Impacts of near-future ocean warming on microbial community composition of the stomach of the soft-bottom sea star Luidia clathrata (Say) (Echinodermata: Asteroidea)

Michelle D Curtis (✉ curtismi@uab.edu)

The University of Alabama at Birmingham   https://orcid.org/0000-0001-8360-8991

Casey D Morrow

The University of Alabama at Birmingham

James B McClintock

The University of Alabama at Birmingham

Research Article

Abstract

There is growing evidence that environmental changes caused by climate change can impact the microbiome of marine invertebrates. Such changes can have important implications for the overall health of the host. In the present study we investigated the impact of chronic exposure to an ambient (28°C) and a predicted mid- (30°C) and end-of-century (32°C) seawater temperature on microbiome modification in tissues of the cardiac stomach of the abundant predatory sea star Luidia clathrata collected in September 2018 from Apalachee Bay, Florida (29°58'N, 84°19'W) in the northern Gulf of Mexico (GOM). Diversity (Shannon index) was lowest among the microbial community of stomach tissue when compared to the microbiome of the artificial sea star feed, and aquarium sand and seawater across all three experimental temperature treatments. Moreover, the stomach microbial community composition was distinct between each of the four sample types. Exposure to the highest experimental temperature treatment (32°C) resulted in a significant modification of the composition of the microbial community in stomach and sand samples, but not in seawater samples when compared to those from the current mean ambient GOM temperature (28°C). Importantly, at the most elevated temperature the stomach microbiome shifted from a Vibrio sp. dominated community to a more diverse community with higher proportions of additional taxa including Delftia sp. and Pseudomonas sp. This microbiome shift could impact the digestive functionality and ultimately the health of L. clathrata, a key soft-bottom predator in the northern GOM.

Introduction

Mean ocean surface temperatures have risen approximately 0.6°C over the last century due to global climate change and are projected to warm up to 4°C by the year 2100 (IPCC, 2019). This warming has already impacted the structure of benthic marine invertebrate communities by forcing shifts in geographic distributions of various species (Schiel et al. 2004; Parmesan 2006), introducing non-native species (Przeslawski et al. 2008), and decreasing fitness of select species subject to thermal stress (Pörtner 2008). Ectothermic marine invertebrates living in shallow waters at or near their upper thermal tolerance are most at risk for deleterious impacts with continued ocean warming (Pörtner 2002; Peck et al. 2008).

Ocean warming has also been linked to increased prevalence of marine pathogens (Harvell et al. 2002) and as a result some organisms are increasingly susceptible to disease. This is particularly the case in marine ectotherms including sea urchins and sea stars (Tracy et al. 2019). For example, the large, multi-armed predatory sea star *Pycnopodia helianthoides* has experienced widescale population declines along the Pacific coast of North America resulting from an outbreak of sea star wasting disease thought to be linked to ocean warming (Harvell et al. 2019). Ocean warming has also been associated with increased morbidity and mortality from disease in the keystone sea star *Pisaster ochraceus* (Bates et al. 2009; Eisenlord et al. 2016; Kohl et al. 2016). Exposure to increased seawater temperature can induce more benign impacts in stars including accelerated growth and elevated feeding rates (Watts and Lawrence 1986, 1990; Eckert et al. 2000; Gooding et al. 2009; Fly et al. 2012; Monaco et al. 2014). However,

sublethal stress impacts from elevated temperature may also occur including higher metabolic demand, decreased immune response (Watts and Lawrence 1986, 1990), and arm autotomy in sea stars (Barker et al. 1992; Haramoto et al. 2007; Barker and Scheibling 2008).

Microbial communities impact a variety of aspects of host physiological function through their influence on host behavior, contribution to metabolic functioning, and maintenance or enhancement of immune response (reviewed in Holmes et al. 2011; Kinross et al. 2011; Vuong et al. 2017). Conversely, microbial community dynamics are affected by the genetics, behavior, and physiological state of the host (Spor et al. 2011; Foster et al. 2015; Gomez et al. 2017). This delicate balance between host-microbiome interactions can be disrupted by changes in host physiology or by changes in the external environment (Lynch and Hsiao 2019). Disruption of this balance can have deleterious consequences for the host, which may in turn lead to significant ecological impacts if the host is an important player in the community. A recent example of host-microbiome discordance with ecological implications is illustrated by the negative impacts of sea star wasting disease on the epidermal microbiome of the keystone sea star *Pisaster ochraceus*. Lloyd and Pespeni (2018) reported a decrease in overall epidermal microbial species richness in *P. ochraceus*, suggesting a shift away from bacterial taxa with functional attributes that supported an immune response to pathogenic microbial typical of sea star wasting disease. In a separate comparative study, disease progression similarly led to a shift in the microbial community structure of the gonad, body wall, tube feet, and pyloric ceca of four species of coralivorous crown-of-thorns sea stars in the genus *Acanthaster* (Høj et al. 2018).

The microbial community of the vertebrate gut also plays a crucial role in host-microbiome dynamics and host health (Kinross et al. 2011; D'Argenio and Salvatore 2015; Shreiner et al. 2015). The same key roles of microbes are likely to occur in marine invertebrate digestive systems. For example, Brothers et al. (2018) found that chronic exposure to an elevated seawater temperature aligned with predicted near-future ocean warming significantly altered the presumptive functionality of the gut microbiome of the common subtropical sea urchin *Lytechinus variegatus*. Importantly, Brothers et al. (2018) found that the predicted microbial metagenomics impacted were those related to host physiological processes of membrane transport and metabolism of amino acids and carbohydrates, suggesting that individuals are likely to face physiological challenges as seawater temperatures continue to rise. Moreover, *L. variegatus* evaluated in this study were collected from the northern Gulf of Mexico (GOM) where population migration northward to escape currently rising seawater temperature is not feasible. If the ultimate outcome of changes in host microbiome structure and functionality is an increase in sub-lethal or lethal impacts on ecologically important species such as sea urchins and sea stars, then there will be important downstream ecological consequences on benthic community structure. Despite the potential significance of rapid anthropogenic climate warming impacting the gut microbiome of a model representative sea urchin that plays a key role in seagrass ecology (Brothers et al. 2018), to date, no studies have similarly examined temperature-sensitivity of the gut (stomach) microbiome of a model sea star.

Luidia clathrata is a key predatory, soft-bottom sea star in the family Luidiidae that has the appropriate attributes to make it a model species for the study of the impacts of ocean warming on the stomach

microbiome. The species is widely distributed and can be found in abundance in near-shore habitats throughout the GOM, as well as nearshore regions of the southeastern Atlantic coast of the U.S. north to North Carolina, coastal environments of northeastern South America, and throughout the Caribbean (Hendler 1995; Lawrence 2013; Lawrence et al. 2013). Its high abundance in shallow (< 40 m depth), protected, bays along the northern GOM, a region projected to experience disproportionately high rates of ocean warming and lacking northward refugia (Przeslawski et al. 2008). Accordingly, *L. clathrata* sampled from this region provide an excellent model for the examination of prospective climate-induced impacts of ocean warming.

In contrast to the complex intestinal tract of the sea urchin *L. variegatus* (Holland 2013), the digestive morphology of *L. clathrata* is comparatively simple. Essentially the digestive system consists of the mouth, cardiac stomach, and five pyloric ceca, the latter used largely in final digestive processes and nutrient storage (Jangoux 1982). Unlike many sea stars that extrude the cardiac stomach from the mouth, members of Luidiidae are generally considered to feed intraorally by depressing the oral disc into the sediment and guiding prey into the mouth with the tube feet (McClintock and Lawrence 1981). Interestingly, McClintock et al. (1983) observed extraoral feeding in *L. clathrata* via a modest extrusion of the cardiac stomach both in the field and under experimental laboratory conditions. In the latter case, powdered food added to sand induced extraoral feeding. A capacity for extraoral feeding distinguishes *L. clathrata* from other luidiid sea stars in that it allows for the exploitation of detrital food resources in addition to infaunal and epifaunal prey.

Despite the established importance of the microbiome in host health and the presumption that the impacts of climate change-induced temperature stress are likely to have negative impacts on aspects of whole animal physiology and behavior, current knowledge of how near-future ocean warming may impact host microbial community structure in marine invertebrates is largely lacking. The purpose of the present study was to experimentally evaluate the potential effects of chronic exposure to predicted near-future seawater temperatures on the stomach microbiome of the common sea star *L. clathrata* sampled from a population in the northern GOM where there no refugia from rising seawater temperatures.

Material And Methods

Acquisition and Husbandry

Adult, non arm-regenerating *Luidia clathrata* (mean arm radius (R) ± SE = 65.2 ± 1.8 mm, n = 30) were collected by standard otter trawl (1 cm mesh size) from Apalachee Bay, Florida (29°58′N, 84°19′W) in early September, 2018. Immediately following collection, each individual was placed individually into a plastic bag containing ambient seawater. The bags containing sea stars were placed into 80-L LoBoy© Styrofoam coolers (10 bagged sea stars/cooler) and shipped overnight to the University of Alabama at Birmingham (UAB). Each cooler was supplied with two 1-lb frozen gel ice packs (ChillPak©, Greiner Bio-One International). Upon arrival at UAB, sea stars were transported to a climate-controlled room. Bags containing individual sea stars and the seawater they were collected from were immediately floated in

seawater in experimental tanks at a temperature of 25°C. The room was placed on a 12:12 h light-dark cycle for the duration of an adjustment period (see below) and throughout the subsequent experiment.

To facilitate gradual acclimatization to experimental tank conditions, the seawater in each plastic bag containing a sea star was replaced with artificial seawater (25°C, salinity 27; previously prepared with Instant Ocean® mixed with reverse-osmosis water filtered through a 4-stage Aquafx Barracuda RO/DI filtration system). Seawater replacements were carried out over a period of 3 h via a series of repeated seawater exchanges. Following this adjustment period, each of the 30 individuals was randomly assigned (using a random number generator in Microsoft Excel) to fifteen 38-L glass aquaria each divided equally (each half = 25.4X12.7X30.5 cm) by a solid plexiglass plate that was sealed in place with standard aquarium silicone sealant. For purposes of clarity, 'half aquaria' will hereafter be referred to as 'separate aquaria'. Accordingly, the final assignment of sea stars was one individual per aquarium. Temperature was maintained in aquaria for each of three temperature treatments by submerging aquaria in large, oval water baths (99X147X61 cm) consisting of 568-L Rubbermaid® structural foam stock pools perched on cement blocks. Water temperature in each of the three water baths was controlled using a submersible aquarium heater (EHEIM Jager 250 W TruTemp). Each aquarium was supplied with a 3-cm deep layer of fine-grain sand (Nature's Ocean® Substrate, Marine White), a re-circulating water pump (Aqueon® Power Filter 10), and air line tubing with an air stone driven by a Coralife® Super Luft SL−65 high pressure aquarium pump.

Sea stars were held in their respective aquaria at 25°C for a two-week holding period without food prior to initiating the experiment. This allowed individuals to adjust to the aquaria and to standardize their nutritional state. Throughout this two-week period and over the subsequent holding period and experiments, water temperature, salinity, and dissolved oxygen were monitored daily using a YSI™ ProPlus meter. Moreover, to evaluate and maintain water quality, nitrate, nitrite, ammonia, and pH levels were measured weekly with an API™ Aquarium Pharmaceuticals liquid test kit. Large (1/3 aquarium volume), water exchanges were conducted weekly, and smaller water exchanges were conducted as necessary.

Following the initial two-week holding period, the water temperature in all three water baths was slowly elevated from 25 to 28°C over a period of 34 days. During this period, and during the subsequent three temperature exposure treatments, each sea star was fed an *ad libitum* diet of formulated sea star feed (formulation of feed provided by Addison Lawrence, Texas A&M University). Feed was proffered to each individual every three days and food not consumed within a 14-h period was removed from each aquarium by gloved hand. During this 34-day temperature adjustment period there were a small number of sea stars in the aquaria that suffered mortality: one in one water bath, and two in each of the other two water baths.

Prior to initiating experimental temperature treatments, three Time-Zero groups of two individuals from each of the three water baths (n = 2X3 water baths = 6 sea stars) were randomly selected using the random number generator in Microsoft Excel to establish a baseline microbial community. The cardiac stomach of each sea star was excised by lifting the stomach tissue with sterile forceps and then cutting it

free with sterile, dissecting scissors. Samples of sand and seawater were simultaneously collected from each aquarium to evaluate their associated microbial communities as controls. The sand samples were each collected by scraping sand into a sterile 50 mL falcon tube. Each sand sample was processed by using a sterilized scoopula to collect approximately 1 g wet sand from the center of each falcon tube and transferring it directly into a sterile Eppendorf tube for DNA extraction. Seawater samples were prepared by vacuum filtering 60 mL of seawater through a sterile, 0.22-μm, Millipore membrane (EMD Millipore Corporation). Stomach, sand, and seawater samples were all immediately frozen and stored at -20°C until DNA extraction.

Over the following 14 days, water temperatures in two of the three water baths were slowly increased to a projected mid-century (30°C) and end-of-century (32°C) ocean temperature based on predictions for the northern GOM (IPCC, 2019). Individuals in the control (ambient) temperature treatment were maintained at 28°C, the present-time mean July-September *in situ* temperature for the northern GOM (National Centers for Environmental Information, NOAA). After this 14-day period, all surviving individuals (n = 7 of 9, 6 of 8, and 4 of 8 for the 28°C, 30°C, and 32°C temperature treatments, respectively) were dissected and cardiac stomach tissue collected and stored as described above. Importantly, all surviving individuals examined in each temperature treatment appeared healthy and were behaviorally vigorous. Sand and seawater samples were re-collected at the end of the experiment as above for later microbiome DNA extractions, along with three samples of the formulated sea star feed.

Microbial DNA Purification and Sequence Analysis

Microbial metacommunity DNA was extracted using a Zymo Research Fecal DNA isolation kit (Catalog # D6010). An amplicon library was constructed with PCR using unique barcoded primers (Kozich et al. 2013; Kumar et al. 2014) to target the hyper variable 4 (V4) region of the 16S rRNA gene (forward primer V4: 5'AATGATACGGCGACCACCGAGATCTACACTATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3'; reverse primer V4:5'-CAAGAGAAGACGGCATACGAGATNNNNNNNAGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3) (Eurofins Genomics, Inc., Huntsville, AL). PCR was conducted with the New England Biolabs LongAmp Taq PCR kit (Catalog # E5200S) under the conditions outlined in Kumar et al. (2014). PCR products were then electrophoresed on a 1.0% agarose/TRIS-borate-EDTA gel, visualized by UV illumination, excised, and purified with a Qiagen QIAquick Gel Extraction Kit (Catalog # 2870) (Kumar et al. 2014).

Purified PCR products were quantified with Pico Green dye, adjusted to a 4 nM concentration, and sequenced on the NextGen sequencing Illumina MiSeq™ platform using an Illumina paired 250-bp kit (Caporaso et al. 2012; Kozich et al. 2013; Kumar et al. 2014). Two samples were excluded from subsequent analysis due to insufficient PCR product volume, bringing final sample numbers for the 28°C, 30°C, and 32°C treatments to (n = 6, 5, and 5, respectively).

The Quantitative Insights into Microbial Ecology package (QIIME2 v2019.10.0; (Bolyen et al. 2019) was used for all downstream data analysis. Demultiplexed sequence data was checked for quality using the DADA2 plugin in QIIME2 (Callahan et al. 2016). Due to a decrease in quality score after 250 nucleotides, the first 13 nucleotides for each forward and reverse read were trimmed and the total read length was

truncated to 250 nucleotides. Chimeric variants were removed and amplicon sequence variants (ASVs) were identified at a 97% similarity threshold according to the DADA2 pipeline (Callahan et al. 2016). Taxonomic assignments were made using reference sequences from the SILVA 132 database (Quast et al. 2012) trimmed to the V4 region with the 515f-806r primers. Representative sequences were classified using the "classify-sklearn" function of the QIIME2 feature-classifier plugin (Bokulich et al. 2018) and non-target sequences (mitochondria, chloroplast, archaea, eukaryota, unknown) were removed prior to analysis. The remaining sequences were subsampled to a maximum depth of 24,573 to account for variation in read depth using the "alpha-rarefacation" analysis command in the diversity plugin for QIIME2. Rarefaction curves for α-diversity in each sample were generated using the "alpha-rarefaction" command in the Qiime2 diversity plugin. The normalized, rarified ASVs were used for all subsequent analysis (Hakim et al. 2015; Brothers et al. 2018).

Statistical Analyses

The "core metrics" command in the diversity plugin for QIIME2 was used to calculate microbial diversity of ASVs within each community (α-diversity) and between communities (β-diversity). Shannon's index values for α-diversity were compared between time zero and 28°C treatments and across all temperature treatments (28°C, 30°C, and 32°C) within sample types (cardiac stomach, sand, seawater, and feed) using Kruskal-Wallis test with Benjamini & Hochberg correction via the "alpha-group-significance" command in the diversity plugin in for QIIME2. β-diversity was calculated using Bray-Curtis dissimilarity values command for cluster analysis and visualized with principal components analysis (PCoA) using the "diversity core-metrics" command in the diversity plugin for QIIME2. Bray-Curtis dissimilarity values for β-diversity were compared for each sample type between Time Zero and 28°C treatments and across all temperature treatments using permutational multivariate analysis of variance (PERMANOVA) via the "beta-group-significance" command in the diversity plugin for QIIME2. A p-value of <0.05 was considered significant for all analyses. A script of all QIIME2 commands used for this study is included as an online resource (see Electronic Supplementary Material 1).

Results

A total of 6,280,565 16S V4 rRNA gene sequences were initially obtained from sequencing. After denoising, quality filtering, and removing non-target sequences the remaining 3,663,121 sequences were assigned to 3,578 ASVs. Rarefaction curves for α-diversity in each sample leveled off and indicated that all sampling was performed to a sufficient depth to represent microbial community diversity. α-diversity (Shannon Index) was highest in feed, and lowest in stomach tissue overall. Generally, α-diversity increased with increasing temperature in sand, decreased in seawater, and decreased in stomach tissue from 28°C to 30°C, with a sharp increase at 32°C (Table 1).

Table 1 Shannon diversity values for all sample types across all temperature treatments. Note: feed samples were taken once at the beginning of the experimental period and all feed was held under constant conditions throughout the experiment.

	Time Zero	28°C	30°C	32°C
Feed	13.54 ± 0.45			
Seawater	4.49 ± 0.95	4.18 ± 0.92	3.53 ± 0.30	3.48 ± 0.43
Sand	5.66 ± 1.43	5.90 ± 0.42	5.96 ± 0.46	6.59 ± 0.43
Stomach	1.70 ± 0.57	1.15 ± 0.70	0.88 ± 0.52	2.14 ± 1.23

Kruskal-Wallis pairwise comparisons of α-diversity revealed no statistically significant change in microbial diversity of any sample type between time zero and 28°C or between any temperature treatment (Table 2).

Table 2 Kruskal-Wallis pairwise significance for seawater, sand, and stomach samples between all temperature treatments. Kruskal-Wallis p-values are shown with Benjamini and Hochberg correction.

	Time Zero − 28°C	28°C − 30°C	28°C − 32°C	30°C − 32°C
Seawater	0.674	0.231	0.130	0.774
Sand	0.573	0.633	0.070	0.102
Stomach	0.140	0.518	0.231	0.072

Vibrio sp., the most abundant ASV overall, represented 40% of the microbial community across all sample types and treatments. In stomach tissue, *Vibrio* sp. made up 94% of the microbial community at 28°C and 30°C and decreased to 66% at 32°C (Fig. 1). Approximately 98% of the bacterial community in stomach tissue at 28°C was made up of four bacterial taxa: *Vibrio* sp. (94.25%), *Carboxylicivirga* sp. (3.01%), *Delftia* sp. (0.36%), and *Halodesulfovibrio* sp. (0.31%) (Fig. 1). Similarly, over 98% of the bacterial community of stomach tissue at 30°C was made up of three bacterial taxa: *Vibrio* sp. (94.40%), *Delftia* sp. (2.62%), and *Pesudomonas* sp. (1.39%) (Fig. 1). The top three bacterial taxa were the same between 30°C and 32°C, but *Vibrio* sp. abundance decreased, *Delftia* sp. and *Pseudomonas* sp. abundance increased, and the top ten most abundant taxa only made up approximately 94% of the total stomach tissue bacterial community (Fig. 1). The most abundant taxa in the bacterial community of stomach tissue at 32°C included: *Vibrio* sp. (66.34%), *Delftia* sp. (11.46%), *Pseudomonas* sp. (4.67%), *Halodesulfovibrio* sp. (3.99%), *Bacteroides thetaiotaomicron* (2.69%), *Bacteroides* sp. (1.73%), *Lachnospiraceae* NK4A136 group (1.07%), *Clostridium butyricum* (0.62%), *Phyllobacterium* sp. (0.55%), and an unidentified member of the kingdom Bacteria (0.37%) (Fig. 1).

The seawater bacterial community was primarily dominated by *Delftia* sp. and *Pseudomonas* sp., both of which increased in abundance from 28°C (29.86% and 18.33%, respectively), to 30°C (35.54% and 22.86%, respectively), to 32°C (39.25% and 25.17%, respectively) (Fig. 1). *Phyllobacterium* sp. and *Methylobacterium* sp. were also present with increasing abundance with warming (3.25% and 1.58%, respectively at 28°C, 4.35% and 2.14%, respectively at 30°C, and 4.77% and 2.60%, respectively at 32°C) (Fig. 1). The most abundant bacterial taxa in sand at 28°C were *Vibrio* sp. (15.42%), *Arcobacter* sp. (6.42%), *Halodesulfovibrio* sp. (5.92%), *Delftia* sp. (5.28%), *Photobacterium* sp. (4.37%), and *Motiliproteus* sp. (3.18%) (Fig. 1). *Vibrio* sp. was also the most abundant taxa in sand at 30°C (10.91%), followed by *Delftia* sp. (6.67%), *Photobacterium* sp. (6.33%), *Propioigenium* sp. (4.85%), *Pseudomonas* sp. (4.27%), and *Shewanella* sp. (3.16%) (Fig. 1). *Photobacterium* sp. and *Vibrio* sp. were also among the top taxa in the sand bacterial community at 32°C (5.95% and 3.63%, respectively), but the most abundant taxa at this temperature was *Motiliproteus* sp. (9.07%) (Fig. 1).

Principal components analysis of Bray-Curtis dissimilarity values showed distinct clustering of microbial community composition by sample type (Fig. 2). Permutational multivariate analysis of variance (PERMANOVA) of Bray-Curtis dissimilarity values revealed significantly different microbial community composition between all sample types (p = 0.001, stomach−sand, stomach−seawater, stomach−feed, sand−seawater, and sand−feed) except for seawater−feed (p = 0.152). Statistically significant changes were observed in microbial community composition between time zero and 28°C for sand and seawater, but not for stomach tissue (PERMANOVA; sand [F = 1.855, q = 0.036, p = 0.030], seawater [F = 3.305, q = 0.009, p = 0.003], and stomach [F = 0.710, q = 0.731, p = 0.731]).

Importantly, comparison of Bray-Curtis dissimilarity values revealed significant changes in stomach tissue microbial community composition associated with warming between 28°C and 32°C (PERMANOVA, F = 2.703, q = 0.045, p = 0.039), but not between 28°C and 30°C (PERMANOVA, F = 1.066, q = 0.322, p = 0.318) or between 30°C and 32°C (PERMANOVA, F = 2.217, q = 0.123, p = 0.115). Significant changes in sand microbial community composition were also detected with warming between 28°C and 32°C and between 30°C and 32°C, but not between 28°C and 30°C (PERMANOVA; 28°C−32°C [F = 2.621, q = 0.020, p = 0.013], 30−32°C [F = 1.874, q = 0.017, p = 0.010], and 28°C−30°C [F = 1.170, q = 0.216, p = 0.208]). No significant difference in microbial community composition of seawater was observed between any of the three temperature treatments (PERMANOVA; 28°C−30°C [F = 1.143, q = 0.252, p = 0.246], 28°C−32°C [F = 1.571, q = 0.115, p = 106], and 30−32°C [F = 1.235, q = 0.097, p = 0.087]).

Discussion

Microbial diversity detected in seawater, sand, and artificial food in the present study were all higher than that detected in the tissues of the cardiac stomach of the star *Luidia clathrata.* This was particularly evident at experimental seawater temperatures of 28°C and 30°C. The highest levels of microbial diversity occurred in seawater and sand (Table 1). This low level of microbial diversity in the stomach tissues of *L clathrata* aligns well with other studies of echinoderms which have reported a generally low microbial

diversity in stomach and digestive tissues of sea urchins (Hakim et al. 2016; Brothers et al. 2018), sea cucumbers (Gao et al. 2014) and sea stars (Jackson et al. 2018). It is also important to note that, similar to the present study, microbial communities characterized from various tissues of 12 species of sea stars have been reported to be distinct from microbial communities of surrounding seawater (Jackson et al. 2018). In the present study, given that extraoral feeding is a common mode of feeding of *L. clathrata* under laboratory conditions (McClintock et al. 1983) and was also observed throughout the present study, it is not unexpected that the composition of the microbial community in stomach tissue was more closely aligned with that of the sand rather than seawater.

In the present study, the dominant class of bacteria making up the bulk of the microbial communities across all sample types in all three experimental temperature treatments was Gammaproteobacteria. The order Vibrionales dominated the stomach microbial community across all temperatures, while Betaproteobacteriales and Pseudomonadales had the highest abundance in seawater across all temperatures. Samples of aquarium sand had the highest diversity with Vibrionales and Betaproteobacteriales being the two most abundant taxa.

The presence of *Vibrio* sp. as the primary component of the stomach microbiome in *L. clathrata* across all three temperature treatments is unique for a sea star. Although high proportion of *Vibrio* spp. have been reported in the intestine of the sea cucumber *Holothuria glaberrima* (Pagán-Jiménez et al. 2019), the ophiuroid *Ophionema* sp. (Dilmore and Hood 1986), and the sea urchins *Strongylocentrotus droebachiensis* and *Tripneustes ventricuosus* (Guerinot and Patriquin 1981), we believe ours is the first study to report this pattern in a sea star. Høj et al. (2018) reported small amounts *Vibrio* sp. in the microbial community of the pyloric cecum of the tropical corallivorous sea star *Acanthaster plancii.* They also reported a significantly higher incidence of Vibrionaceae-related taxa in diseased individuals than in healthy individuals. Jackson et al. (2018) did not detect *Vibrio* sp., but reported *Tenericutes* as the most abundant microbial taxa in the pyloric caeca of the sea stars *Pycnopoida helanthoides, Crossaster papposus, Orthasterias koehleri, Pteraster tesselatus, Linckia guildingii, Pentaceraster* spp., and *Linckia laevigata* from the coasts of Washington, USA and Queensland, Australia. A 10- to 20-fold increase in *Vibrio* sp. and *Alteromonas* sp. was observed in the microbial communities of food pellets ingested by the sea urchin *Lytechinus variegatus* held chronically under conditions of near-future warming (30°C) (Brothers et al. 2018). An opposite trend was observed in the present study, as abundance of *Vibrio* sp. declined in stomach tissues of *L. clathrata* chronically exposed to an end-of-century warming temperature of 32°C.

High proportion of bacteria from the Vibrionaceae family is common in gut and stomach tissues of mussels and marine arthropods (Preheim et al. 2011), particularly in crabs (Huq et al. 1986). Additionally, marine copepods reared in an aquarium setting have been shown to have a higher abundance of *Vibrio* in their gut than copepods from the natural environment (Sochard et al. 1979). Food availability has been reported to affect the gut microbiome of the shrimp *Penaeus monodon* with higher abundance of *Vibrio* in the gut of individuals fed to those fed a restricted diet (Simon et al. 2020). Furthermore, Lokmer and Wegner (2015) found thermal stress, but not exposure to *Vibrio* infection, significantly altered the

microbiome of the hemolymph of the temperate pacific oyster *Crassostrea gigas*. As some *Vibrio* spp. are typically heat-resistant, rapidly growing opportunists (Bourne et al. 2016), it will be interesting to further examine the role of this taxa in the stomach microbiome of luidiid and non-luidiid sea stars to determine if their presence is indicative of disease or stress, or if *Vibrio* spp. are common members of gut bacterial reservoir communities as seen in other echinoderms and invertebrates more generally (Webster and Thomas 2016; Jackson et al. 2018).

The shift in the stomach microbial community composition of *L. clathrata* from ~94% *Vibrio* sp. dominated (as observed in the 28°C and 30°C treatments) to higher proportions of *Delftia* sp. and *Pseudomonas* sp. in the highest temperature treatment (32°C) is noteworthy. This significant shift in the composition of the stomach's microbial community at the predicted end-of-century temperature for the northern GOM is consistent with that observed in the sea urchin *Lytechinus variegatus* (Brothers et al. 2018). However, the temperature induced trends for the major microbial taxa present are reversed in these two echinoderms. Brothers et al. (2018) reported that under conditions of near-future warming, the proportion of *Vibrio* sp. in the gut of *L. variegatus* increased relative to the other microbial taxa. In the present study, the opposite trend in *Vibrio* sp. occurred with relative abundance trending down with increasing temperature. *Pseudomonas* is known to possess antibacterial activity (Jayatilake et al. 1996) with one strain reportedly having inhibitory properties against marine pathogenic *Vibrio* (Chythanya et al. 2002). It is unknown whether the shifts in the microbial communities we observed in the stomach of *L. clathrata* held at elevated temperature are reversible with a reduction in temperature. However, there is evidence this may not be the case. For example, Ramsby et al. (2018) reported a disruption in the microbial community of the tropical sponge *Cliona orientalis* at 29°C and bleaching of *Symbiodinium* algae at 32°C, were both irreversible when the sponge was returned to its natural temperature of 23°C.

Microbial community composition of the tissues of marine invertebrates are critically important both in the context of resistance to disease (Egan and Gardiner 2016; Lloyd and Pespeni 2018). Gudenkauf and Hewson (2015) reported high proportions of both *Vibrio* sp. and *Pseudomonas* sp. in body-wall tissues of the Pacific coast sea star *Pycnopodia helianthoides* infected with sea star wasting disease. In contrast, these same microbial taxa were reported in lower proportions in the body-wall tissues of the Pacific keystone sea star *Pisaster ochraceus* (Paine 1968) over the documented progression of sea star wasting disease (Lloyd and Pespeni 2018). Gudenkauf and Hewson (2015) suggested that *Vibrio* sp. *and Pseudomonas* sp. might represent an active portion of the holobiont that increases rapidly in proportion when host tissues are compromised. *Delftia,* a genus of gram-negative facultative opportunistic pathogen in the family Comamonadaceae, is known to inhabit marine environments. To date, *Delftia* has not been reported in sea stars, although this group of gram+ bacteria has been detected in a non-pathogenic form in adults of the hermatypic coral *Acropora gemmifera* (Zhou et al. 2017). Additionally, *Delftia acidovorans* has been shown to act as an opportunistic pathogen in European eels held in captivity, co-infecting the eels along with *Pseudomonas anguilliseptica* (Andree et al. 2013).

In addition to considering the effect of stress-induced gut microbiome modification on overall health, it is also important to consider the potential effects on nutrient processing and metabolism. Brothers et al.

(2018) detected a significant shift in predicted metabolic and membrane transport functions in the gut microbiome under elevated temperature stress. This observation underlies the importance of understanding how climate change-induced stressors such as ocean warming will affect the gut microbiome and its associated functions. Although little is yet known about the role of the digestive system microbial community in the processes of digestion and metabolism in sea stars, symbiotic gut bacteria are known to play a role in amino acid provision for some terrestrial invertebrates including the potato psyllids (Thao et al. 2000; Arp et al. 2014) and enchytraeid worms (Larsen et al. 2016).

While the present study focused on adult sea stars, it is noteworthy that the microbial community of sea star larvae is distinct from that of the external environment and can display shifts with dietary changes associated with larval growth and development (Carrier et al. 2018). Bipinnaria larvae of *Luidia* sp. collected from plankton samples in the Northern Atlantic displayed a well-established microbial community with the majority of bacteria isolated to the developing gastric region (Bosch 1992). Bosch (1992) surmised that these symbiotic bacteria were likely associated with digestive processes in the developing larvae. To date, the microbiome of bipinnaria and brachiolaria larvae of *L. clathrata* is unknown. George et al. (1990) suggested that bacteria, in addition to dissolved nutrients and vitellogenic reserves, might have played a role in nutrition of early development of *L. clathrata* bipinnaria larvae during their laboratory-based experiment. The findings of Bosch (1992) and George et al. (1990) warrant further exploration of the community structure and functional role of the microbiome across larval, juvenile, and adult life history phases in sea star development.

The simple anatomical structure and basal phyletic nature of the genus *Luidia* provides a unique opportunity for developing an understanding of gut microbiome dynamics in sea stars. Although members of *Luidia* lack the pyloric stomach found in higher sea star taxa, in addition to predatory foraging *L. clathrata* exhibits extraoral feeding (McClintock et al. 1983). This behavior is unique among the Paxillosid sea stars, affording *L. clathrata* the potential advantage of exploiting detrital food resources in addition to infauna and epifauna. Moreover, it suggests a possible evolutionary link between luidiid sea stars and higher taxa with more complex digestive anatomy (McClintock et al. 1983). Whether the microbial community associated with the gut of *L. clatharata* is also representative of an evolutionary branch between the guts of intraoral and extraoral sea stars remains to be determined.

As has been generally reported in the literature to date, gut microbial communities in healthy individuals under non-stressful conditions have been reported to be relatively stable. Lloyd and Pespeni (2018) reported disruption of microbial community stability and decrease in microbial richness in the epidermal body wall of the sea star *Pisaster ochraceus* with progression of sea star wasting disease. This stability was similarly observed in the gut microbial community of the sea urchin *Lytechinus variegatus* exposed to near-future ocean warming (Brothers et al. 2018). Stomach bacterial community structure in *L. clathrata* in the present study was altered by exposure to projected near-future warming. This is consistent with the presumption stress allows for disruption of microbial community structure as opportunistic pathogenic bacteria flourish at the expense of previously stable communities comprised of a few bacterial taxa. Stressors such as increased seawater temperature are known to disrupt microbial

community structure in marine environments and organisms by either increasing or decreasing overall diversity (e.g., Brothers et al. 2018; Li et al. 2018; Ramsby et al. 2018; Alma et al. 2020; Botté et al. 2020; Greenspan et al. 2020).

Conclusions

In the present study we report the first account of a sea star gut microbiome community reacting to projected ocean warming in a common sea star. Moreover, this is the first description of the gut microbiome in a sea star from a primitive Asteroid (family Luidiidae). The microbial community of the tissues of the cardiac stomach of the common soft bottom sea star *Luidia clathrata* was determined to be distinct from that of formulated feed, sand, and seawater that to which sea stars in the experimental study were exposed. Of these four sample types, the stomach microbial community had the lowest overall diversity, with the order Vibrionales, *Vibrio* in particular, dominating the microbiome across all three temperature treatments (28, 30, 32°C). The shift in stomach microbiome to include higher proportions of *Delftia* and *Pseudomonas* at 32°C is of note as *Vibrio, Delftia,* and *Pseudomonas* can all be opportunistic pathogens with some *Pseudomonas* having activity against some marine pathogenic *Vibrio.* Further studies should be conducted to examine the microbial communities of *Luidia* and other sea stars from the natural environment to see if *Vibrio*-domination is common in natural microbial communities to determine how much the base community observed in this study might have been influenced by acclimatization to aquarium conditions.

Declarations

Compliance with Ethical Standards

Ethics approval Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of interest/Competing interests

The authors have no conflicts of interest or competing interests to declare.

Data/Code availability

Data will be provided upon reasonable request to the corresponding author [MDC]. A document outlining the code used in analysis of the data for this project has been included as an electronic supplementary file.

Authors' contributions

MDC and JBM conceived the study. MDC conducted the experiments, analyzed the data, and provided the early draft of the manuscript. CDM oversaw and facilitated the microbiome sample processing and data generation. MDC, JBM, and CDM worked together on later drafts to develop this manuscript.

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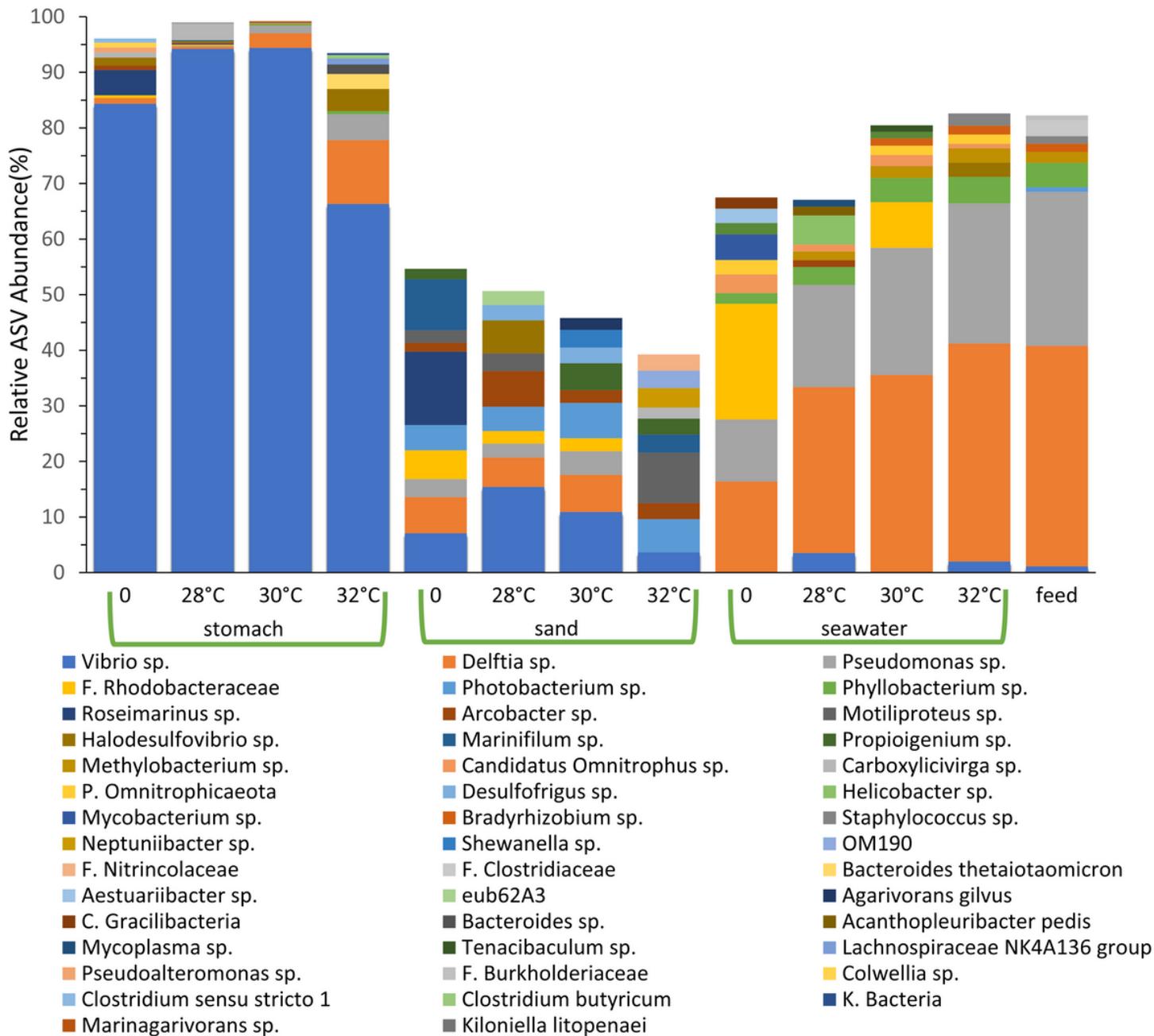
Figures

Figure 1

Relative abundance of the ten most abundant amplicon sequence variants (ASVs) from samples of feed and Luidia clathrata stomach, seawater, sand, and feed at time zero and after two-week exposure to warming. ASVs binned and taxonomy assigned using Qiime2, data sorted and stacked bar chart constructed using Excel. Taxa represented by the most resolved level based on SILVA9 (v132) taxonomic classification
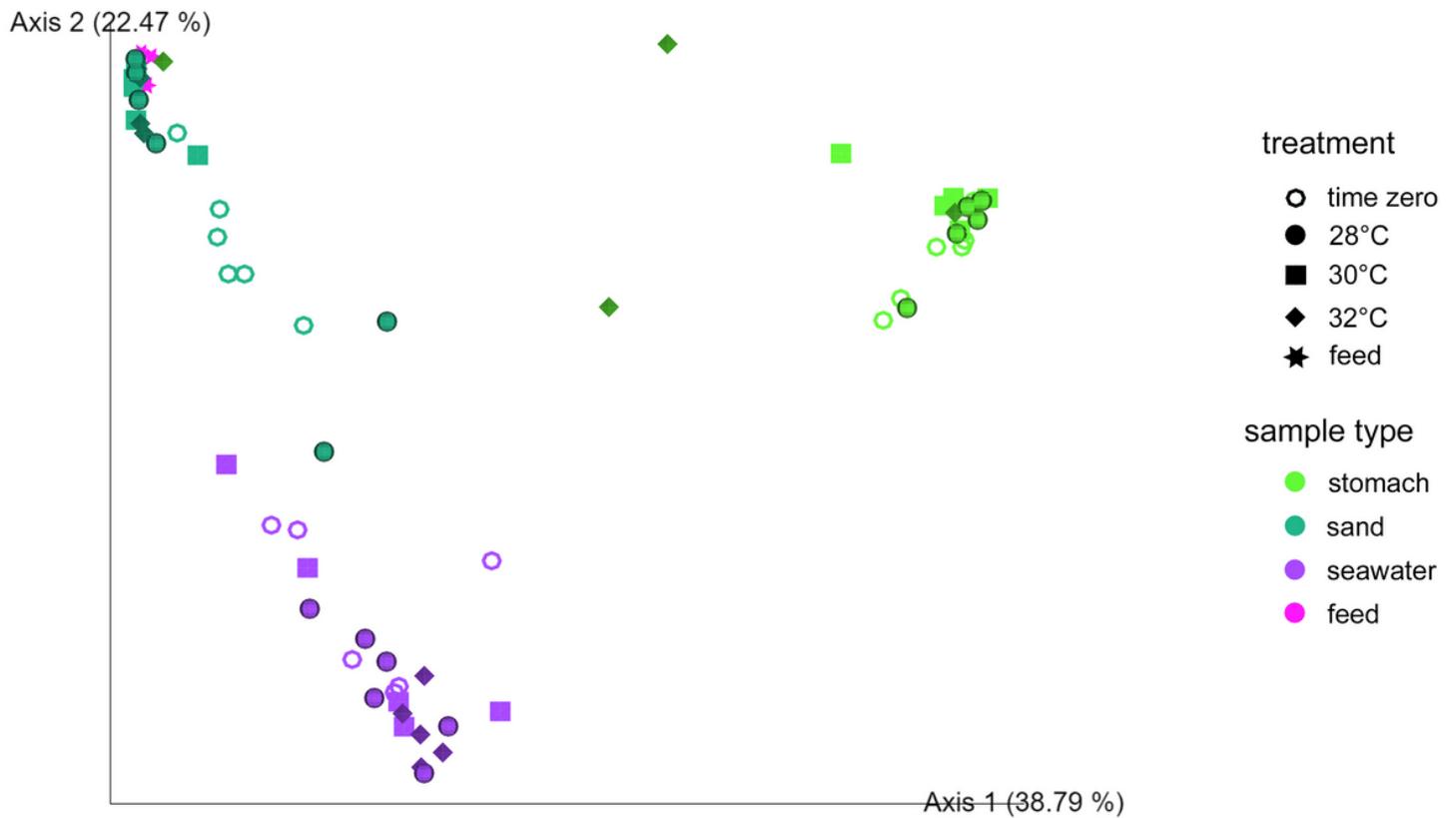
Figure 2

2-dimensional principal component analysis (PCoA) plot of Bray-Curtis dissimilarity for feed, and stomach, sand, and seawater samples for Luidia clathrata at time zero and held under 28°C, 30°C, and 32°C for 2 weeks. Axis 1 explains 38.79% variance and axis 2 explains 22.47%. Graphed with Qiime2

Supplementary Files

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