Host- microbiome interactions in a changing sea: the gill microbiome of an invasive oyster under drastic changes in temperature

Running title– Symbiotic bacteria of invasive oyster and climate change

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Abstract

The gill tissue of bivalve mollusks hosts rich symbiotic microbial communities that may contribute to the host wellbeing. *Spondylus spinosus* is a Lessepsian invasive oyster to the eastern Mediterranean Sea that has become highly abundant, while constantly expending its range northwestward. Using 16S rRNA gene amplicon sequencing we examined how temperature affects the gill microbiota of *S. spinosus,* and the oysters themselves, in a series of experiments: exposing the oysters to the current annual seawater temperature range; to the colder temperature of the western Mediterranean Sea; and to elevated temperature as predicted under global warming scenarios. The bacterial genus *Endozoicomonas* dominated the communities of the *S. spinosus*, mainly upon exposure to winter-like temperatures. Exposure to elevated seawater temperature resulted in a significant change in the bacterial communities, while the oysters maintained normal functioning, suggesting that the oyster may survive a seawater warming scenario. Exposure to colder winter temperature typical to the western Mediterranean Sea resulted in health deterioration of the oysters, emergence of opportunistic pathogens, and a decline in the relative abundance of *Endozoicomonas,* suggesting that *S. spinosus* might not survive in the cold western Mediterranean Sea. The findings indicate that gill bacteria are greatly affected by temperature, which could consequently restrict the range expansion of this and other invasive oysters.

Keywords: *Endozoicomonas* / Lessepsian migration / Mediterranean Sea / microbiome / oyster microbiota / *holobiont*

Introduction

Organisms in all ecosystems around the world, including marine ones, are crucially affected by temperature. Consequently, global warming is considered one of the major environmental concerns, posing a threat to marine biodiversity, as well as contributing to the invasion of marine species, particularly that of tropical species into subtropical and temperate seas (1). Invasive species affect native habitats and indigenous communities, and therefore are considered the second most serious threat to biodiversity, following habitat destruction (2). The Mediterranean Sea is enduring a "tropicalization" process in which seawater temperature is constantly rising (3), followed by an increase in the introduction and spread of invasive tropical species. The main vector of this tropicalization process has been the opening of the Suez Canal in 1869, connecting the Mediterranean and the Red Sea (4). This has led to a continuing massive unidirectional invasion of Red Sea organisms, known as the Lessepsian migration (4). The principle water current in the Eastern Mediterranean Sea moves counterclockwise (5), which causes a passive dispersal of species from the Mediterranean opening of the Suez Canal northwestwards.

A prominent Lessepsian invader is the bivalve oyster *Spondylus spinosus* Schreibers, 1793, which was first documented in the Eastern Mediterranean in 1988 (6). It has since expanded north-west to Lebanon, Cyprus, Turkey, and Greece (7–11). *S. spinosus* forms dense bed-like aggregations along the Israeli Mediterranean coast at a depth of 2-40 m and has the potential therefore to upset the equilibrium of the invaded ecosystem, alter its topography, and replace and even eradicate native species

(6,11,12). Bivalves are considered ecosystem engineers, not only due to their complex hard shell structure, which can supply a refuge and substrate for epibionts (13), but also due to their filter-feeding ability, affecting water turbidity, which in turn may trigger changes in the biota (14). *S. spinosus* is consequently considered as one of the worst invasive species in the Mediterranean Sea (15).

The possession of suitable symbiotic microbiota is crucial for a species' success, as they may provide vital functions, such as supplying their host with nutrients, assisting in food digestion (16,17), and protecting their host against pathogens (18). Oyster microbiota may play a substantial role during larval settlement and metamorphosis, as has already been demonstrated for other bivalve species (19–22). Furthermore, the microbiota may assist the host organism during an invasion process (23). The relatively rich microbiota in bivalve gill tissue (17,23) are considered to be stable autochthonous communities integrated within the host tissues, unlike the microbiota in other organs, such as the gonads and digestive system, in which the microbiota are generally variable and allochthonous (23,24). In addition, the gill tissue in bivalves can be enriched with symbiotic bacteria (17,23), harboring specialized cells hosting symbiotic bacteria, termed bacteriocytes (24).

Symbiotic microbiota functionality is likely to be a critical factor for the invasion success. We have previously shown a high degree of conservation in *S. spinosus* gill microbiomes in the Eastern Mediterranean and the Red Sea, which is indicative of a co-invasion by the host and its symbionts (25), although significant seasonal differences in the bacterial communities were noted. One of the greatest impediments in the process

of biological invasion is that of contending with temperature differences between the source region and the invaded one (26). Bacterial growth is highly sensitive to changes in temperature, whose optima may vary among the different species. Thus, because the microbiome may be more sensitive to temperature shifts than its host, this may determine the distributional range of the invasive holobiont.

Although the temperature in the indigenous region of *S. spinosus*, the Red Sea, may fluctuate annually between 21-28°C (27), along the Israeli Mediterranean coastline this oyster encounters a broader range of 16-31°C (28). Since the microbiome is assumed to contribute to invasion success, its response to shifts in temperature is necessary in order to understand and contend with potential further invasions and trajectories of range expansion. Here we experimentally tested how the temperature regime of the invaded region may affect the microbiota of an invasive species. We also examined the effect of two temperature scenarios on the holobiont, including the possibility of further spread of the oyster into the colder central-western Mediterranean Sea, and its response under a predicted warmer temperature as a result of global warming (29).

Materials and Methods

Four experiments were conducted in order to examine the effect of temperature on the *S. spinosus* holobiont (Table 1.A). Two experiments were designed in order to determine the effect of the current seasonal temperature regime along the Israeli

Mediterranean coast as following: (1) a ‘warming experiment' conducted during February- March 2016 (winter) and (2) a ‘cooling experiment'- during August-September 2016 (summer) (Table 1.A). A 'global-warming experiment' (October-November 2016), incorporated a 2°C higher temperature than the ambient summer temperature, as anticipated under a global warming scenario (29). An 'extreme-cold experiment’ (April 2016), was under seawater temperature of 11°C, prevailing during winter in the shallow western Mediterranean Sea,

Experiment design

Using SCUBA- diving, oysters were removed from the substrate with a hammer and chisel at the Sdot-Yam site (32°29′26.0′N 34°53′09.4′E), on the Israeli Mediterranean coast (3–6 m depth), then placed in buckets filled with seawater and immediately transferred to the marine laboratory at Mevo'ot Yam, Mikhmoret, where the controlled temperature experiments were conducted (Fig. 1, Table 1.B). For each experiment five oysters were randomly retrieved, immediately sacrificed for microbial analysis, and assigned as an inception group (time 0). Sampling procedure comprised dissecting the oysters under sterile conditions, surgically removing the gills, placing the gill tissue in a cooler box (on ice) and its immediate transfer to Tel Aviv University, where it was stored at -20°C until further processing (see ahead). All the oysters, except the inception group, were divided into two aquaria: an experimental (300 l) and a control one (150 l). Each aquarium was supplied with a flow-through of 3 l/min, while maintaining temperature, pH (8.3), and salinity (3.8%) corresponding to ambient Mediterranean conditions. Temperature was monitored four times a day (HOBO Pendant®

Temperature/Light 8K Data Logger, ONSET). Ammonia, nitrite, nitrate, and phosphate levels were monitored twice a week (API® test kit). The oysters were maintained under ambient temperature for one week of acclimation in order to eliminate the possible effect of collection or introduction into the experimental system. Subsequently, four to five oysters were randomly retrieved for assessment as an acclimation group in order to determine whether captivity had influenced the bacterial communities. The control aquarium constantly maintained under ambient conditions, while the experimental group was exposed to a modified temperature at a rate of 2°C per day until reaching the desired value (Table 1.A). The oysters were exposed to the target temperature for two weeks, after which five to ten oysters were sacrificed as described above to determine possible effect on the bacterial communities. Concomitantly, five oysters were sampled from the control aquarium. The final phase was a adjustment of temperature in the experimental aquarium at a rate of 2°C per day until returning to the ambient seawater temperature, followed by keeping the oysters at ambient temperature for a recovery period of an additional two weeks, after which five to eight oysters were sacrificed for assessment as a recovery group. Concurrently, five oysters from the control aquarium were sacrificed for assessment as a control-recovery group. The 'extreme cold' experiment (see above), lacked a recovery phase, due to insufficient oyster supply.

DNA extraction

At Tel Aviv University bacterial genomic DNA from the gill tissue was extracted using the PowerSoil DNA extraction kit (MoBio, CA, USA). All gill samples were homogenized

using 24 tube vortex apparatus at maximum speed for 10 minutes, and then processed according to the manufacturer's protocol, and DNA samples were then stored at –20°C.

16S rRNA gene amplicon sequencing and sequence analysis

Total DNA of each sample was PCR-amplified using universal prokaryotic primers CS1-341F 5'- ACACTGACGACATGGTTCTACANNNNCCTACGGGAGGCAGCAG and CS2-806R 5'- TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT of the 16S rRNA gene from 144 DNA samples. Twenty-nine PCR cycles (95°C 15 sec., 53°C sec. 15, 72°C 15 sec.) were conducted using PCR mastermix KAPA2G Fast™ (KAPABiosystems); and successful amplification was verified by agarose gel electrophoresis. DNA samples were shipped at 4ºC to the Chicago Sequencing Center of the University of Illinois for paired-end deep sequencing of the PCR products on an Illumina MiSeq platform, for determination of bacterial community composition.

Demultiplexed raw sequences were quality filtered (removing bases with a PHRED quality score<20), length filtered (discarding sequences <380bp) and merged using PEAR (30). Data were then processed using the Quantitative Insights into Microbial Ecology (QIIME) software package (31). VSEARCH (32) was used for dereplication and Operational Taxonomic Units (OTUs) picking at 99% identity; to reduce spurious OTU formation,nly sequences repeating more than five times (100% similarity) were allowed to form new OTUs. Chimeric OTUs (identified by uchime_ref using the gold.fa database) were removed. OTUs were then assigned a taxonomy using the UCLUST (33) algorithm and Silva v128 database. The UniFrac based distance matrix, obtained from QIIME, was exported to PAST, a statistical data analysis package (34). Analysis of

similarity (ANOSIM) (35) was calculated, and principal coordinates analysis (PCoA) was performed based on weighted and unweighted UniFrac distances. Statistical significance threshold was α=0.05 for all tests.

Results

A total of 2 254 548 high-quality amplicon reads with an average read length of 429 base pairs were obtained from 144 oysters in the experiments as follows: warming- 33, cooling- 40, global warming- 49 and extreme cold- 22. Each experiment yielded an average of 15,442 reads per sample (range: 20 to 33,008/ sample). A total of 1,840 OTUs were initially recovered, but eight chloroplast OTUs were removed, leaving a total of 1,832 OTUs for the subsequent analyses.

Though 50 distinct bacterial phyla were represented in the gill tissues of *S. spinosus*, the phylum Proteobacteria dominated, representing 67±18% (n=144) of the total microbiota. Within the Proteobacteria, bacterial communities were primarily composed of the classes γ- proteobacteria (68±22%) and α- proteobacteria (19±7%). *Endozoicomonas*, of the γ- proteobacteria class, was the most abundant genus (25±25%) of gill bacterial communities.

Warming experiment

Invasive species originating from the northern Red Sea need to contend with a higher summer water temperature in the Mediterranean Sea than that prevailing in their indigenous one. Oysters were collected in February 2016 (seawater temperature 17 °C),

and divided into experimental and control aquaria. The former was exposed to a gradual increase in temperature up to 31°C, followed by a recovery period during which the temperature was gradually reduced to the ambient temperature of 19°C (March 2016) (Table 1.B). The control groups were designed similarly to those of the previous experiment (see above). All oysters used in this experiment demonstrated normal behavior.

Principal coordinates analyses (PCoA) based on weighted or unweighted UniFrac distances (Fig. 2) both indicated a distinct separation of the summer-like group from the six ambient groups. The recovery group yielded a similar bacterial composition to that of the ambient groups. Pair-wise comparisons of all oysters collected during the experiment exhibited significant differences between the summer-like group and the ambient groups grouped together (ANOSIM P= 0.02, R= 0. 34, P= 0.02, R= 0.25, for weighted and unweighted UniFrac, respectively. Supplementary Table S1).

A relative abundance of 44±14% was noted for γ-proteobacteria in the inception group (17°C), which decreased to 33±20% in the experimental summer-like group. Nonetheless, the relative abundance of γ-proteobacteria increased along with a minor ambient temperature rise of 3°C during the experiment (acclimation: 72±20%, control: 61±27%, recovery: 57±19%, control-recovery: 72±21%, Supplementary Fig. S3). These findings suggest that the extended period of gradual warming had led at least in part, to this increase. The bacterial communities of the summer-like group were more diverse than those of the ambient temperature groups (Fig. 3.A), with median Shannon diversity values of 4.21 vs. ≤3.12. Members of the Cloacimonetes phylum were uniquely

detectable in the summer-like group, reaching a relatively high median relative abundance of 5±3%. The abundance of the order Xanthomonadales (class γ-proteobacteria) increased in the summer-like group (3±2%) and remained similar during the recovery process (relative abundance of 5±3%).Additionally, prevalent OTUs in the summer-like group included members belonging to *Desulfovibrio* (5±3%; phylum γ-proteobacteria), the family *Lentimicrobiaceae* (phylum Bacteroidetes, 6±2%) and the genus *Bacteroides* (4±3%). The genus *Ruegeria* (class α-proteobacteria) featured a high relative abundance in the summer-like group (4±3%), nevertheless, was also present in lower abundance in the recovery group.

Endozoicomonas was the major determinant for the differences between the summer-like group and the oysters that were held under winter ambient temperature (LEfSe - Linear discriminant analysis effect size, Supplementary Figure S4) (36). Members of the *Endozoicomonas* genus were previously shown to be more abundant in gills of *S. spinosus* during winter than during summer (25). It was therefore expected that they would decrease as temperature increased. Indeed, *Endozoicomonas* level was 36±15% in the inception group (winter) while in the summer-like group it was 19±24%. *Endozoicomonas* returned to its initial level of 36±30% in the recovery group, whereas in the control group that remained under the ambient temperature throughout the experiment, its level was even higher (58±33%, Fig. 3.B). These findings indicate that substantial changes take place in the bacterial community of *S. spinosus* in response to increased temperatures.

Cooling experiment

The first experiment sought to assess the effect of a decrease in water temperature that a Red Sea gill microbiome would encounter upon invading the Mediterranean Sea. Oysters were collected during August 2016 (ambient seawater 30°C) and divided between an experimental and a control aquarium (Fig. 1, Table 1.A). The oysters were exposed to a gradual decrease in water temperature to a winter value of 17°C, followed by a recovery period in which the temperature was gradually adjusted to reach the ambient seawater temperature (29°C, September 2016, Table 1.B). The control groups comprised a group of oysters sampled immediately after acclimation and two additional groups maintained under ambient seawater temperature that were sampled concurrently with the experimental group. All oysters in this experiment demonstrated normal behavior, exhibiting immediate valve closing as a response to external stimuli; their soft tissues were intact and displayed normal coloration; and their muscles contracted as a reaction to touch.

Principal coordinates analysis (PCoA) based on UniFrac distances demonstrated only a weak separation of the experimental winter-like group from the control samples. The inception (summer) group differed from the experimental winter-like group (ANOSIM P= 0.04, R= 0.68, see Supplementary Figure S5, Supplementary Table S2), again indicating temperature significantly affect bacterial communities.

The γ- proteobacteria was the most dominant class in the gill microbiomes. Its relative abundance in oysters sampled immediately after collection (inception group) was 22±6%, and it increased under the lower temperatures (winter-like, 17°C: 38±16%). An increase in relative abundance of γ- proteobacteria was noted after a 2°C decrease in ambient seawater temperature following inception, in the control animals (31±6%), as well as in the recovery (34±21%) and control-recovery ones (42±8%) (Supplementary Figure S6). The abundance of cyanobacteria increased in oysters held in the aquaria compared to those examined immediately after being retrieved from the sea; while *Ruegeria* did not change much throughout the experiment (Fig. 4). The genus *Endozoicomonas* of the γ- proteobacteria contributed 13±12% of the microbiota, and was present in all samples but one from the recovery group.

Global-warming experiment

Given the substantial effect on the gill microbiome composition as a result of temperature change, the current study addressed the issue of microbiome modification during global warming. Oysters were collected during October 2016 (seawater temperature 29°C), and exposed to the unprecedented high water temperature values of 32°C and 33°C. The oysters were then allowed a recovery period during which the temperature was gradually adjusted to the ambient seawater temperature of 20°C. The control groups were as in the previous experiments (see above).

Principal coordinates analysis (PCoA) based on unweighted UniFrac (Fig. 5) indicated a distinct separation between the two experimental temperatures and the ambient groups. Pair-wise comparisons of all oysters collected throughout the experiment revealed significant differences between the 32°C/ 33°C groups and all other groups (ANOSIM P≤ 0.034, R=0.66), with the exception of the control groups (first-control and second-control), respectively (ANOSIM P≤ 0.36, R=0.57. Supplementary Table S3).

The relative abundance of bacterial OTUs (Fig. 6) indicated that each group was dominated by different bacterial taxa. For instance, some of the oysters from the acclimation group were enriched with OTUs associated to the family *Marinilabiaceae* (phylum Bacteroidetes); the genus *Arcobacter* (class ε-proteobacteria) reached high levels in most of the oysters from the two extreme temperature groups; and the genus *Halomonas* (class γ-proteobacteria) was more abundant in the recovery group. Although *Endozoicomonas* tend to decrease under exposure to warmer temperatures, its relative abundance was nearly unaffected by the extreme temperature of 32-33°C.

The exposure of oysters to extreme temperatures, matching those of the predicted global warming for the present century, significantly altered the bacterial communities. However, in contrast to our expectations, the extreme warm temperature did not affect the relative abundance of *Endozoicomonas,* nor the physical condition of the oyster host.

Extreme-cold experiment

During the extreme cold experiment oysters were exposed to Western Mediterranean winter seawater temperature typical to the coastal sites of Spain and northern Italy (37,38), in order to determine the possible effects of this temperature on *S. spinosus* microbiota, if indeed its invaded range will include that region. This experiment took place during winter, when ambient seawater temperature was 20°C. Oysters in the experimental aquarium were gradually exposed to 11°C. The control groups were as in the previous experiments (see above). All five oysters from the experimental group (11°C) revealed stress symptoms, manifested in keeping their valves closed most of the time. Two of them hardly responded to any external stimulus. The other 17 control oysters remained normal throughout the experiment.

The relative abundance of bacterial OTUs (Fig. 7) revealed several unique patterns: two out of five oysters in the inception group (1 and 2) and one from the acclimation group (4) featured a similar pattern, in which the genus *Klebsiella* (family Enterobacteriaceae, class γ-proteobacteria) was highly abundant. Along with this genus were the *Enterobacteriaceae*: *Esherichia-Shigella* and *Morganella*, and the Firmicutes: *Veillonella* and *Streptococcus*. These three oysters also exhibited a relatively low abundance of *Endozoicomonas. Endozoicomonas* was highly abundant in the other three oysters in the inception group that exhibited a low abundance of *Enterobacteriaceae*.

Strikingly, the two stressed oysters from the experimental group did not possess any *Endozoicomonas*, whereas this genus was present in all other oysters throughout the experiment. All the oysters under the extreme temperature featured certain distinct bacteria at relatively high abundance compared to the other groups, and the relative

abundance of these taxa in the two stressed oysters was even higher. These included the genera *Arcobacter* and *Vibrio* (class γ-proteobacteria), the family *Colwelliaceae* (class γ-proteobacteria), and the genus *Pseudoalteromonas* (class γ-proteobacteria). These findings indicate that the oysters were stressed by the cold water temperature, leading some putatively beneficial members of the microbiome to be reduced to below detection level, while genera associated with opportunistic pathogens of invertebrates, such as *Vibrio*, increased.

Discussion

Bacterial dynamics are significantly affected by temperature

In this study we used the Lesspesian migrant *S. spinosus* oysters as a model organism in order to examine how exposure to a broad range of temperatures can affect the dynamics and composition of bacterial community, and consequently the geographical expansion-range of an invasive host species. First, we studied how normal seasonal temperature fluctuations of the invaded habitat influence the bacterial community dynamics. As expected, the temperature-shifted bacterial communities demonstrated substantial changes in composition in both the cooling and warming experiments. However, in the warming experiment, the summer-like group was more diverse in bacterial species than the ambient temperature group, comprising several unique bacterial taxa and a unique abundance pattern of several other taxa. An intriguing example of this is the genus *Ruegeria*, which prospered in the summer-like group and then remained relatively abundant in the recovery (ambient winter temperature) group.

Interestingly, *Ruegeria* was also relatively abundant throughout the cooling experiment which took place during summer. This finding may imply that *Ruegeria* is a summer-associated genus and, therefore, oysters in the cooling experiment already possessed a reservoir of this genus, which was maintained throughout that period. In the warming experiment *Ruegeria* increased in oysters following exposure to summer-like temperature and was then maintained at a high level. These findings offer direct support for a previous study carried out under natural conditions, which suggested that seasonality influences the bacterial community composition of *S. spinosus* oysters in the Mediterranean Sea (25).

The dominant oyster-associated bacterial genus Endozoicomonas *is significantly affected by temperature changes*

It has been previously suggested that oyster-associated *Endozoicomonas* species tend to thrive in their natural habitat during winter (25,39). Accordingly, in the warming experiment the expected pattern was observed in which the relative abundance of *Endozoicomonas* decreased upon exposure to the peak summer temperature of 31°C, and increased when exposed to cold winter temperatures of 17°C. Notably, 41 days after onset of the cooling experiment, the ambient water temperature had already dropped from 31°C to 29°C. In contrast to our expectations, oysters that were kept under ambient temperature throughout this entire period (control recovery group) revealed a greater increase in the relative abundance of *Endozoicomonas* than the winter-like group (17°C, Fig. 4). This suggests that the length of exposure to a given temperature may affect the bacterial dynamics and stability. Consequently, we could

potentially have observed a greater increase in the relative abundance of *Endozoicomonas* in winter-like group had it been exposed to 17°C for a comparable period of time to that of the control group. Nevertheless, optimal temperature for *Endozoicomonas* might not be either winter or summer temperature, but somewhere in between. It was previously suggested that the optimal growth temperature of *Endozoicomonas montiporae* in the laboratory is 25°C (40), and for *Endozoicomonas elysicola* an optimal range of 25-30°C (41). In agreement with these studies, in the warming experiment, the relative abundance of *Endozoicomonas* was observed to return to its initial level after the recovery group had been returned to ambient conditions (18°C, Fig. 3.B).

The ability of the oyster host and Endozoicomonas *symbionts to survive global warming*

The average annual water temperature elevation rate between the years 1982–2012 was 0.035±0.007°C in the Mediterranean Sea and 0.05°C in the Levantine basin alone (42). Currently, annual water temperature along the Israeli Mediterranean coast fluctuates between 16°C in winter and 31°C in summer (25). According to several scenarios, Mediterranean Sea temperatures are predicted to rise by the end of the 21st century by 0.5–2.6°C, with maximum warming expected to occur in the Ionian and Levantine sub-basins (42). It is often argued that ocean warming will result in an accelerated rate of marine invasions (43–45), which in turn will lead to the replacement and eradication of native species (46). Therefore, understanding the effects of global warming on invasive species and predicting how they will respond to a continuous warming trend is highly important. The current study examined how exposure of

invasive oysters to an elevation of 2°C in maximal seawater temperature would affect the oyster host and its associated microbiota. The results indicate that such a shift in seawater temperature significantly alters the bacterial communities in the oyster gill tissue. For instance, the genus *Arcobacter* was relatively prevalent in the two experimental extreme warm temperature groups. This genus is a common genus inhabiting several bivalve species (47), includes several known pathogens of humans and animals (48,49), and was also reported in high abundance in diseased corals (50). An additional example is the genus *Halomonas,* which was abundant in the recovery group. *Halomonas* has been previously described in various marine organisms such as sea urchins (51), and in the gill tissue of ascidians (52), and was suggested to be a pathogen of larvae of the Chilean scallop *Argopecten purpuratus* (53). Previous studies indicated that elevated seawater temperature may produce a change in the bacterial communities of marine hosts (54), and may lead to the emergence of pathogens or activation of their virulence genes (55–57). Thus, a similar induction of opportunistic pathogens in *S. spinosus* driven by temperature elevation might be expected. However, all oysters in the global warming experiments did not demonstrate any signs of physical deterioration and maintained a normal functionality. Moreover, *Endozoicomonas* relative abundance was not markedly affected by temperature elevation. This genus is the dominant genus in the gills of these oysters and a core member of many other marine invertebrate including sponges (58,59), corals (40,60,61) and other mollusks (23,41,62,63). Previous studies have demonstrated that maintaining stable bacterial communities may assist the host in preventing disease, as has been suggested for the corals *Montipora aequituberculata* (64) and *Porites astreoides* (65). Therefore, we

consider that *S. spinosus* oysters will survive further global warming, perhaps at the expense of more heat-sensitive oyster species.

Oysters and Endozoicomonas *bacteria are seriously impacted by cold temperature of the Western Mediterranean Sea*

As a response to climate change organisms are predicted to shift their geographical ranges toward colder environments (66). The north-western region of the Mediterranean Sea is its coldest part, with temperatures that may reach 11 °C in winter (37,38). As water temperatures increase, it is expected that it will become increasingly suseptible to species range expansion. Most of the invasive thermophilic species in the Mediterranean Sea are of Red Sea origin (67%), being introduced first into the eastern basin and later expanding their ranges into the western basin (67). We were therefore intrigued by the question of whether *S. spinosus* would be able to endure the cold temperatures of the western basin and consequently expand its range further westward. In the extreme cold experiment, several oysters displayed a decline in the relative abundance of *Endozoicomonas*, with a concomitant increase in relative abundance of several other taxa. In the experimental group, this was characterized by an increase in *Arcobacter*, *Vibrio* and *Pseudoalteromonas,* while in the inception group *Klebsiella*, *Escherichia-Shigella*, *Morganella*, *Veillonella* and *Streptococcus*. The latter genera are known to inhabit various marine organisms (68,69), and some of their members are suspected pathogens (70–72). It is therefore suggested that a decline in the relative abundance of *Endozoicomonas* may provide an opportunity for pathogens to colonize the oyster. This hypothesis is supported by prior studies in corals that have indicated

that a reduction in the relative abundance of *Endozoicomonas* is characterized by the opportunistic colonization of pathogens (73,74). Alternatively, these bacterial community shifts could represent an overall physiological deterioration in the oyster's health due to exposer to colder temperature. Indeed, in contrast to their exposure to the warmer temperature, a noticable health deterioration was noted when oysters were exposed to 11°C. All five oysters exposed to such cold temperature (experimental group) closed their valves for most of the time, unlike normal ones that kept their valves mostly open. Two of these experimental group oysters were also lethargic and no longer responded to any physical stimuli. The remaining 17 oysters of the extreme cold experiment were considered healthy throughout the experiment. The physical deterioration was accompanied by the emergence of distinct bacterial taxa, which could be considered as opportunistic pathogens including *Arcobacter* (50), *Vibrio* (56), *Colwelliaceae* (75), and *Pseudoalteromonas* (76). Strikingly, the two severely affected oysters hosted an even higher relative abundance of these genera, while not retaining any detectable *Endozoicomonas*. Nevertheless, the other three moderately affected oysters of the extreme temperature group had high relative abundance of *Endozoicomonas*. This suggests that the oyster host is more susceptible to the cold temperature than is its *Endozoicomonas* symbiont, and that deterioration due to exposure to cold temperature starts first with the host and only later affects its symbiotic bacteria. The reduction or complete absence of *Endozoicomonas* in affected animals has been described previously, for example in corals (73,74). These findings imply that oysters would not be able to survive a longer exposure to colder temperatures, and therefore might not

survive in the Western Mediterranean Sea, as long as winter water temperature there drops to such a level.

In summary, the current findings indicate that global warming will allow the marine invasive oyster to retain its current dominance in the Eastern Mediterranean, while colonizing coastal areas further north-west of its current geographic range. However, as long as the winter water temperatures remain as low as 11°C, north-west regions of the Mediterranean are expected to remain uncolonized by *S. spinosus*, and possibly by other Red-Sea invasive oysters. The results indicate that the microbial symbionts can be dramatically affected by temperature shifts which in turn could determine the invasion range expansion of invasive species, either by promoting bacterial pathogenesis or by impacting beneficial mutualists. This study provides an experimental framework to examine an invasive holobiont, including the dynamics of its microbial symbionts. Such studies will allow informed predictions regarding future invasion trajectories in the marine environment. Performing similar studies on invasive species, can also help improve the management of biodiversity in the Mediterranean Sea in particular, and other invaded seas in general.

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Competing Interests

The authors declare no conflict of interest.

Author Contributions

YRD, YB and UG conceived and designed this study. Field and laboratory work were conducted by YRD, and data analys by YRD and LR. YRD, YB and UG wrote the paper.

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Data Accessibility and Benefit-Sharing Statement

All metadata, taxonomic composition tables and Unifrac matrices of this study will be archived in Dryad

Figure Legends

Table 1.A. Experiments inducing different temperature regimes from the ambient on the invasive oyster *Spondylus spinosus.* **B.** Sampling groups in the experiments inducing different temperature regimes from the ambient on the invasive oyster *Spondylus spinosus.*

Figure 1. Schematic representation of the design of the experiments inducing different temperature regimes from the ambient on the invasive oyster *Spondylus spinosus.* Numbers indicate samples taken at the different time-points.

Figure 2. Principal coordinates analysis (PCoA) of (a) weighted and (b) unweighted unifrac matrix presenting microbial communities of *Spondylus spinosus* in the warming experiment.

Figure 3. Warming experiment*:* **A.** Relative abundance of bacterial taxa in gills of *Spondylus spinosus*. Each color represents a distinct genus-level OTU. Only taxa that constituted >5% of an individual sample and were present in at least two samples are presented, and the rest are indicated as 'others'. **B.** Relative abundance of *Endozoicomonas* in the different groups.

Figure 4. Relative abundance of bacterial taxa in gills of *Spondylus spinosus* along the cooling experiment. Each color represents a distinct genus-level OTU. Only taxa that constituted >5% of an individual sample and were present in at least two samples are presented, and the rest are indicated as 'others'

Figure 5. Principal coordinates analysis (PCoA) of unweighted unifrac matrix presenting microbial communities of *Spondylus spinosus* in the global warming experiment.

Figure 6. Relative abundance of bacterial taxa in gills of *Spondylus spinosus* along the global warming experiment. Each color represents a distinct genus-level OTU. Only taxa that constituted >5% of an individual sample and were present in at least two samples are presented, and the rest are indicated as 'others'

Figure 7. Relative abundance of bacterial taxa in gills of *Spondylus spinosus* along the extreme cold experiment. Each color represents a distinct genus-level OTU. Only taxa that constituted >5% of an individual sample and were present in at least two samples are presented, and the rest are indicated as 'others'.