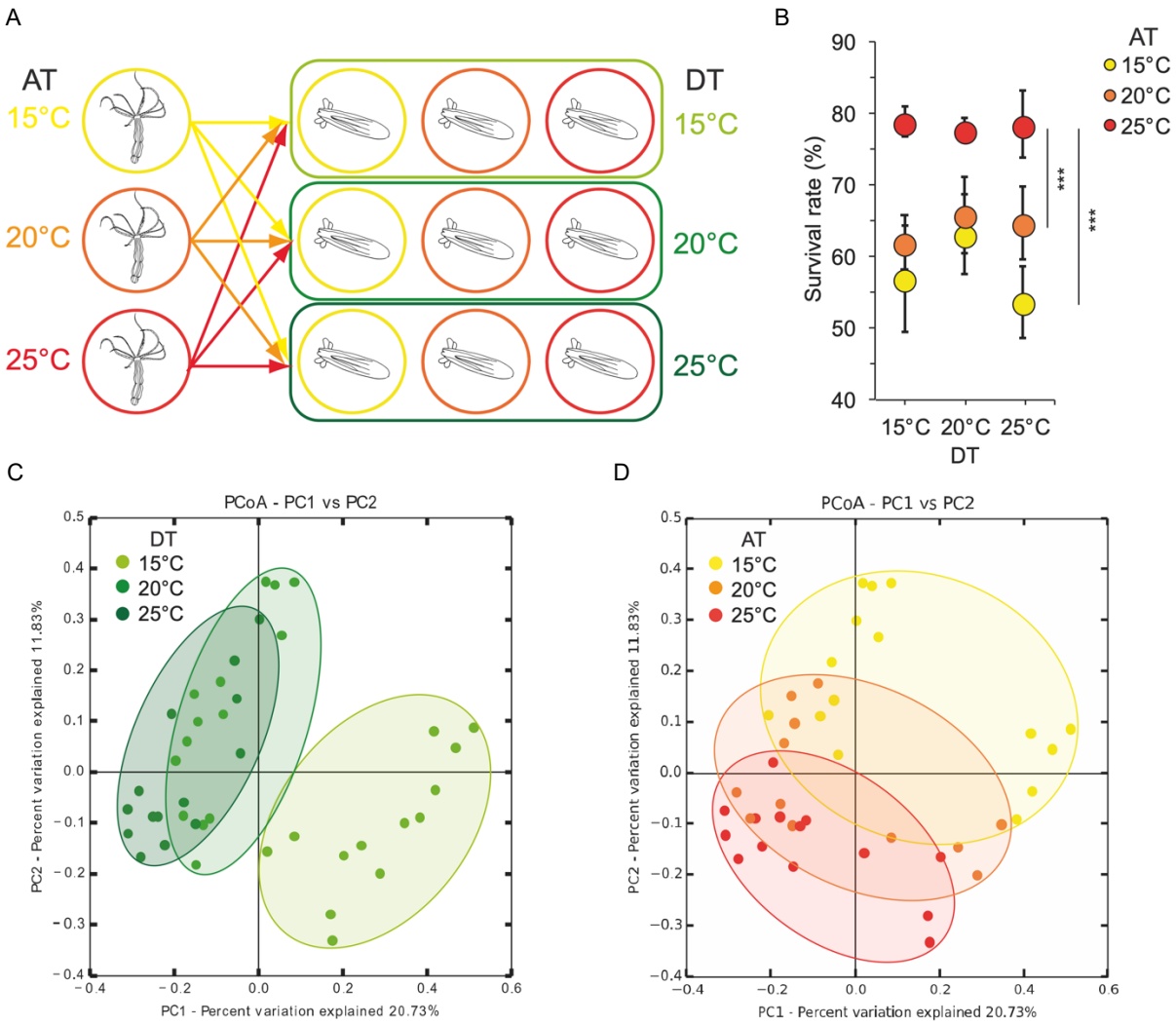

Figure 16. Transmission of thermal tolerance to the offspring. (A) Experimental scheme: acclimated females from each AT were induced for sexual reproduction. All oocyte-packs were fertilized with the sperms from a single male polyp from the standard conditions. After fertilization, each embryo pack was split into 3 parts and placed at different DT (15, 20, or 25 °C). After 1 month of development, survival rate and bacterial colonization were analyzed. (B) Offspring survival rate (ratio between the initial number of oocytes and survived juvenile polyps were calculated), a Kruskal-Wallis test was performed followed by Dunn´s post-hoc comparisons (n = 10; H = 32.658; ***p < 0.001), average values with error bars indicating SE are presented. (C) PCoA (based on Binary-Pearson metric, sampling depth = 24500) illustrating similarity of bacterial communities according with F1 DT; (D) PCoA (based on binary-Pearson metric, sampling depth = 24500) illustrating similarity of bacterial communities according with F0 AT. In (C) and (D) the same data are plotted, but with different color code.

Table 3. Statistical analysis determining the influence of F0 females' AT and F1 DT on bacterial colonization of F1 polyps (number of permutations = 999).

		Adonis		Anosim	
parameter	**beta-diversity metric**	***R2***	***P value***	***R***	***P value***
	Binary-Pearson	0.170	0.001	0.373	0.001
	Bray-Curtis	0.133	0.001	0.237	0.001
F0 AT	Pearson	0.139	0.001	0.166	0.002
	Weighted-Unifrac	0.093	0.024	0.091	0.010
	Unweighted-Unifrac	0.116	0.001	0.148	0.005
	Binary-Pearson	0.262	0.001	0.696	0.001
	Bray-Curtis	0.260	0.001	0.621	0.001
F1 DT	Pearson	0.338	0.001	0.542	0.001
	Weighted-Unifrac	0.300	0.001	0.408	0.001
	Unweighted-Unifrac	0.211	0.001	0.413	0.001

Discussion

Long-term acclimation promotes heat tolerance in *N. vectensis*

The ability of marine animals to adapt to future thermal scenarios is of pivotal importance for the maintenance of biodiversity and ecosystem functioning. Recent studies indicate that sessile marine animals, like corals, sponges or anemones, could adapt more rapidly to climate change than expected (Guest et al., 2012; MJH Oppen et al., 2015; Puisay et al., 2018; SR Palumbi et al., 2014; Torda et al., 2017; Yu et al., 2020). Recent and long-term observations in the field displayed higher heat tolerance of corals pre-exposed to thermal stress compared to unexposed ones and showed that wild populations are slowly becoming less sensitive than they were in the past (Jury & Toonen, 2019; Sully et al., 2019; Thomas et al., 2018). In our study, the host's thermal resistance showed an increase with the acclimation time. It is important to point out that the standard culture temperature for *N. vectensis* in the lab is 20 °C. The animals maintained at 20 °C, therefore, have been acclimated to this condition for a long time and this might explain their highest survival at 40 woa. Interestingly, the animals acclimated at 15 °C showed at both time points 100 % mortality, indicating that these animals would not be able to survive extreme high temperature events.
Our results are consistent with other studies that investigated the acclimation capacity of corals in lab experiments. Pre-acclimated individuals of *Acropora pruinosa*, a scleractinian coral, showed lower signs of bleaching when exposed to successive heat stress, in comparison to the not-pre-acclimated ones (Yu et al., 2020). Also in the field, *Acropora hyacinthus* showed less mortality after heat stress when acclimated to wide temperature fluctuations, than when acclimated to less variable environments (Oliver & Palumbi, 2011). These different resistances are correlated to adaptive plasticity in the expression of environmental stress response genes (Kenkel & Matz, 2017) and the presence of an advantageous microbiota (Barker, 2018), but a causative relation was not shown in both cases. Our study separates the contribution of the microbiota to temperature acclimation from host effects by performing microbial transplantation experiments on a single host genotype.

Microbiota plasticity promotes metaorganism acclimation

Shifts in the composition of bacterial communities associated with marine animals in response to changes in environmental factors (*i.e.,* temperature, salinity, pH, light exposure, oxygen, CO_2 concentrations, etc.) have been demonstrated in numerous studies (D. Bourne et al., 2008; Carrier & Reitzel, 2017; Koren & Rosenberg, 2006; Littman et al., 2011; Mortzfeld et al., 2016; Thurber et al., 2009; van Oppen & Blackall, 2019; Ziegler et al., 2017). In some cases these changes in microbiota composition correlated with a higher fitness of acclimated animals (Ziegler et al., 2017), but causal connections are rare. An experimental replacement of a single bacterium and subsequent demonstration of acquired heat tolerance by the host was only shown in aphids (Moran & Yun, 2015).

To infer if and to what extent the acclimated microbiota confers thermal resistance, we performed transplantation experiments of microbiota from acclimated animals to non-acclimated ones. These experiments proved that polyps transplanted with the microbiota from animals acclimated at 25 °C for 132 woa acquired a higher thermal tolerance than those transplanted with the 15 °C acclimated microbiota. It is important to point out that the animals selected as recipients for this experiment were all clones of the same age, size and shared the same life history, since they came from the same culture box, and belonged to the same clonal line as the acclimated donors. With this experimental setup, we were able to disentangle microbiota contribution to thermal acclimation from host genotype effects and proved that acclimated bacteria can act as heat tolerance promoting bacteria.

Microbial community acclimation is a highly dynamic process that began in the first few weeks after the environmental change, and most adjustments in bacterial diversity occurred by 84 woa. Afterward, the microbial community likely reached a stable and homeostatic state. Previous studies on corals (Ainsworth et al., 2015; Hester et al., 2016) demonstrated the presence of a “core microbiota”, defined as a group of microbial species that are either persistent over time and/or in different environments or locations and are less sensitive to changes in the surrounding environment. Members of the core microbiota may not necessarily represent the most abundant groups of the community but are hypothesized to exert pivotal functions for the maintenance of holobiont homeostasis. In contrast, there is a “dynamic microbiota” that varies by species, habitat, and life stage and is likely a product of stochastic events

or a response to changing environmental conditions (Hester et al., 2016). Also in *N. vectensis,* it appears that during the acclimation process, a core microbiota remained stable in all acclimated polyps, while a more dynamic part of the microbiota changed, either increasing or decreasing the abundance of certain species. The increase in α-diversity indicates either the acquisition of new bacterial species from the environment or a higher evenness in species abundances, where OTUs that were rare at the beginning of the experiment and lower temperature, became more abundant and thus detectable. The acquisition of new bacterial species during lab experiments appears unlikely since the polyps are isolated from their natural environment. Nevertheless, the acclimated animals are not maintained under sterile conditions and thus an exchange of microbial species with the culture medium and from the food supply cannot be excluded. As already pointed out in numerous studies (D. G. Bourne et al., 2016; Pollock et al., 2019; Zilber-Rosenberg & Rosenberg, 2008), higher microbial diversity enhances the ability of the host to respond to environmental stress by providing additional genetic diversity.

In addition to the changes in species composition and relative abundances, the associated microbial species can evolve much more rapidly than their multicellular host (N. S. Webster & Reusch, 2017). Rapidly dividing microbes are predicted to undergo adaptive evolution within weeks to months (Elena & Lenski, 2003). Therefore, an adaptation of the host can also occur via symbiont acquisition of novel genes (Hehemann et al., 2010), via mutation and/or horizontal gene transfer (N. S. Webster & Reusch, 2017). Even if the abundance of a certain bacterial species did not change significantly between the different ATs, it is possible that it acquired new functions during the acclimation process and adapted to the new conditions.

Alphaproteobacteria and Gammaproteobacteria constitute the main microbial colonizers of corals (Ainsworth et al., 2015; D. G. Bourne, 2005) and *N. vectensis* (Leach et al., 2019; Mortzfeld et al., 2016). The increased thermal tolerance of animals acclimated at high temperatures is often associated with an increase in the abundance of these bacterial classes in the associated microbiota (Pootakham et al., 2019; N. Webster, 2016). In thermally stressed animals, Alphaproteobacteria constitute an important antioxidant army within the coral holobiont (Van Alstyne et al., 2006) and together with members of the Gammaproteobacteria significantly inhibited the growth of coral pathogens (*e.g., V. coralliilyticus* and *V. shiloi*) (Reshef et al., 2006; Rypien et

al., 2010). They are also known to exert nitrogen fixation in endosymbiosis with marine animals, providing the host with additional nutrient supply (Blazejak et al., 2005; Dubilier et al., 1999; Rincón-Rosales et al., 2009). In our study, Alphaproteobacteria significantly increased in abundance in the animals acclimated at high temperature and most of the bacterial OTUs significantly overrepresented in the animals transplanted with the 25 °C acclimated microbiota belong to the Alpha- and Gammaproteobacteria. Among these OTUs are members of the genera *Sulfitobacter, Francisella,* and *Vibrio*, and one Flavobacteriia OTU of the genera *Muricauda*. All these bacterial groups are known pathogens or symbionts of multicellular organisms (Rosenberg & DeLong, 2013). In particular, *Sulfitobacter* is an endosymbiont of vestimentiferans inhabiting hydrothermal vents, where it performs sulfite oxidation (Kimura et al., 2003); *Francisella* is an intracellular pathogen of mammals and various invertebrates and it is supposedly capable of ROS scavenging (Melillo et al., 2010; Rabadi et al., 2016). Members of the Flavobacteriaceae are key players in biotransformation and nutrient recycling processes in the marine environment, known intracellular symbionts of insects and intracellular parasites of amoebae (McBride, 2014). All these characteristics make them promising candidates for providing thermal tolerance to the host.

Changes in host gene expression may confer acclimation

Acclimation is generally thought to be driven by shifts in gene expression (Bay & Palumbi, 2015; SR Palumbi et al., 2014). Microbial transplantation experiments allowed us to measure the contribution of bacterial plasticity to host acclimation separately from genetic factors. Although we also observed significant adjustments in the transcriptomic response to thermal acclimation in *N. vectensis*, we were unable to assess the contribution of changes in gene expression to thermal acclimation. The adjustments in gene expression are most likely a combination of acclimation to the new temperature condition and the changed microbial colonization.

This hypothesis is supported on one hand by the observed adjustments of gene expression involved in innate immune responses in acclimated animals. In *N. vectensis* polyps acclimated to the higher temperature, we observed a downregulation of genes involved in innate immunity. Previous studies on *Hydra* showed that the cnidarian innate immune system actively controls the composition and the

homeostasis of the associated microbiota and that such associations are both species-specific and life-stage specific (Augustin et al., 2010, 2017; Franzenburg et al., 2013; Fraune et al., 2009). Animals challenged by unfavorable environmental conditions (high temperature in this case), may suppress their immune reaction to favor the establishment of new symbionts. In corals, it has been shown that non-acclimated individuals expressed stronger immune and cellular apoptotic responses than acclimated ones, and disease-related metabolic pathways were significantly enhanced in the former (Yu et al., 2020). Moreover, the immune system is sensitive to environmental change (D. G. Bourne et al., 2016), and colonization by beneficial symbionts might lead to the suppression of the host immune response (van Oppen & Blackall, 2019). Elements of the innate immune system, including several members of the interleukin signaling cascades and the transcription factor NF-kB, have been characterized in *N. vectensis* and are hypothesized to play similar roles as their vertebrate homologs (Brennan et al., 2017; Reitzel et al., 2008; Sullivan et al., 2009; Wolenski et al., 2011). Interestingly, a GO term comprising genes implicated in viral processes were upregulated in the animals acclimated at 15 °C, suggesting a possible higher susceptibility of these animals to infections and a possible implication to their lower viability.

On the other hand, the upregulation of genes involved in steroid biosynthesis and metabolism in animals acclimated to high temperature may indicate the contribution of steroid signaling in the regulation of phenotypic plasticity (Gáliková et al., 2011; Taubenheim et al., 2021), *e.g.,* in body size regulation and reproduction rate in response to different temperatures. The enhanced production of small RNAs (sRNAs) in the animals acclimated to high temperature, and the upregulation of processes involved in chromatin remodeling in animals acclimated at 15 °C, suggest a general change in transcriptional and translational regulations at these two extreme conditions, which might take part in acclimation. Chromatin remodeling processes are implicated in epigenetic modifications and thus possibly inheritable by the offspring (Becker & Workman, 2013).

A recent publication (Barno et al., 2021) analyzed coral-associated bacteria proteomes and detected potential host epigenome-modifying proteins in the coral microbiota. This, in concert with specific symbionts inheritance, may constitute an additional mechanism for thermal resistance transmission along with generations and may explain the significantly higher fitness of the 25 °C acclimated animals' offspring.

Acquired higher fitness is transmitted to the next generation

The capacity of a species to survive and adapt to unfavorable environmental conditions does not only rely on the adaptability of adults but also on the survival of the early life stages. Even if the adults can acclimate to periodic heat waves and seasonal temperature increases, their offspring may have a much narrower tolerance range (Chua et al., 2013; Ericson et al., 2012; Reitzel et al., 2013; Sheppard Brennand et al., 2010). There is evidence that offspring of marine species, including fishes, mussels, echinoderms and corals can acclimatize to warming and acidifying oceans via transgenerational plasticity (Bernal et al., 2018; Clark et al., 2019; JM Donelson et al., 2012; Miller et al., 2012; Munday, 2014; Ryu et al., 2020; Veilleux et al., 2015; Zhao et al., 2018). Both transmission of epigenetic modifications (Eirin-Lopez & Putnam, 2019; Fallet et al., 2020; HM Putnam, 2015; L Daxinger, 2010; Ptashne, 2013; Ryu et al., 2020) and microbiota-mediated transgenerational acclimatization (MJH Oppen et al., 2015; N. S. Webster & Reusch, 2017) may be involved in this process.

A recently published work showed that *N. vectensis* polyps acclimated to high temperature transmit thermal resistance to their offspring (Rivera et al., 2021). In our experiments, we moved a step further by exploring the potential contribution that the microbiota may have in the inheritability of this plasticity. We fertilized oocytes of acclimated females with sperm of a single male to keep the genetic variability as low as possible, and cultured the offspring in a full factorial design at 15 °C, 20 °C and 25 °C. As expected, offspring originating from females acclimated at 25 °C showed the highest survival rate. These results confirmed that polyps acclimated to high temperature transmit a higher overall fitness to their offspring. The fact that offspring from genetically identical female polyps show differences in overall fitness suggests either the vertical transmission of specific beneficial bacteria, the transmission of epigenetic modifications, or a combination of both.

For many marine invertebrates, vertical transmission of microbial symbionts is assumed (Hirose & Fukuda, 2006; JL Padilla-Gamiño, 2012; Sharp et al., 2007; Sipkema et al., 2015). In particular, species that undertake internal fertilization and brood larvae, tend to preferably transmit their symbionts vertically, whereas broadcast spawners and species that rely on external fertilization are thought to mainly acquire

their symbionts horizontally (Apprill et al., 2009; Lesser et al., 2013; Sharp et al., 2012). Bacteria may also be transmitted to the gametes by incorporation into the mucus that surrounds oocyte and sperm bundles (Ceh et al., 2012; Leite et al., 2017; Ricardo et al., 2016). Alternatively, the gametes may acquire bacteria immediately after release by horizontal transmission through the water, which contains bacteria released by the parents (van Oppen & Blackall, 2019). A recent publication showed that *N. vectensis* adopts a mixed-mode of symbiont transmission to the next generation, consisting of a differential vertical transmission from male and female parent polyps, plus a horizontal acquisition from the surrounding medium during development (Baldassarre et al., 2021). Consistent with the results of this study, our results suggest vertical transmission of heat tolerance-promoting bacteria.

Acclimated microbiota, a source for assisted evolution

Microbial engineering is nowadays regularly applied to agriculture and medicine to improve crop yields and human health (Epstein et al., 2019). Pioneering theoretical works, including the Coral Probiotic Hypothesis (Reshef et al., 2006) and the Beneficial Microorganisms for Corals concept (Peixoto et al., 2017), suggested that artificial selection on the microbiota could improve host fitness over time frames short enough to cope with the actual and future rates of climate changes. Some studies have started microbial engineering on corals as a restoration/conservation option for coral reefs subjected to environmental stresses (Chakravarti et al., 2017; Damjanovic et al., 2017, 2019). Recently, corals exposed to experimental warming and inoculated with consortia of potentially beneficial bacteria were shown to bleach less when compared to corals that did not receive probiotics (Rosado et al., 2019). It needs to be pointed out that microbiota-mediated transgenerational acclimatization (MJH Oppen et al., 2015; N. S. Webster & Reusch, 2017) is of pivotal interest because it would be a suitable target for manipulations in the perspective of future assisted evolution programs (MJH Oppen et al., 2015; Rosado et al., 2019).

In this study, we proved that long-term acclimation induces enormous changes in the physiology, ecology and even morphology of genetically identical animals. Animals exposed to high temperatures can acclimate and resist heat stress, and this resistance can be transmitted to non-acclimated animals by microbiota transplantation and most likely also to the next generations. We were able to pinpoint specific bacterial groups

that may be responsible for different thermal tolerances of their hosts and may be good candidates for future assisted-evolution experiments.

CONTRIBUTION OF MATERNAL AND PATERNAL TRANSMISSION TO BACTERIAL COLONIZATION IN *NEMATOSTELLA VECTENSIS*

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Abstract

Microbial communities confer multiple beneficial effects to their multicellular hosts. To evaluate the evolutionary and ecological implications of the animal-microbe interactions, it is essential to understand how bacterial colonization is secured and maintained during the transition from one generation to the next. However, the mechanisms of symbiont transmission are poorly studied for many species, especially in marine environments, where the surrounding water constitutes an additional source of microbes. *Nematostella vectensis*, an estuarine cnidarian, has recently emerged as model organism for studies on host-microbes interactions. Here, we use this model organism to study the transmission of bacterial colonizers, evaluating the contribution of parental and environmental transmission to the establishment of bacterial communities of the offspring. We induced spawning in adult male and female polyps of *N. vectensis* and used their gametes for five individual fertilization experiments. While embryos developed into primary polyps, we sampled each developmental stage and its corresponding medium samples. By analyzing the microbial community compositions of all samples through 16S rRNA gene amplicon sequencing, we showed that all host tissues harbor microbiota significantly different from the surrounding medium. Interestingly, oocytes and sperms are associated with distinct bacterial communities, indicating the specific vertical transmission of bacterial colonizers by the gametes. These differences were consistent among all the five families analyzed. By overlapping the identified bacterial ASVs associated with gametes, offspring and parents, we identified specific bacterial ASVs that are well supported candidates for vertical transmission via mothers and fathers. This is the first study investigating bacteria transmission in *N. vectensis,* and among few others on marine spawners that do not brood larvae. Our results shed light on the consistent yet distinct maternal and paternal transfer of bacterial symbionts along the different life stages and generations of an aquatic invertebrate.

Introduction

Multicellular organisms originated in a world dominated by unicellular organisms. Thus, the current-day relationships of animals and microbes, from parasitism to mutualism, evolved most likely from ancient unicellular eukaryote–bacterial interactions (Bosch & McFall-Ngai, 2021; McFall-Ngai et al., 2013). In aquatic environments these relationships are essential components of animal health and physiology, influencing the nutrient cycling (Lema et al., 2012; Raina et al., 2009; Santos et al., 2014; Wegley et al., 2007), gut development (Rawls et al., 2004), resistance against pathogen colonization (Fraune et al., 2015; Jung et al., 2009; Krediet et al., 2013), osmoregulation and oxidative stress responses (D. G. Bourne et al., 2016; Lesser, 1996; Peixoto et al., 2017; Rosado et al., 2019), as well as larvae settlement and metamorphosis (Dobretsov & Qian, 2004; Hadfield, 2010; Huang et al., 2012; Tran & Hadfield, 2011).

Given the importance of these relationships, it is essential to understand how bacterial colonization is secured and maintained during the transition from one generation to the next (Bosch & McFall-Ngai, 2021). There are two ways animals acquire their bacterial symbionts, horizontal transmission, in which the bacterial symbionts are acquired from the environment, and vertical transmission in which the bacterial symbionts are transferred via the gametes or by direct contact with the parents. In most animals a combination of both mechanisms (mixed mode transmission) contributes to the establishment of early life bacterial colonization (Bright & Bulgheresi, 2010). While vertical transmission of bacterial symbionts facilitates the evolution and maintenance of mutualistic relationships (Bosch & McFall-Ngai, 2021; Koga et al., 2012), horizontal acquisition requires efficient host selection mechanisms to ensure appropriate bacterial colonization (Franzenburg et al., 2013; Nyholm & McFall-Ngai, 2004). For microbes that are transmitted horizontally, symbiotic life is facultative and free-living populations serve as reservoirs for colonization (Bright & Bulgheresi, 2010). In the marine environment, such free-living populations occur both in shallow and deep waters (Aida et al., 2008; Gros et al., 2003; Harmer et al., 2008) and, in some cases, are replenished by the release of symbionts from the host itself (Salerno et al., 2005). These bacteria provide a diverse pool of potential colonizers for horizontal acquisition and could confer advantages under changing environmental conditions (Hartmann et al., 2017).

As marine invertebrates have great diversity of life history, reproductive and developmental modes, they exhibit diverse modes of bacterial transmission (Russell, 2019). An accredited idea among authors was that species that brood their larvae, transmit symbionts to the next generations through direct contact of the parents with the offspring (Bernasconi et al., 2019; Bright & Bulgheresi, 2010; Di Camillo et al., 2012), while transmission in broadcast spawners, is dominated by horizontal transfer of bacteria from the surrounding water (van Oppen & Blackall, 2019). As more studies are being conducted, there are more evidences that do not necessarily support this idea (Apprill et al., 2012; Björk et al., 2019; Leite et al., 2017; Nussbaumer et al., 2006; Oliveira et al., 2020). Many broadcast spawners pass their symbionts to their offspring by incorporating them into the mucus that envelops oocyte and sperm bundles (Ceh et al., 2012; Leite et al., 2017; Ricardo et al., 2016) and a wide spectrum of mixed-mode transmission in brooders and in free and broadcast spawners is revealed (Bernasconi et al., 2019; Damjanovic, Menéndez, Blackall, & Oppen, 2020; Damjanovic, Menéndez, Blackall, & van Oppen, 2020; Fieth et al., 2016; Sipkema et al., 2015).

N. vectensis is an anthozoan cnidarian that lives burrowed in sediments of estuarine areas and is widely used as model organism in eco-evo-devo studies (Fraune et al., 2016; Hand & Uhlinger, 1994). *N. vectensis* reproduces both sexually and asexually and its full life cycle can be maintained under laboratory conditions. It is gonochoric and sexual reproduction is triggered by changes in light exposure, food intake and increased temperature. Adult females release several hundreds of oocytes embedded in gelatinous sacks, while adult males release sperms directly into the surrounding water (Fritzenwanker & Technau, 2002; Hand & Uhlinger, 1992; Stefanik et al., 2013). The embryos develop inside the gelatinous sack and, within one to two days, ciliated planula larvae hatch from the oocytes and are released into the water where they remain freely swimming until settlement. After settlement, the planulae metamorphose into primary polyps. Under optimal conditions, the sexual maturity is reached within three to four months (Hand & Uhlinger, 1992).

Previous studies showed that adult *N. vectensis* harbors a specific microbiota whose composition changes in response to different environmental conditions and among geographic locations (Mortzfeld et al., 2016). In addition, sampling of different body regions (physa, mesenteries and capitulum) of the adult revealed a specific microbiota for each region, with specific dominance of spirochaetes bacteria within the capitulum

(Bonacolta et al., 2021). Also changes in the diel lighting cycle induced differences in composition and relative abundance in *N. vectensis* microbiome (Leach et al., 2019). It has also been observed that different life stages (larva, juvenile and adult) host specific microbiota (Domin et al., 2018; Mortzfeld et al., 2016).

In this study, we aimed at understanding how microbial symbionts are transmitted through generations and established in early life stages. Through metabarcoding of the 16S rRNA gene, we analyzed the microbial community composition in separated pairs of adult female and male polyps, and their corresponding newly released gametes, planula larvae and primary polyps. The comparisons to the corresponding medium microbiota allowed us to identify bacterial species that are specifically host associated and putatively maternally and paternally transmitted to the offspring.

Materials and methods

Animal culture

N. vectensis anemones were F1 offspring of CH2XCH6 individuals collected from the Rhode River in Maryland, USA (Fritzenwanker & Technau, 2002; Hand & Uhlinger, 1992). Adult polyps were cultured in *N. vectensis* medium (NM) composed of 12.5 ppt artificial sea water (Red Sea) and maintained at 18 °C in the dark. They were fed five times a week with freshly hatched *Artemia* brine shrimps (Ocean Nutrition Sep-Art Artemia Cysts). Embryos were raised at 21 °C in the dark and planulae or polyps were collected.

Spawning induction and fertilization

Five adult female polyps (labelled with numbers) and five adult male polyps (labelled with letters) were starved for 4 days prior to fertilization at standard conditions (18 °C, in the dark), to avoid contamination from the food. A day before induction the animals were washed three times with sterile NM (autoclaved and filtered on 0.22 µm membrane) and separated into sterile six well plates. The induction was performed by exposing both males and females to increased temperature (25 °C) and light for 13h. After spawning, the adults were washed three times with sterile NM, snap-frozen in liquid nitrogen and stored at -80 °C until DNA extraction. As control, the media where each female was kept before and during induction was filtered on a 0.22 µm membrane and stored at -80 °C for DNA extraction. Sperms and oocyte sacks were individually collected into 1.5 ml tubes. Half of the sperms from each male polyp was collected for DNA extraction and the other half was used for fertilization of the oocytes. The sperms for DNA extraction were washed 3 times in sterile NM and stored at -80 °C until processing. Each oocyte sack (here referred as oocytes) was washed three times in sterile NM and cut in two halves using a sterile scalpel; one half was collected for DNA extraction and the other half was fertilized. Four days post fertilization (dpf), fertilized oocytes developed into planulae. Half of the planulae from each oocyte sack was washed three times with sterile NM and collected for DNA extraction, the other half was kept in the incubator for further development. Ten dpf, planulae developed

into primary polyps that were washed three times with NM and collected for DNA extraction (**Figure 1**).

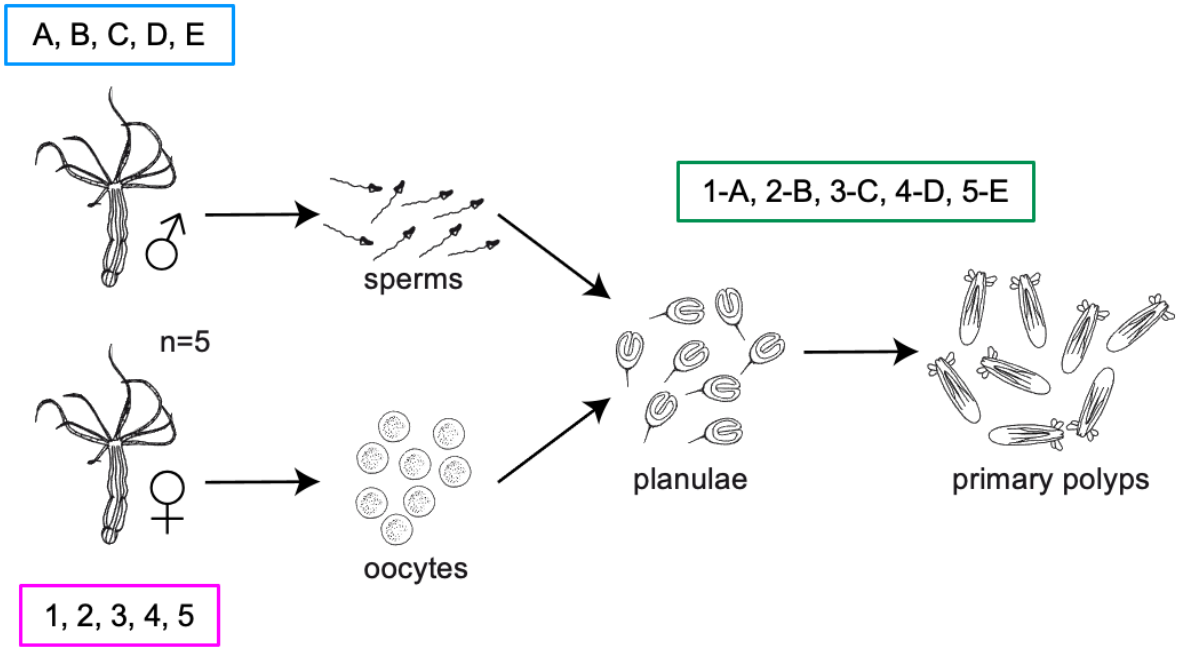

Figure 17. Experimental design. Five pairs of parent polyps were induced for spawning. Their gametes and offspring were collected separately and used for further development and 16S rRNA gene sequencing.

DNA extraction and 16S rRNA sequencing

The gDNA was extracted from adult animals, oocyte sacks, sperms, planulae and primary polyps, with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) as described in the manufacturer's protocol. DNA was eluted in 100 µl elution buffer. The eluate was kept frozen at -20 °C until sequencing. For each sample the hypervariable regions V1 and V2 of bacterial 16S rRNA genes were amplified. The forward primer (5´- **AATGATACGGCGACCACCGAGATCTACAC** XXXXXXXX TATGGTAATTGT AGAGTTTGATCCTGGCTCAG-3´) and reverse primer (5´- **CAAGCAGAAGACGGCATACGAGAT** XXXXXXXX AGTCAGTCAGCC TGCTGCCTCCCGTAGGAGT -3´) contained the Illumina Adaptor (in bold) p5 (forward) and p7 (reverse) (Fadrosh et al., 2014). Both primers contain a unique 8 base index (index; designated as XXXXXXXX) to tag each PCR product. For the PCR, 100 ng of template DNA (measured with Qubit) were added to 25 µl PCR reactions,

which were performed using Phusion Hot Start II DNA Polymerase (Finnzymes, Espoo, Finland). All dilutions were carried out using certified DNA-free PCR water (JT Baker). PCRs were conducted with the following cycling conditions (98 °C—30 s, 30 × [98 °C—9s, 55 °C—60s, 72 °C—90s], 72 °C—10 min) and checked on a 1.5% agarose gel. The concentration of the amplicons was estimated using a Gel Doc TM XR+ System coupled with Image Lab TM Software (BioRad, Hercules, CA USA) with 3 µl of O'GeneRulerTM 100 bp Plus DNA Ladder (Thermo Fisher Scientific, Inc., Waltham, MA, USA) as the internal standard for band intensity measurement. The samples of individual gels were pooled into approximately equimolar sub-pools as indicated by band intensity and measured with the Qubit dsDNA br Assay Kit (Life Technologies GmbH, Darmstadt, Germany). Sub-pools were mixed in an equimolar fashion and stored at -20 °C until sequencing. Sequencing was performed on the Illumina MiSeq platform with v3 chemistry (Rausch et al., 2016). The raw data are deposited at the Sequence Read Archive (SRA) and available under the project ID PRJNA737505.

Analyses of bacterial communities

A total of 35 samples belonging to five separated families (five mothers, five fathers, five sperm batches, five oocyte sacks, five planulae batches, five primary polyps batches and five medium controls) were submitted for 16S RNA gene sequencing. The 16S rRNA gene amplicon sequence analysis was conducted through the QIIME2 2021.4 platform (Bolyen et al., 2019). Sequences with 100% identity were grouped into ASVs and clustered against the SILVA RNA reference database (Quast et al., 2013; Yilmaz et al., 2014). Denoising and quality filtering were performed through the DADA2 plugin implemented in QIIME2 (Callahan et al., 2016, p. 2). A sample with less than 7000 reads was removed from the dataset, being considered as outlier. For the successive analysis, the number of ASVs per sample was normalized to 7000 reads.
Alpha-diversity was calculated using the Faith's PD, evenness, dominance and the total number of observed ASVs metrics implemented in QIIME2. Statistical significance was tested through the non-parametric Kruskal-Wallis test through QIIME2 and JASP 0.14.1 (JASP Team, 2020).
Beta-diversity was calculated in QIIME1 (Caporaso et al., 2010) and QIIME2 according with the different beta-diversity metrics available (Binary-Pearson, Bray-Curtis,

Jaccard, Weighted-Unifrac and Unweighted-Unifrac). Statistical values of clustering were calculated using the nonparametric comparing categories methods PERMANOVA and Anosim implemented in QIIME2.

Bacterial ASVs associated with specific conditions were identified through LEfSe (Segata et al., 2011). LEfSe uses the non-parametric factorial Kruskal-Wallis sum-rank test to detect features with significant differential abundance, with respect to the biological conditions of interest; subsequently LEfSe uses Linear Discriminant Analysis (LDA) to estimate the effect size of each differentially abundant feature. To identify vertically transmitted bacteria ASVs, we performed pairwise comparisons of the surrounding medium microbiota with the microbiota of the *N. vectensis* samples. We were then able to infer ASVs associated with each animal life stage and therefore putative bacterial candidates for vertical transmission**.** The results obtained from LEfSe analyses were cross-checked against the outcomes of a logical test based on presence/absence data, performed directly on the ASV table generated for each sampled family. We assumed that a maternally transmitted bacterium should be present simultaneously in the microbiota of mother polyps, oocytes and planulae but absent from the medium and a paternally transmitted bacterium should be present in father polyps, sperms and planulae but absent from the medium. Bacterial ASVs that might be acquired horizontally from the medium by the early life stages were filtered by their concurrent presence in planula or primary polyps and medium and absence in the gametes.

Results

Microbiota associated with *N. vectensis* tissues is distinct from surrounding medium

The experimental setup allowed us to assign gametes and offspring to their parent polyps in order to identify bacterial colonizers that are likely transmitted vertically among separated animal families and to differentiate them from the surrounding medium (**Figure 1**).

The sequencing was successful for 34 samples. After filtering and denoising, 2325 different ASVs were detected, with the number of reads per sample ranging between 678748 and 7026 (**Table S1**). Beta-diversity analyses revealed that bacterial communities from the surrounding medium were distinct from those associated with host tissue (**Figure 2A, Table 1**), indicating a specific bacterial colonization in all life stages of *N. vectensis*. In contrast, alpha-diversity analyses revealed no significant differences between the bacterial communities of the medium to the bacterial communities associated with host tissues (**Figure 2B, Table S2**). Although the medium showed a similar species richness compared to the animal tissues (**Figure 2B**), bacterial species in the medium showed lower diversity at the phylum level compared to those associated with the host (**Figure 2C**), *e.g.,* Spirochaetota and Firmicutes are absent in the medium. In comparison, the host bacterial communities showed a higher evenness and lower dominance (**Figure 2C, Table S2**) and harbored uniquely more bacterial species than the medium, with an overlap of 161 ASVs shared between both medium and host (**Figure S1**). Given that the life stages of *N. vectensis* associate with specific bacterial communities distinct from the environment, a portion of these is likely to be non-random.

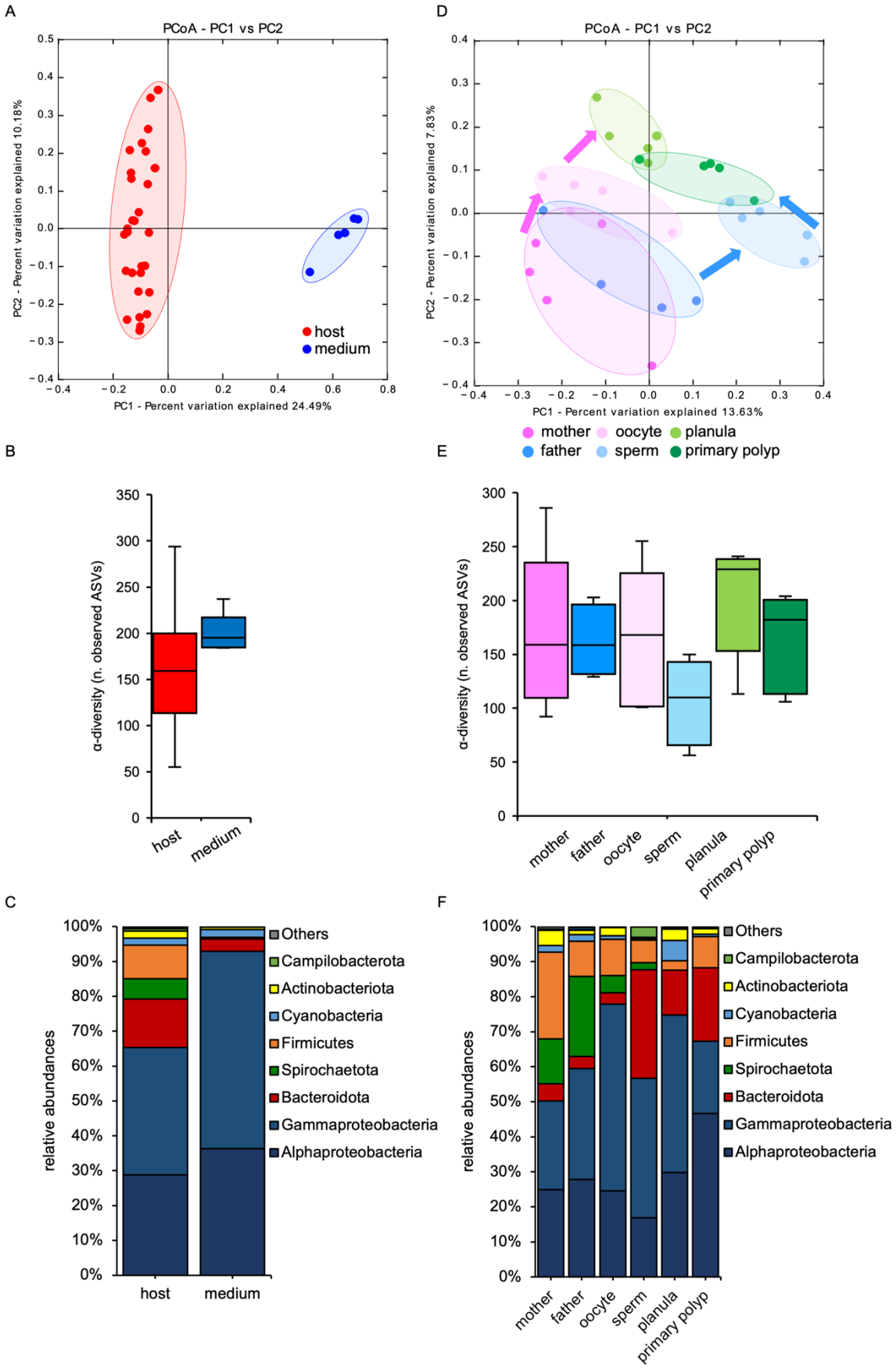
A
PCoA - PC1 vs PC2
PC2 - Percent variation explained 10.18%
PC1 - Percent variation explained 24.49%
host
medium
D
PCoA - PC1 vs PC2
PC2 - Percent variation explained 7.83%
PC1 - Percent variation explained 13.63%
mother
father
oocyte
sperm
planula
primary polyp
B
α-diversity (n. observed ASVs)
host
medium
E
α-diversity (n. observed ASVs)
mother
father
oocyte
sperm
planula
primary polyp
C
relative abundances
host
medium
Others
Campilobacterota
Actinobacteriota
Cyanobacteria
Firmicutes
Spirochaetota
Bacteroidota
Gammaproteobacteria
Alphaproteobacteria
F
relative abundances
mother
father
oocyte
sperm
planula
primary polyp
Others
Campilobacterota
Actinobacteriota
Cyanobacteria
Firmicutes
Spirochaetota
Bacteroidota
Gammaproteobacteria
Alphaproteobacteria

Figure 18. Microbiota diversity analyses among sample source and life stage. (A) PCoA (based on Binary-Pearson metric, sampling depth = 7000) illustrating similarity of bacterial communities based on sample source; (B) alpha-diversity (observed ASVs) comparison of medium and animal tissue samples (max rarefaction depth = 7000, num. steps = 10); (C) relative abundance of main bacterial groups between host and medium samples; (D) PCoA (based on Binary-Pearson metric, sampling depth = 7000) illustrating similarity of bacterial communities based on developmental stage; (E) alpha-diversity (observed ASVs) comparison by developmental stage (max rarefaction depth = 7000, num. steps = 10); (F) relative abundance of main bacterial groups among different life stages.

Table 3. Beta-diversity statistical tests comparing the different sample sources, the developmental stages and the families. Statistical analyses were performed (methods ANOSIM and PERMANOVA, number of permutations = 999) on each of the pairwise comparison distance matrices generated.

		ANOSIM		**PERMANOVA**	
parameter	**beta-diversity metric**	***R***	***p-value***	***pseudo-F***	***p-value***
Host vs medium	Binary-Pearson	0.987	0.001	10.130	0.001
	Bray-Curtis	0.940	0.001	9.757	0.001
	Jaccard	0.978	0.001	6.048	0.001
	Weighted-Unifrac	0.198	0.069	5.800	0.001
	Unweighted-Unifrac	0.865	0.001	6.559	0.001
Life stage	Binary-Pearson	0.408	0.001	1.926	0.001
	Bray-Curtis	0.488	0.001	2.682	0.001
	Jaccard	0.458	0.001	1.544	0.001
	Weighted-Unifrac	0.468	0.001	3.553	0.001
	Unweighted-Unifrac	0.339	0.001	1.614	0.001
Family	Binary-Pearson	0.004	0.420	1.041	0.347
	Bray-Curtis	0.000	0.465	0.984	0.470
	Jaccard	-0.030	0.682	1.020	0.353
	Weighted-Unifrac	-0.031	0.698	0.978	0.467
	Unweighted-Unifrac	-0.031	0.683	0.975	0.573

Specific bacterial communities colonize oocytes and sperms

Analyzing the associated bacterial communities of *N. vectensis* according with their life stages revealed a clear clustering (**Figure 2D, Table 1**). Interestingly, the bacterial communities of sperms and oocytes were distinct from those of all the other life stages

and from each other, indicating distinct mechanisms shaping the bacterial colonization of gametes (ANOSIM: R = 0.444, p = 0.003, **Figure 2D**), and a specific transmission from male and female parent polyps. Both planulae and primary polyps harbored similar bacterial communities (**Figure 2D**) that clustered between the sperms and oocytes samples, suggesting the contribution of maternal and paternal transmitted bacteria to early life stages colonization. While no significant differences in the bacterial alpha-diversity could be detected, sperms harbored bacterial communities with a slightly lower bacterial alpha-diversity compared to all the other samples (**Figure 2E, Table S3**).

In contrast, no clustering (**Figure S2A, Table 1**) and no differences in alpha-diversity (**Figure S2B, Table S4**) were evident according to family status.

Looking at the different bacterial groups, greater abundances of Spirochaetota (between 23.2 ± 17.2% and 5.2 ± 5.9%) and Firmicutes (between 27.3 ± 13.6% and 10.4 ± 7.8%) were evident in the adults and in the oocytes, while Bacteroidota were relatively more abundant in the sperms and in the offspring (between 27.6 ± 29.2% and 12.4 ± 12.4%). The abundance of Alphaproteobacteria increased from the adults (23.4 ± 10.9% and 27.2 ± 13.9% respectively) through the primary polyps, in which they reached the maximum abundance (46.4 ± 9.3%) (**Figure 2F**). These differences among life stages and sexes suggest a differential transmission of specific bacterial groups through the gametes.

Offspring bacterial colonizers originate from oocytes, sperms and surrounding medium

By using the LEfSe algorithm (Segata et al., 2011) and the pairwise comparisons between host tissues and surrounding medium, we identified 15 ASVs that were significantly more abundant in mother polyps, oocytes and planulae (**Figure 3A, Table S5**) and 5 ASVs that were significantly more abundant in father polyps, sperms and planulae (**Figure 3B, Table S5**), than in the surrounding medium. By overlapping these results with those obtained from the presence-absence calculations (**Table S1**), we were further able to filter the LEfSe results to those candidates that were completely absent from the medium in any of the animal families (**Table S6**). We ended up with 13 ASVs potentially transmitted by the mother and 5 ASVs potentially transmitted by the father (**Figure 3C**). The ASVs potentially transmitted by both mother and father polyps belong to the classes Alpha- and Gammaproteobacteria (**Table S6**).

In addition, one ASV potentially transmitted by mother polyps is a member of the Firmicutes phylum, while father polyps potentially transmit one member of the Bacteroidota phylum (**Figure 3C**). The high resolution of the presence-absence analysis, allowed us to also point out a slight variability in bacterial transmission that occurs between different families (**Table S6**).

In a final step we aimed at detecting bacterial ASVs that are present in the offspring and in the medium but not in the gametes, and thus, likely acquired horizontally from the medium. For planulae and primary polyps, we detected 24 and 25 ASVs respectively, with an overlap of 10 ASVs shared between both developmental stages (**Table S6**). In both planulae and primary polyps, these bacteria belonged to the classes Alpha- and Gammaproteobacteria, and the phyla Bacteroidota and Actinobacteriota. In addition, the primary polyps shared with the medium also four members of the Firmicutes (**Figure 3D**). These results suggest that each developmental stage is able to associate with different and specific environmental symbionts.

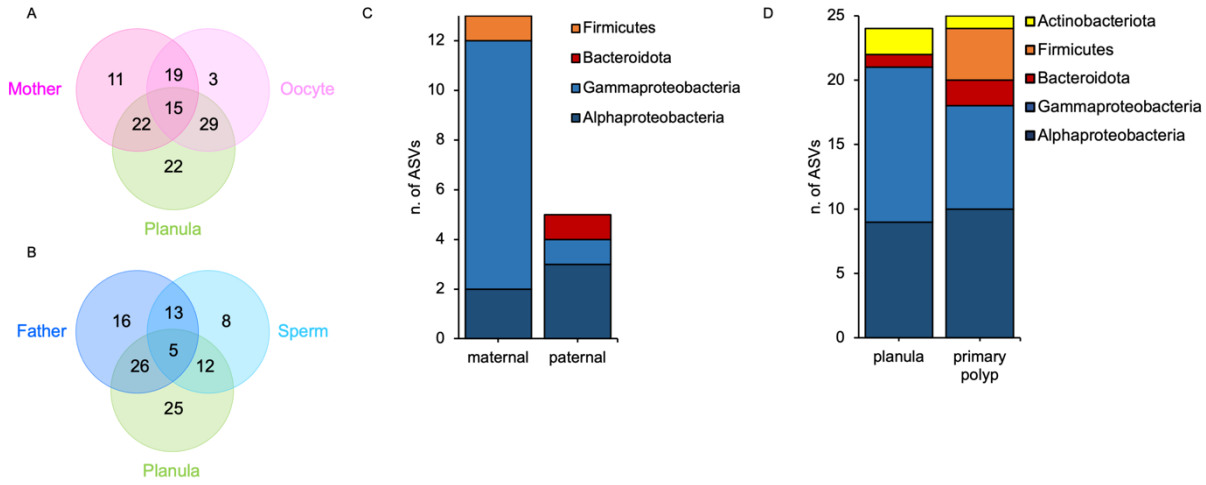

Figure 19. Vertical and horizontal contributions to offspring microbiota. (A) Venn diagram showing the number of ASVs shared between mothers, oocytes and planulae; (B) Venn diagram showing the number of ASVs shared between fathers, sperms and planulae; (C) Bar-chart of parentally transmitted bacterial ASVs divided by major groups; (D) Bar-chart of environmentally transmitted bacterial ASVs divided by major groups.

Discussion

Maternal and paternal transmission of bacterial symbionts

A reliable transfer of specific symbionts is required to maintain the bacterial associations across generations. Thereby, vertically transmitted bacteria may represent beneficial symbionts, necessary for animals' development and physiology, bacteria that lack a free-living stage, or more simply, bacteria that are present in the open water in too low abundance to be recruited (Bright & Bulgheresi, 2010; Salerno et al., 2005). So far, few studies have undertaken a detailed comparison of microbial communities of parents, gametes and progeny in marine organisms (Bernasconi et al., 2019; Bright & Bulgheresi, 2010; Damjanovic, Menéndez, Blackall, & Oppen, 2020; Damjanovic, Menéndez, Blackall, & van Oppen, 2020; Igawa-Ueda et al., 2021; Leite et al., 2017; Oliveira et al., 2020; Quigley et al., 2017, 2018, 2019; Sharp et al., 2012; Sipe et al., 2000).

Our results, consistent with results obtained in former studies on broadcast spawning corals (Quigley et al., 2017; Sharp et al., 2010), indicate that *N. vectensis* transmits microbial symbionts to its offspring mainly maternally. Current knowledge is limited on the contribution of male parents to the progeny microbiota (Damiani et al., 2008; De Vooght et al., 2015; Watanabe et al., 2014); nevertheless, some examples exist regarding marine invertebrates (JL Padilla-Gamiño, 2012; Kirk et al., 2013; Leite et al., 2017; Sharp et al., 2010; Usher et al., 2005). In our study it seems that also male polyps transmit specific bacteria to the next generation.

As suggested from previous studies on corals, we hypothesize that in *N. vectensis* mother polyps incorporate bacterial colonizers into the mucus bundles that surround the oocytes (Ceh et al., 2012; Leite et al., 2017; Ricardo et al., 2016), while the sperms may acquire the bacteria before fertilization by horizontal transmission through water, which contains bacteria released by the parents (van Oppen & Blackall, 2019). This strategy is less specific than strict vertical transfer, however, not as non-specific as random horizontal acquisition of seawater communities (Ceh, van Keulen, et al., 2013).

Our results are supported by fluorescence in situ hybridization (FISH) approaches applied on coral larvae and gametes, indicating the presence of bacteria in the

ectoderm of brooded larvae (Sharp et al., 2012), but not inside gametes, embryos and larvae of several broadcast spawners (Sharp et al., 2010).

Offspring microbiota results from mixed-mode bacterial transmission

N. vectensis male and female polyps transmit different bacterial species through their gametes, with the oocytes contributing the most to the bacterial assemblage of the planulae, thus indicating distinct selecting forces. For instance, through the oocyte bundles, the mothers might provide the developing embryos with specific antimicrobial peptides (Fraune et al., 2010) and, in the case of *N. vectensis* also with nematosomes, multicellular motile bodies with putative defense function (Babonis et al., 2016). In vertebrates, like birds, fishes, and reptiles, passive immunity is transmitted through the deposition of antibodies in eggs (Grindstaff et al., 2003) and the fertilization envelope of fish eggs has demonstrated bactericidal activity (Kudo & Inoue, 1989).

Although not yet demonstrated, the bigger size of oocytes and the presence of a cytoplasm, in comparison to the sperm, may offer more room for carrying symbionts on the surface and/or intracellularly; sperms on the other hand, may carry strict symbionts in the nucleus (Usher et al., 2005; Watanabe et al., 2014). Additionally, the offspring can be partly colonized post-spawning through the uptake of microbial associates released by the parents into the surrounding seawater, as previously described (Apprill et al., 2009; Ceh et al., 2012; Ceh, van Keulen, et al., 2013; Sharp et al., 2010).

As already observed, the early life stages and adult microbiota in *N. vectensis* differ significantly (Domin et al., 2018; Mortzfeld et al., 2016). Our results suggest that a portion of the early life stages microbiota is the result of a parental transmission while another part is horizontally acquired from the environment during ontogeny. This hypothesis is supported also by the tendency of higher bacterial species richness associated with the early developmental stages in comparison to that of the adults. Higher alpha-diversity in the early life stages has been described also in other studies on marine animals (Epstein, Torda, et al., 2019; Fieth et al., 2016; R. A. Littman, Willis, & Bourne, 2009; Mortzfeld et al., 2016; Nyholm & McFall-Ngai, 2004) indicating an host filtering window during which the microbiota is shaped to a more stable community. This can relate with the different ecological (*e.g.,* pelagic vs benthonic, motile vs sessile, preying vs filter-feeding) and/or developmental requirements across

the life stages of animals that have complex life cycles (Bosch & McFall-Ngai, 2021; Mortzfeld et al., 2016; H. M. Putnam, 2021). *N. vectensis'* life cycle includes a pelagic, freely swimming, not feeding planula larva and benthonic, sessile, preying primary polyp and adult stages. Therefore, it is easy to imagine that the symbiotic microbial community is impacted by the deep ecological and morphological changes during host development and that specific bacterial species may provide different benefits to the different life stages. As already pointed out (Fraune et al., 2010), organisms in which embryos develop outside the mother's body, in a potentially hostile environment, use a "be prepared" strategy involving species-specific, maternally produced antimicrobial peptides for protection. These antimicrobial peptides not only have bactericidal activity but also actively shape and select the colonizing bacterial community (Fieth et al., 2016; Fraune et al., 2010). It is likely that also *N. vectensis* is able to employ different mechanisms to shape and control its symbiotic microbiota in a life stage-specific manner.

Consistently with previous studies (Berg et al., 2020; Deines et al., 2017; Gilbert et al., 2012; Glasl et al., 2019; Goldsmith et al., 2018; Hernandez-Agreda et al., 2017; Sullam et al., 2018), our results showed that between different parents a slight variability of vertically and environmentally transmitted bacteria exists, highlighting the potential impact of host genotype and stochastic events on symbiotic community establishment of offspring.

The hologenome theory of evolution (Zilber-Rosenberg & Rosenberg, 2008) proposed that microbiome mediated plasticity of the host phenotype can be under selection pressure and may contribute to animal adaptation. Vertical transmission of the microbiota could therefore facilitate transgenerational adaptation of animals to changing conditions (N. S. Webster & Reusch, 2017), while horizontal transmission may mitigate some of the deleterious consequences of obligate host-association/strict vertical transmission (*e.g.,* genome degradation and reduction of effective population size) (Russell, 2019). Concertedly, vertical transmission may secure the maintenance of coevolved beneficial bacteria, while horizontal acquirement of new bacterial partners increases the flexibility of beneficial effects under changing environmental conditions. Future studies should compare the function of vertically transmitted and horizontally acquired bacterial associates, providing important insights into the potential of microbial communities to promote animal adaptation to changing environmental conditions.

DISCUSSION

In a perspective of dramatic global changes taking place, is urgent to understand how organisms that are sessile might adapt in time-lapses as short as an individual's lifespan and if and how this adaptation is transmittable to the next generations or to individuals that didn't experience such challenging conditions before. In this context, symbiotic microorganisms are gaining more and more attention as possible target to foster rapid evolution of multicellular species (*e.g.,* reviewed in (Marangon et al., 2021). Micro-symbionts are expected to adapt faster than their hosts, due to shorter generation times and much greater population sizes (Bang et al., 2018). These associations are mediated by communication through various chemical compounds. This interkingdom cell-to-cell signaling involves small molecules such as hormones that are produced by eukaryotes and hormone-like small molecules that are produced by bacteria (Pacheco & Sperandio, 2009). Bacterial signals are also able to modulate signal transduction and immune responses of animal hosts, and, conversely, host hormones can modulate bacterial gene expression (Kravchenko et al., 2008) and so, promote metaorganism assembly and resilience (Pietschke et al., 2017). Host adaptation may thus be supported by bacterial horizontal gene transfer (HGT) (exchange of genetic material, including mobile genetic elements (MGEs) such as plasmids, transposons, and phages), symbiont shuffling (proportional changes of microbiome members) and switching (loss or acquisition of microbes) and mutations in bacterial genomes (Bosch & Miller, 2016; Fraune et al., 2016; McFall-Ngai et al., 2013; Reshef et al., 2006; Theis et al., 2016; Voolstra & Ziegler, 2020; N. S. Webster & Reusch, 2017). In particular, microbial symbionts may provide their hosts with rare nutrients (Bruno et al., 2003; Ceh, Kilburn, et al., 2013), defensive molecules (Fraune et al., 2015; Garren et al., 2016), compounds able to promote and induce larval settlement and metamorphosis (Tran & Hadfield, 2011; N. S. Webster et al., 2004); and, specifically under heat stress, with HSP (heat shock proteins), ROS (reactive oxygen species) scavenging enzymes (Barshis et al., 2013; Diaz, 2016; Hamdoun et al., 2007) or even mycosporine-like amino acids (MAAs) with anti-UV protection function (Dunlap & Shick, 1998; T. Banaszak et al., 2000). Furthermore, beneficial symbionts can outcompete microbial opportunists and/or pathogens that might be favored from challenging environmental conditions by occupying their specific niches

or by secreting antimicrobial peptides (CJ Krediet et al., 2013; Gignoux-Wolfsohn et al., 2017; Gil-Turnes et al., 1989; Krediet et al., 2013; Ritchie, 2006).

Since Reshef and colleagues developed the “Coral Probiotic Hypothesis” (Reshef et al., 2006), a wide range of studies has investigated the possibility to sustain endangered natural population by manipulating their associated microbiota. Assisted evolution is defined as the process whereby human intervention is used to accelerate the rate of naturally occurring evolutionary processes (Foo & Byrne, 2016). In this kind of studies, the symbionts are subjected to ocean change stressors in the laboratory to identify strains with enhanced tolerance (MJH Oppen et al., 2015), larvae and juveniles can then be inoculated with these stress-tolerant symbionts. Although these inoculation experiments returned promising results, some points still need to be addressed, *e.g.*, which microbial taxa are essential for metaorganism functioning, and which are functionally redundant, and which are the best delivery approaches to reach the target, with minima inoculum loss and environmental impact (Peixoto et al., 2021). Simple metazoan models can help understand the mechanisms behind the adaptation process and detecting tools to sustain the natural populations dealing with it. In this study we investigated, through a bigger dataset than used in (Mortzfeld et al., 2016), how the lab culturing under both even and distinct conditions may impact the microbiota composition of animals with a different genetical and biogeographic background. We found out that changes in environmental conditions (*i.e.,* provenance geographic location, sampling season, field vs lab culturing, different culture temperatures) induce specific physiological responses in animals originated from different natural populations and in their associated microbiota. We could detect a generalized loss of alpha- and beta-diversity when transitioning from the field to the lab and under extreme seasons and temperatures. Nevertheless, below these major differences, a minor specific biogeographic and individual fingerprint in the microbiota composition was always maintained, even after long periods of captivity and acclimation. In the light of these evidences, we wanted to assess the impact of a long-term acclimation process on the physiology and the microbiota composition in genetically identical animals and investigate the role that the microbiota itself might play in this process. We saw that, although originated from the same clonal strain, adult animals showed significant differences in survival, reproduction rate, size, transcriptomic profiles and heat-shock tolerance when acclimated at different thermal conditions. Interestingly this last characteristic could be transmitted to unacclimated

offspring via microbiota vertical transmission and to unacclimated germ-free adults via microbiota transplantation. To assess the specificity and consistency of these bacterial associations we investigated their dynamics between different life stages, gametes and generations. We could prove that each *N. vectensis* life stage is associated with a specific microbial community that is consistent between independent replicates and therefore, is not-random. In addition, male and female parent polyps transmit different and specific symbiont species to the offspring, while a portion of the microbiota is acquired horizontally from the environment. Furthermore, in a complementary study from our team, has recently been shown that bacterial colonizers of *N. vectensis* are initially selected by the host and when recolonization is induced in adults, it follows the ontogenetic colonization pattern. Successively, the subsequent colonization is determined by bacteria-bacteria interactions (Domin et al., 2022).

Despite these intriguing outcomes, many points still require to be addressed: i) where are the microbiota elements allocated on the host body (*i.e.,* gametes, early stages and adults, males and females)? ii) What specific functions are the associated symbionts exerting (*e.g.,* out-competition of pathogens, secretion of protective compounds, etc.)? iii) Which is the degree of plasticity and functional redundancy within the *N. vectensis* associated microbial community? In particular, is the acclimation support provided by few strictly associated species or by the community as a whole and/or are several symbiont species interchangeable under changing conditions? iv) What are the weight and the role of other players, *e.g.,* viruses and archaea within the associated community? v) What are the relative proportions between host and microbiota contribution in the acclimation process? vi) Will the great plasticity of *N. vectensis* and its microbiota be sufficient to cope with the upcoming dramatic environmental changes, and/or will certain populations be more susceptible than others? vii) In the perspective of assisted evolution approach for *N. vectensis* natural populations, would the inoculation with resistance-conferring symbionts be feasible and effective?

All these points are of pivotal interest at the purpose of taking advantage of the model metaorganism *N. vectensis* in order to gain general knowledge and develop strategies applicable also on other challenged species.

CONCLUSIONS

In this study we confirmed the suitability of *N. vectensis* as a plastic metaorganism, capable of adapting to rapidly changing conditions and that its symbiotic microbiota is actively involved in the process. We demonstrated that the associations between *N. vectensis* and its symbionts are specific, consistent but also actively shaped by the host in response to developmental and environmental cues. We showed that, although interfertile, *N. vectensis* geographically distant natural populations are subjected to local adaptation processes that also impact the microbiota composition and that individuals with different biogeographic and genetic background show different susceptibility to changing conditions. Furthermore, we have been able to experimentally transfer the thermal resistance of acclimated animals to unacclimated ones via microbiota transplantation alone. These results support the hypothesis that recolonizing individuals of natural populations subjected to challenging environmental conditions, with thermal resistance-conferring bacterial strains, might represent an efficient strategy for the sustaining of endangered ecosystems.

AUTHOR CONTRIBUTIONS

1. *Genotype–environment interactions determine microbiota plasticity in the sea anemone Nematostella vectensis*

Laura Baldassarre, Adam M. Reitzel, Sebastian Fraune

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Participation in	**Author initials***
Study design	SF/AR/**LB**
Experimentation	**LB**, AR
Data analysis, interpretation	**LB**/SF
Manuscript writing	**LB**, SF
Manuscript reviewing	**LB**, SF, AR

2. *Microbiota mediated plasticity promotes thermal adaptation in the sea anemone Nematostella vectensis*

Laura Baldassarre, Hua Ying, Adam M. Reitzel, Sören Franzenburg, Sebastian Fraune

Nature Communication, 13, 3804, https://doi.org/10.1038/s41467-022-31350-z

Participation in	**Author initials***
Study design	SF/**LB**/AR
Experimentation	**LB**, SöF
Data analysis, interpretation	**LB**/HY, SF
Manuscript writing	**LB**, SF
Manuscript reviewing	**LB**/SF, HY, AR

3. *Contribution of Maternal and Paternal Transmission to Bacterial Colonization in Nematostella vectensis*

Laura Baldassarre and Shani Levy, Rinat Bar-Shalom, Laura Steindler, Tamar Lotan, Sebastian Fraune

Frontiers in Microbiology, 12, 1664-302X, https://doi.org/10.3389/fmicb.2021.726795

Participation in	**Author initials***
Study design	SF/LS/RB-S, **LB**, SL, TL
Experimentation	SL, RB-S, **LB**
Data analysis, interpretation	**LB**, SF

Manuscript writing	**LB**, SF
Manuscript reviewing	**LB**, SF, LS, RB-S, SL

*Responsibility decreasing

Additional published manuscripts that I contributed to:

- *The Role of DNA Methylation in Genome Defense in Cnidaria and Other Invertebrates*

Hua Ying, David C. Hayward, Alexander Klimovich, Thomas C.G. Bosch, **Laura Baldassarre**, Teresa Neeman, Sylvain Forêt, Gavin Huttley, Adam M. Reitzel, Sebastian Fraune, Eldon E. Ball, David J. Miller

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