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Salinity and Temperature Effects on Physiological Responses of *Vibrio fischeri* from Diverse Ecological Niches

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Abstract

Vibrio fischeri is a bioluminescent bacterial symbiont of sepiolid squids (Cephalopoda: Sepiolidae) and monocentrid fishes (Actinopterygii: Monocentridae). *V. fischeri* exhibit competitive dominance within the allopatrically distributed squid genus *Euprymna*, which have led to the evolution of *V. fischeri* host specialists. In contrast, the host genus *Sepiola* contains sympatric species that is thought to have given rise to *V. fischeri* that have evolved as host generalists. Given that these ecological lifestyles may have a direct effect upon the growth spectrum and survival limits in contrasting environments, optimal growth ranges were obtained for numerous *V. fischeri* isolates from both free-living and host environments. Upper and lower limits of growth were observed in sodium chloride concentrations ranging from 0.0% to 9.0%. *Sepiola* symbiotic isolates possessed the least variation in growth throughout the entire salinity gradient, whereas isolates from *Euprymna* were the least uniform at <2.0% NaCl. *V. fischeri* fish symbionts (CG101 and MJ101) and all free-living strains were the most dissimilar at >5.0% NaCl. Growth kinetics of symbiotic *V. fischeri* strains were also measured under a range of salinity and temperature combinations. Symbiotic *V. fischeri* ES114 and ET101 exhibited a synergistic effect for salinity and temperature, where significant differences in growth rates due to salinity existed only at low temperatures. Thus, abiotic factors such as temperature and salinity have differential effects between free-living and symbiotic strains of *V. fischeri*, which may alter colonization efficiency prior to infection.

Introduction

Years of research with various members of *Vibrionaceae* have shown temperature and salinity to be integral agents in governing *Vibrio* population dynamics [1,2], physiological stress responses [3], and evolution [2,4]. For example, brackish, coastal, and pelagic waters are each uniquely inhabited by distinct *Vibrio* populations [2]. Since temperature and salinity gradients are known to change over these environments, their variability can determine the fitness of each unique *Vibrio* population. Furthermore, *Vibrio* species found in freshwater are prominent since they possess a low Na^+ requirement for growth and starvation survival (e.g., *Vibrio cholerae* and *Vibrio mimicus* [2]). In addition, members of the Vibrionaceae occur naturally

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in the digestive tract and on the skin surface of marine animals [1]. In general, the genus *Vibrio*—along with their close relatives such as *Photobacterium*—are thought to be especially adapted to engaging in pathogenic and benign host-microbe interactions, with these symbiosis traits probably having a deep and ancient common ancestry, arising independently numerous times during the evolution of Vibrionaceae [5].

The association between sepiolid squids (Mollusca: Cephalopoda) and *Vibrio fischeri* has become a model system for studying the physiological and molecular signaling between hosts and their bacterial symbionts. The association is mutualistic, since the bacterially produced bioluminescence provides camouflage for the squid hosts in a cryptic behavior termed counterillumination [6]. Interestingly, *V. fischeri* is also a bioluminescent symbiont of monocentrid fishes, including the genera *Monocentris* and *Cleidopus* [7]. Moreover, some strains of *V. fischeri* that are free-living are unable to develop a light organ association with squid or fish hosts, making them symbiotically incompetent [8]. *V. fischeri* isolated from monocentrid fishes are only capable of colonizing sepiolid squids in laboratory experiments at a reduced efficiency and possess a lower carrying capacity within cephalopod hosts [9]. Prior data has also demonstrated that symbiotically incompetent *V. fischeri* and those colonizing *Euprymna, Sepiola*, and monocentrid fishes are genetically distinct from each other [5, 10-13].

All extant species of *Euprymna* (Cephalopoda: Sepiolidae) are largely allopatric and distributed in the Indo-West Pacific [14]. Previous research has shown that *V. fischeri* strains native to one *Euprymna* species will out-compete conspecific symbionts that are non-native [9,15]. These and other data suggest that host specialization and competitive dominance may be the result of symbionts locally adapting to *Euprymna* species in their environment. However, observations of the stratigraphical distributions of *V. fischeri* and *V. logei*, two symbiotic species found in Mediterranean sepiolids, determined that temperature and not host squids established *Vibrio* distribution in the Mediterranean Sea [16,17]. Numerous *Sepiola* species exist sympatrically in the Mediterranean, and most of these host species simultaneously co-occur with both *V. fischeri* and *V. logei* [16,18]. Thus, contrary to the competitive dominance observed in *Euprymna*, *Vibrio* symbionts in *Sepiola* are host generalists [16]. Other studies with bivalve and vertebrate hosts have also demonstrated that salinity and temperature influence host colonization [19-22], abundance and distribution [23-25], physiological state and survival [26-28], and the adhesive capabilities to host epithelia [29]. Hence, previous research has made it apparent that salinity and temperature influence all life cycle stages of *Vibrio* species, including the biogeography of free-living cells, host attachment with subsequent proliferation during symbiosis, and the alternative evolutionary trajectories available to different host ranges.

Especially important is the question as to how evolutionary lifestyle as a host generalist, host specialist, fish versus squid symbiont, and free-living cell influences growth limits of *V. fischeri* to abiotic factors such as salinity and temperature. Studying the microbial growth of marine symbionts constantly experiencing shifts between marine free-living and host phases is critical since the osmotic pressure can change dramatically between these two environments [30,31]. This subject definitely needs to be addressed with the remarkable illumination in recent years that virulence and osmoregulation possess links within pathogenic *Vibrio* species [32]. Therefore, we studied the physiological performances of various *V. fischeri* strains isolated from different host and free-living environments over a gradient of NaCl concentrations ranging from 0.0-9.0% to observe any correlations between *V. fischeri* lifestyle and osmotic effects on microbial growth. We also measured synergistic effects of salinity and temperature on *V. fischeri* growth to determine if these factors had any influence on bacterial fitness during competition with one another.

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Methods

Bacterial Strains, Media, and Culture Maintenance

Table 1 lists collection sites, squid hosts, and isolated strains of *V. fischeri* used in this study. Once isolated from squid light organs [9], strains were stored at -80°C in cryo-vial tubes containing a final concentration of 20% glycerol in either seawater tryptone (SWT: 70% 32 ppt Instant Ocean artificial seawater, 0.5% Bacto tryptone, 0.3% Bacto yeast extract, 0.3% glycerol, pH 7.5-8.0; [33]) or Luria-Bertani high salt liquid media (LBS: 1.0% Bacto tryptone, 0.5% Bacto yeast extract, 2% NaCl, 0.3% glycerol, 50 mM Tris-HCl, pH 7.5; [34]). The day before each experiment, strains from the -80°C freezer stock were streaked for use onto SWT agar plates (1.5%) to isolate single colonies.

Bacterial Growth over Salinity Gradients

To study *V. fischeri*'s ability to grow over a wide salinity range, isolates were acquired from diverse niches (Table 1), namely as obligately free-living (i.e., symbiotically incompetent and unable to colonize a fish or squid host), fish symbionts (procured from *Monocentris* and *Cleidopus*), squid-host generalist symbionts (isolated from *Sepiola* squid), and squid-host specialist symbionts (isolated from *Euprymna* squid). Individual colonies of each strain from SWT plates were used to inoculate 18×150 mm test tubes containing 5 mL SWT. These test tubes served as starter cultures for the experiment. Tubes were incubated at 28°C while shaking at 225 rpm for 16 h. Thereafter, 10 μL of each overnight starter culture was used to inoculate test tubes containing 5 mL of fresh SWT liquid media. The subsequent cultures were incubated at 28°C and shaken at 225 rpm for 3 h. After 3 h of growth, a Uvikon XL spectrophotometer was used to measure optical density (OD_{600}) of all cultures. Cultures were then inoculated into test tubes containing 5 ml LBS with salinities spanning 0.0-9.0% NaCl. All cultures began at the same initial cell density of 5×10^5 colony forming units (CFU)/mL. NaCl concentrations were increased by 1.0% NaCl, except between 0.0-1.0% and between 6.0-7.0%. Increments between these concentrations were at 0.1%, since these were the minimum and maximum limits of growth for all cultures. Test tubes were placed in a shaker for 24 h at 28°C and 225 rpm. Optical density (OD_{600}) readings of each culture were measured at each concentration (n=5).

Monoculture Growth Studies on *V. fischeri* from *Euprymna* Hosts

Strains of *V. fischeri* isolated from various *Euprymna* species (Table 1) were grown at different temperatures (12°C, 28°C, and 32°C) and salinities (24, 32, and 38 ppt) to observe how these parameters affect generation times in SWT. Salinity was measured using a refractometer (ATAGO® Co., LTD, Japan). These particular temperatures and salinities were chosen since they are representative of the environments *V. fischeri* encounters in nature outside the host. Three-hour cultures of each strain were grown in the same manner as the optical density-salinity gradient studies.

After 3 h of growth, a Uvikon XL spectrophotometer was used to take optical density measurements (OD_{600}) of each culture. Cultures were inoculated in triplicate into 125-mL flasks containing 50 mL of SWT to bring the initial cell density to 5×10^5 CFU/mL. Salinity and temperature were measured at the following settings: 24 ppt/12°C, 24 ppt/28°C, 24 ppt/32°C, 32 ppt/12°C, 32 ppt/28°C, 32 ppt/32°C, 38 ppt/12°C, 38 ppt/28°C, and 38 ppt/32°C. We also examined the effect of nutrient-limiting media on growth rates using minimal ribose media [35] with two symbiotic *V. fischeri* strains, ES114 and EM17. Flasks (125 mL) were aerated at 225 rpm and maintained at the appropriate temperatures for three hours prior to inoculation to guarantee the media was at the correct temperature. OD_{600} measurements were measured from each of the flask cultures every 30 min for 8 h to obtain growth curves for each strain. OD_{600} measurements were natural log transformed to calculate each strain's generation time. Since the experiment was designed with a two-way factorial (or two crossed factors) in a

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completely randomized design, our analysis used a two-way ANOVA with interaction and when the interaction was present, means of the factor combinations were separated by pairwise *t* tests. When interaction was not present, the means at the three temperatures and salinities were separated by pair-wise *t* tests (α=0.05).

V. fischeri ES114 and *V. fischeri* EM17 Competition Growth Studies

To search for the possibility of antagonism or allelopathy between strains, competition growth experiments were completed with *V. fischeri* ES114 and EM17, since both had similar growth rates across the entire range of salinities and temperatures examined. Triplicate 125-mL flasks with 50 mL SWT were co-inoculated with equal numbers (50:50 ratio) of both strains. The initial cell densities of *V. fischeri* ES114 and EM17 were each half of the monoculture inoculations (2.5×10^5). This was to achieve the same starting total cell population as in the monoculture growth studies. Salinity of the SWT media and temperature at which they were incubated were as follows: 24 ppt/12°C, 32 ppt/28°C, and 38 ppt/32°C, which represented low, intermediate, and high conditions. Flasks were aerated at 225 rpm and maintained at the experimental temperatures for 3 h prior to inoculation to guarantee the media was at the correct temperatures. Cell enumeration of each strain was ascertained through plate counts by sampling from each of the replicate flasks once every hour. Bacterial ratios were obtained by counting the number of visibly luminous colonies in the dark (EM17), and subsequently the total number of colonies in the light (EM17+ES114). The difference between the two counts yields the total number of *V. fischeri* ES114 colonies. Since *V. fischeri* ES114 is not visibly luminous, this allows quantification of both strains when grown together [36]. The competition growth rate data was then subjected to *t* tests to detect any significant differences using the software package SAS. The usage of either optical density (OD_{600}) or cell density (CFU/mL) yielded similar growth rates for identical strains; therefore, we used the cell density as an approximation of each strain.

Results

Effects of Salinity Gradients on *V. fischeri* from Different Ecological Niches

V. fischeri native to *Euprymna* species exhibited variable growth throughout the salinity gradient compared to *Sepiola* strains (Figs. 1 and 2). This trend was especially important at lower salinities (<2.0 NaCl, Fig. 1), even when comparing those data to non-squid strains (Fig. 3). In contrast, *V. fischeri* isolated from *Sepiola* squids exhibited the most uniform growth throughout the entire salinity gradient (Fig. 2). *V. fischeri* ET401 and EB12 were the least able to grow at low salinity (<1.0% NaCl) of all the strains isolated from host animals. Conversely, non-squid *V. fischeri* exhibited more variability at high salinities (>5.0% NaCl, Fig. 3). No bacterial growth was observed for any of the strains at either the low or high ranges measured in this study (0.0, 7.0, 8.0, or 9.0% NaCl; Figs. 1, 2, and 3).

Of all the strains, *V. fischeri* ES114 demonstrated the best overall growth at low salinities (<3.0% NaCl) and *V. fischeri* ET401 at higher salinities (>4.0% NaCl). The one exception was free-living *V. fischeri* CB37, which exhibited the best overall growth over most of the salinity range (Fig. 3). Of all the strains examined, *V. fischeri* ATCC 7744 (a free-living isolate) had the most constrained growth across the entire salinity gradient. Therefore, host generalists *V. fischeri* (from *Sepiola* species) were the least different from each other in their ability to grow from 0.0-9.0% NaCl when comparing them to *V. fischeri* from the host genus *Euprymna* (host specialists) and non-squid niches (fish symbionts and obligately free-living bacterioplankton).

Temperature and Salinity Growth Studies of *V. fischeri* from *Euprymna*

Growth rates of the Australian and Japanese *V. fischeri* were tested to assess if any correlations existed between generation times for symbionts as result of being isolated from the same host

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species (Fig. 4) or the same geographical location (Fig. 5). Mean generation times for strains at each salinity-temperature combination examined (α=0.05) are shown in Table 2. Generation times with dissimilar letters (a, b, c, or d) within the column of each strain were significantly different from each other, whereas growth rates possessing the same letter are statistically equivalent. For example, all values with the letter "a" are equal to one another, and all values with the letter "b" are the same. However, all generation times with the letter "a" are statistically different from those with the letter "b". Temperature significantly affected all five *V. fischeri* strains, whereas a significant salinity result was detected only in *V. fischeri* ES114 and ET101. A significant synergistic interaction between temperature and salinity was also observed within these same two strains (Figs. 4 and 5). At 12°C, growth rates for all strains were significantly lower than those at 28°C and 32°C, while growth rates between these two later temperatures were similar (Figs. 4 and 5). Significant salinity effects for *V. fischeri* ES114 and ET101 were observed only at 12°C. Increasing the salinity at this temperature led to more rapid generation times for *V. fischeri* ET101 (Table 2). Mean generation times for the competition growth studies of *V. fischeri* ES114 and EM17 in nutrient-rich SWT were not significantly different from monoculture generation times of these two strains in the same media at higher salinity and temperature conditions (Table 3). However, a significant difference was observed between the competition and monoculture generation times at 24 ppt/12°C (Fig. 6). Monoculture generation times of *V. fischeri* ES114 and EM17 in minimal ribose media were significantly slower than those in SWT for the same temperature (Table 3). Generation times (as noted with different letters, Table 3) were also significantly different from each other.

V. fischeri EM17 and ET401 generation times behave most similarly to each other than to any other *Vibrio* symbionts across different temperature and salinity conditions (Table 2), as neither displayed a significant salinity-temperature interaction. Salinity and temperature had a significant interaction on the growth rates of *V. fischeri* ET101 and ES114. Similar to *V. fischeri* EM17 and ET401, these two strains have similar generation times at 28°C and 32°C. However, salinity had a dissimilar significant effect on *V. fischeri* ET101 and ES114 growth rates at 12°C. Although *V. fischeri* ET401, ET101, and EM17 are the only strains to annually experience temperatures as low as 12°C (Table 4), they seem to lack growth rates that are uniquely adapted for those temperatures. No significant difference in generation times among *Vibrio* symbionts was observed at 12°C.

Comparably, *V. fischeri* ES114 is from Hawaii, where temperature is nearly constant: the difference between surface temperature and that of 100 m below sea level is only a few degrees centigrade (www.nodc.noaa.gov), yet *V. fischeri* ES114 generation time at 28°C is not significantly faster than the other symbionts (Table 3). In this regard, *V. fischeri* EM17 and ET401 are derived from environments extensively variable in temperature throughout the year. *V. fischeri* ES114 experienced a significant salinity effect and salinity-temperature interaction on its growth rate where *V. fischeri* EM17 and ET401 did not, implying the microbial physiology of *V. fischeri* ES114 is more sensitive to variable environments. However, this does not necessarily allude to the conclusion that *V. fischeri* EM17 and ET401 are better adapted to variable environments.

Discussion

V. fischeri is a cosmopolitan microbe with a ubiquitous distribution in oceans, estuaries, brackish waters, and marine sediments throughout the world [37], as either part of free-living bacterioplankton or as a mutualistic symbiont [7,38]. Although most *V. fischeri* strains are "facultative" symbionts with cyclical free-living and mutualistic lifestyles, *V. fischeri* strains exist that persist strictly as members of the bacterioplankton and are symbiotically incompetent, essentially becoming obligately free-living [8]. Clearly, *V. fischeri* is establishing its worldwide dissemination through oceanic water currents as host animals are known to be limited in their

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dispersal ability [10,11]. Ocean temperatures can range between -1.0°C and 30°C with salinities ranging between 5 to 38 ppt (www.nodc.noaa.gov) [39]. Previous studies investigating the microbial ecology of luminous bacteria suggests that species composition of a particular environment was largely determined by patterns of temperature, salinity, nutrient concentration, solar radiation, and other abiotic factors [7,40-43].

Although this idea may continue to hold for microfloral planktonic communities, more recent research has demonstrated that selective pressures in marine bioluminescent bacteria for specificity toward their host fishes and cephalopods with light organs can preside over normal evolutionary physiological requirements. For instance, *Photobacterium leiognathi* typically is more abundant as a free-living microbe in warmer waters; however, this species can be found as a symbiont in both temperate-water and tropical leiognathid fishes [7]. *V. fischeri* itself is usually a temperate-water species but can be found in hosts inhabiting both tropical and temperate waters [38]. This provides evidence that the distribution, ecology, and evolution of luminescent bacterial species in marine environments can be partially driven by symbiosis as opposed to abiotic factors.

Previous work has demonstrated luminous *Vibrio* species colonizing light organs of the Mediterranean genus *Sepiola* was determined by temperature and not squid-host specificity [16,18]. Alternatively, *V. fischeri* symbionts colonizing the squid genus *Euprymna* from the Indo-west Pacific were primarily determined by host specificity [9,15]. Such outcomes governed by abiotic or host specificity may be dependent on the number of hosts available, utilization of different host animals, and whether hosts are allopatric or sympatric. Additionally, the host animal can directly influence symbiont abundance and distribution via seeding the oceanic water column with bacteria through daily venting cycles [44].

Due to the complexity of host interactions and abiotic factors in directing the community structure of marine luminescent microorganisms such as *V. fischeri*, roles of both ecological determinants need further investigation to better understand how this microbe resides in the diverse niches it occupies [7,17]. Our study measured the effects of salinity and temperature on growth rates (i.e., generation times) of *V. fischeri* from several *Euprymna* species in nutrient-rich media (Table 2). Nutrient-rich media have previously been used to simulate a host environment when studying effects of salinity and temperature on the microbial physiology of *Vibrio* species [26]. Since growth rates of microorganisms have characteristics that represent underlying physiological processes of single cells (e.g., biosynthesis of macromolecules), understanding how abiotic factors influence *Vibrio* generation times will facilitate the illumination of the cellular events responsible for changes in microbial populations during symbiosis [45].

V. fischeri ET101 encounters considerable variation in temperature (Table 4), and growth appears more sensitive to changes in salinity and temperature than *V. fischeri* ES114. Correlations between significant effects on generation times by abiotic factors and constant/variable environments are absent. Finally, *V. fischeri* ET101 and ET401, two *E. tasmanica* symbionts isolated from squid from two distinct locations (Table 1) has demonstrated no detectable competitive dominance [9]. However, both *V. fischeri* isolates are genetically distinguishable [11]. *V. fischeri* ET101 was isolated from *E. tasmanica* inhabiting Melbourne, Victoria and *V. fischeri* ET401 was isolated from *E. tasmanica* living in Townsville, Queensland (Table 1). Waters near Victoria typically range in temperature from about 12-17° C, whereas Queensland is much warmer (24-26°C). *V. fischeri* ET101 and ET401 possess generation times that were uniquely affected by salinity and temperature (Fig. 4). For instance, *V. fischeri* ET401 possess the fastest logarithmic growth at low salinity and low temperature (24 ppt/12°C), yet *V. fischeri* ET101 has the most rapid generation time at high salinity and low temperature (38 ppt/12°C; Table 2, Fig. 4). Salinity only has this growth-altering effect at

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12°C and not at the higher temperatures. This outcome may be the result of underlying differences in regional physiological adaptations.

V. fischeri EB12 and EM17 are isolates from two Japanese hosts, *E. morsei* and *E. berryi*. *E. morsei* tends to occur in cooler temperate waters of northern Japan, while *E. berryi* is more frequently found in southern Japan's temperate warm waters, including along the coast of China [46]. Distributions of *E. morsei* and *E. berryi* do overlap partially. Nevertheless, these hosts are believed to be sibling species that are either reproductively isolated or hybridization is rare [47]. Similar to *V. fischeri* ET101 and ET401, generation times of Japanese strains may respond differently to salinity and temperature due to physiological differences resulting from evolution within their respective thermal niches. At low salinity and low temperature (24 ppt/12°C), *V. fischeri* EM17 had a shorter generation time than *V. fischeri* EB12. As the salinity increased at low temperature (12°C), the generation times between these two strains became more similar (Table 2, Fig. 5). Although *V. fischeri* EB12 infects *E. berryi*, which is restricted to sub-temperate/warm waters (17-25°C), this strain may also experience temperatures as low as 2° C during its free-living planktonic phase in northern Japanese waters.

V. fischeri ES114 and EM17 both have generation times of approximately 30 min at 32 ppt/ 28°C in SWT media, but *V. fischeri* ES114 still out-competes EM17 in *E. scolopes* under similar environmental conditions [15]. Interestingly, a “competition” effect was observed at 24 ppt/12°C; both strains grew significantly slower in the presence of the other. Additionally, *V. fischeri* EM17 generation time was more negatively affected by the presence of *V. fischeri* ES114 than ES114 was by EM17 at 24 ppt/12°C (Fig. 6). Hence, microbial allelopathy may at least be partially responsible for competitive dominance in *Euprymna*, especially at lower temperatures (e.g., 12°C). *Vibrio* symbionts have quite similar generation times over the temperatures and salinities examined in nutrient-rich media, yet native strains still out-compete non-native ones under laboratory conditions that approximately simulate natural habitats. If these results can be extrapolated to the nutrient-rich environment of the host, competitive dominance in *Euprymna* would not be solely the result of native *Vibrio* symbionts possessing faster generation times or growth rate constants (growth parameters *g* and *k*) than non-native ones while in the host. Rather, *V. fischeri* strains may be more actively competing against one another via faster generation times when they are part of the free-living bacterioplankton, where the oceanic water column serves as a semi-starving environment relative to the host. Growth rates of *V. fischeri* ES114 and EM17 in minimal media demonstrate that symbionts can have differential growth rates when nutrients may be more limiting. The possibility remains that competitive dominance may be a combination of faster growth rate parameters and microbial amensalism, as these two phenomena are not mutually exclusive.

Despite the presence of competitive dominance of native *V. fischeri* during *Euprymna* colonization [15], population genetic surveys of host squid and *V. fischeri* symbionts suggest secondary colonizations occur [11], whereby previously allochthonous strains become established in a novel animal host. Particular attributes of the *Vibrio*-sepiolid squid symbiosis engender native symbionts of *Euprymna* spp. susceptible to at least partial displacement by non-native invaders. Attachment and proliferation of *V. fischeri* within axenic squid hatchlings emerging from eggs can be initiated and completed with as little as ten cells [8], creating severe symbiont founder effects and genetic bottlenecks during the colonization of the hosts each generation. Symbiont populations may undergo considerable genetic drift upon acquiring new hosts, exposing residential symbionts to potential deleterious effects of Muller's ratchet [48, 49]. An upper bound then crystallizes and confines the magnitude of adaptation that native symbionts achieve to their hosts, diminishing the likelihood of a permanent evolutionarily enduring advantage gained by native vibrios over non-native ones in host colonization. Specific morphological changes are triggered within squid hatchlings upon colonization by *Vibrio* cells, and these immense transformations continue to occur throughout the early stages of the

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symbiosis [50], which make continuous and serial re-colonization from free-living symbionts less probable after maturity of the association.

These properties could permit invading non-native *V. fischeri* to retain occupancy of foreign *Euprymna*, despite the prevailing presence of competitive dominant strains in an area, providing the non-native symbionts arrive and settle into host animals first. Non-native *V. fischeri* initially outnumbering native strains is key to this scenario to offset competitive dominance. Low temperature environments (i.e., winters, cold temperate climates) appear to foster conditions and alternative salinities where allochthonous vibrios could accomplish this inside hosts during early stages of symbiosis by exploiting their faster generation times (Table 2), if optimal conditions were sustained. Perhaps localities combining low temperature, low salinity, and semi-starvation with free-living bacterioplankton in estuaries and deltas during cold periods provide the best opportunities to function as reservoirs for non-native *Vibrio* symbionts. Although marine environments normally range in salinity from 3.3-3.7% [40], investigating the effects of salinity on *V. fischeri* growth is important considering this species ability to invade and thrive within novel hosts and environments is related to its capacity to manage stress [51].

The genus *Vibrio* includes some of the most common culturable marine bacteria, but the general ecology of *Vibrio* in the oceans still remains poorly characterized [52]. Furthermore, the physical, chemical, and biological variation that serves as the impetus for the adaptation and diversification of *Vibrio* species is yet to be well described. This study demonstrates different *V. fischeri* strains have various ranges of salinity that they are able to tolerate and grow (Figs. 1, 2, 3). Surprisingly, slight changes in salinity (e.g., Δ0.1% NaCl≈Δ1 ppt) led to dramatic changes in growth. As a result, the most prevalent strain within a given geographical region can be quite dynamic, depending on the level of salinity fluxes that occur as a consequence of local water currents and seasonal changes. In some instances, sudden and sharp demarcations existed where a particular strain grew significantly and where it did not grow at all, indicating gradual zones of decreasing growth are not always present with subtle changes in salinity. Hence, minute salinity changes in the marine environment could dramatically influence host squid and *V. fischeri* symbiotic relationships (Figs. 1, 2, 3). Some overlap existed in the physiological response among different strains isolated from *Euprymna*, *Sepiola*, and non-squid (i.e., obligately free-living and fish symbionts). These overlapping salinities may represent where different strains can coexist simultaneously in the open ocean. Obligately free-living strains failed to grow above 6.0% NaCl. Therefore, undertaking a symbiotic lifestyle may select for *V. fischeri* more adapted to higher salinities, although this needs to be further investigated. *V. fischeri* CB37 was the only symbiotically incompetent strain able to grow above 6.0% NaCl (Fig. 3).

Understanding how temperature and salinity modulate the biogeographical distribution of *Vibrio* populations can allow us to predict whether these populations are greatly influenced by abiotic pressures. For example, *V. fischeri* ET101 and ET401 both occur in Australian waters. The former grows faster at 24 ppt/12°C and the later at 38 ppt/12°C. The two strains have similar generation times at all other combinations of salinity and temperature. The salinity 38 ppt is not a common salinity in their habitat (Table 4). Stabilizing selection of growth rates at high and low salinities at 12°C is not a suitable explanation of their cooccurrence within *E. tasmanica*. Instead, a more plausible hypothesis is *V. fischeri* ET401 maintains its population by growing more quickly within this host at low salinity and is more prone to expulsion from the light organ through ventilation cycles, while *V. fischeri* ET101 maintains its presence by colonizing the squid more efficiently throughout the host's temperature and salinity ranges. Therefore, *V. fischeri* ET101 would be less likely to be expulsed into the water column. This is analogous to an *r*-selected (ET401) versus *K*-selected (ET101) strategy of survival. Further

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studies investigating intra-strain variation throughout large environmental gradients are planned in future studies.

Although microbial growth was principally sensitive at high and low NaCl concentrations, other parameters such as oxygen concentration, trace metals, radiation, hydrostatic pressure, and stress responses affect marine microbial populations and need to be considered [40]. For instance, stress is known to affect the quality of organic carbon produced by vibrios living in simple, microbial loop foodwebs. This phenomenon influences the quality of carbon available to other trophic levels [53]. Illumination in recent years that the multitude of microbial physiological responses to stress (e.g., heat shock, starvation) are coupled and cross-talk elevates the complexity and provides fertile ground for intriguing research [54]. Future work will examine the extent to which *V. fischeri* symbionts are capable of adapting to different environmental niches (extreme temperatures, low/high salinities, and feast/famine nutrient conditions) and whether the evolutionary potential to adapt to these environments are correlated with those *Vibrio* symbionts (e.g., strains, species) most abundant in the habitats, leading to a greater understanding of microbial diversity, speciation, and evolution.

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References

1. Farmer, J., III; Janda, JM. Family Vibrionaceae. In: Brenner, D.; Krieg, NR.; Staley, JT., editors. Bergy's manual of systematic bacteriology. Vol. vol 2. Springer; New York, NY: 2001. p. 491-555.
2. Urakawa, H.; Rivera, ING. Aquatic Environment. In: Thompson, FL.; Austin, B.; Swings, J., editors. The biology of Vibrios. ASM; Washington, D.C.: 2006. p. 175-189.
3. McDougald, D.; Kjelleberg, S. Adaptive responses to Vibrios. In: Thompson, FL.; Austin, B.; Swings, J., editors. The biology of Vibrios. ASM; Washington, D.C.: 2006. p. 133-155.
4. Bartlett, DH. Extremophilic Vibrionaceae. In: Thompson, FL.; Austin, B.; Swings, J., editors. The biology of Vibrios. ASM; Washington, D.C.: 2006. p. 156-171.
5. Nishiguchi MK, Nair VA. Evolution of pathogenicity and symbioses in Vibrionaceae: a combined approach using molecules and physiology. Int J Syst Bacteriol 2003;53:2019–2026.
6. Jones BW, Nishiguchi MK. Counterillumination in the bobtail squid, *Euprymna scolopes* (Mollusca: Cephalopoda). Mar Biol 2004;144:1151–1155.
7. Ruby EG, Lee K-H. The *Vibrio fischeri-Euprymna* scolopes light organ association: current ecological paradigms. Appl Environ Microbiol 1998;64:805–812. [PubMed: 16349524]
8. Ruby EG. Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. Annu Rev Microbiol 1996;50:591–624. [PubMed: 8905092]
9. Nishiguchi MK. Host recognition is responsible for symbiont composition in environmentally transmitted symbiosis. Microbiol Ecol 2002;44:10–18.
10. Kimbell JR, McFall-Ngai MJ, Roderick GK. Two genetically distinct populations of bobtail squid, *Euprymna scolopes*, exist on the island of O'ahu. Pac Sci 2002;56:347–355.
11. Jones BW, Lopez JL, Huttenberg J, Nishiguchi MK. Population structure between environmentally transmitted Vibrios and bobtail squids using nested clade analysis. Mol Ecol 2006;15:4317–4329. [PubMed: 17107468]
12. Boettcher KJ, Ruby EG. Occurrence of plasmid DNA in the sepiolid squid symbiont *Vibrio fischeri*. Current Microbiology 1994;29:279–286.
13. Lee K-H, Ruby EG. Competition between *Vibrio fischeri* strains during initiation and maintenance of a light organ symbiosis. J Bacteriol 1994;176:1985–1991. [PubMed: 8144466]

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14. Nesis, KN. Cephalopods of the world. T.F.H.; Neptune City, N.J: 1982.
15. Nishiguchi MK, Ruby EG, McFall-Ngai MJ. Competitive dominance among strains of luminous bacteria provides an unusual form of evidence for parallel evolution in sepiolid squid-Vibrio symbioses. Appl Environ Microbiol 1998;64:3209–3213. [PubMed: 9726861]
16. Nishiguchi MK. Temperature affects species distribution in symbiotic populations of Vibrio. Appl Environ Microbiol 2000;66:3550–3555. [PubMed: 10919820]
17. Jones BW, Maruyama A, Ouverney CC, Nishiguchi MK. Spatial and temporal distribution of the Vibrionaceae in coastal waters of Hawaii, Australia, and France. Microbiol Ecol 2007;54:314–323.
18. Fidopiastis PM, Boletzky Sv, Ruby EG. A new niche for *Vibrio logei*, the predominant light organ symbiont of squids in the genus *Sepiola*. J Bacteriol 1998;180:59–64. [PubMed: 9422593]
19. Kaspar CW, Tamplin ML. Effects of temperature and salinity on the survival of *Vibrio vulnificus* in seawater and shellfish. Appl Environ Microbiol 1993;59:2425–2429. [PubMed: 8368832]
20. Marco-Noales E, Biosca EG, Amaro C. Effects of salinity and temperature on long-term survival of the eel pathogen *Vibrio vulnificus* biotype 2 (serovar E). Appl Environ Microbiol 1999;65:1117–1126. [PubMed: 10049871]
21. Amaro C, Biosca EG, Fouz B, Alcaide E, Esteve C. Evidence that water transmits *Vibrio vulnificus* biotype 2 infections to eels. Appl Environ Microbiol 1995;61:1133–1137. [PubMed: 7793914]
22. Motes ML, DePaola A, Cook DW, Veazey JE, Hunsucker JC, Garthright WE, Blodgett RJ, Chirtel SJ. Influence of water temperature and salinity on *Vibrio vulnificus* in Northern Gulf and Atlantic Coast oysters (*Crassostrea virginica*). Appl Environ Microbiol 1998;64:1459–1465. [PubMed: 9546182]
23. Jiang SC. *Vibrio cholerae* in recreational beach waters and tributaries of Southern California. Hydrobiol 2001;460:157–164.
24. Jiang SC, Fu W. Seasonal abundance and distribution of *Vibrio cholerae* in coastal waters quantified by a 16S-23S intergenic spacer probe. Microbiol Ecol 2001;42:540–548.
25. Louis VR, Russek-Cohen E, Choopun N, Rivera ING, Gangle B, Jiang SC, Rubin A, Patz JA, Huq A, Colwell RR. Predictability of *Vibrio cholerae* in Chesapeake Bay. Appl Environ Microbiol 2003;69:2773–2785. [PubMed: 12732548]
26. Hijarrubia MJ, Lazaro B, Sunen E, Fernandez-Astorga A. Survival of *Vibrio vulnificus* under pH, salinity and temperature combined stress. Food Microbiol 1996;13:193–199.
27. Hoff KA. Survival of *Vibrio anguillarum* and *Vibrio salmonicida* at different salinities. Appl Environ Microbiol 1989;55:1775–1786. [PubMed: 2475069]
28. Wolf PW, Oliver JD. Temperature effects on the viable but non-culturable state of *Vibrio vulnificus*. FEMS Microbiol Ecol 1992;10:33–39.
29. Bordas MA, Balebona MC, Zorrilla I, Borrego JJ, Morinigo MA. Kinetics of adhesion of selected fish-pathogenic *Vibrio* strains to skin mucus of Gilt-Head Sea Bream (*Sparus aurata* L.). Appl Environ Microbiol 1996;62:3650–3654. [PubMed: 8837420]
30. Xu C, Ren H, Wang S, Peng X. Proteomic analysis of salt-sensitive outer membrane proteins of *Vibrio parahaemolyticus*. Res Microbiol 2004;155:835–842. [PubMed: 15567278]
31. Xu C, Wang S, Ren H, Lin X, Wu L, Peng X. Proteomic analysis on the expression of outer membrane proteins of *Vibrio alginolyticus* at different sodium concentrations. Proteomics 2005;5:3142–3152. [PubMed: 16021606]
32. Sleator RD, Hill C. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol Rev 2001;26:49–71. [PubMed: 12007642]
33. Nealson KH. Isolation, identification, and manipulation of luminous bacteria. Methods Enzymol 1978;57:153–165.
34. Dunlap PV. Regulation of luminescence by cyclic AMP in cya-like and crp-like mutants *Vibrio fischeri*. J Bacteriol 1989;171:1199–1202. [PubMed: 2536674]
35. Lupp C, Hancock REW, Ruby EG. The *Vibrio fischeri* sapABCDF locus is required for normal growth, both in culture and in symbiosis. Arch Microbiol 2002;179:57–65. [PubMed: 12471505]
36. Nishiguchi, MK.; Ruby, EG.; McFall-Ngai, MJ. Phenotypic bioluminescence as an indicator of competitive dominance in the *Euprymna*-*Vibrio* symbiosis. In: Hastings, JW.; Kricka, JW.; Stanley,

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PE., editors. Bioluminescence and chemiluminescence: molecular reporting with photons. Wiley; New York, NY: 1997. p. 123-126.

37. Nishiguchi, MK.; Jones, BW. Microbial biodiversity within the Vibrionaceae. In: Seckback, J., editor. Origins, evolution, and the biodiversity of microbial life. Kluwer; Dordrecht, The Netherlands: 2004. p. 531-548.
38. Nishiguchi MK, Lopez JE, von Boletzky S. Enlightenment of old ideas from new investigations: the evolution of bacteriogenic light organs in squids. Evol Dev 2004;6:41–49. [PubMed: 15108817]
39. Atlas, RM.; Bartha, R. Microbial ecology: fundamentals and applications. Cummings; Menlo Park, CA: 1998.
40. Yetinson T, Shilo M. Seasonal and geographic distribution of luminous bacteria in the eastern Mediterranean Sea and the Gulf of Elat. Appl Environ Microbiol 1979;37:1230–1238. [PubMed: 16345404]
41. O'Brien CH, Sizemore RK. Distribution of the luminous bacterium *Beneckea harveyi* in a semitropical estuarine environment. Appl Environ Microbiol 1979;38:928–933. [PubMed: 16345465]
42. Ruby EG, Nealson KH. Seasonal changes in the species composition of luminous bacteria in nearshore seawater. Limnol Oceangr 1978;23:530–533.
43. Shilo M, Yetinson T. Physiological characteristics underlying the distribution patterns of luminous bacteria in the Mediterranean Sea and the Gulf of Elat. Appl Environ Microbiol 1979;38:577–584. [PubMed: 16345442]
44. Lee K-H, Ruby EG. Effect of the squid host on the abundance and distribution of symbiotic *Vibrio fischeri* in nature. Appl Environ Microbiol 1994;60:1565–1571. [PubMed: 16349257]
45. White, D. The physiology and biochemistry of the Prokaryotes. Oxford University Press; Oxford, UK: 2000.
46. Norman MD, Lu CC. Redescription of the southern dumpling squid *Euprymna tasmanica* and a revision of the genus *Euprymna* (Cephalopoda: Sepiolidae). J Mar Biol Assoc UK 1997;77:1109–1137.
47. Nateewathana A. The Sepiolidae (Cephalopoda) of the Andaman Sea, Thailand, with description of *Euprymna hyllebergi* sp. nov. Phuket Mar Biol Cent Spec Pub 1997;17:465–481.
48. Rispe C, Moran NA. Accumulation of deleterious mutations in endosymbionts: Muller's ratchet with two levels of selection. Am Nat 2000;156:425–441.
49. Moran NA. Accelerated evolution and Muller's ratchet in endosymbiotic bacterial. Proc Nat Acad Sci USA 1996;93:2873–2878. [PubMed: 8610134]
50. McFall-Ngai MJ. Consequences of evolving with bacterial symbionts: insights from the squid-*Vibrio* association. Annu Rev Ecol Syst 1999;30:235–256.
51. Bennett AF, Lenski RE. Experimental evolution and its role in evolutionary physiology. Am Zool 1999;39:346–362.
52. Giovannoni SJ, Sting U. Molecular diversity and ecology of microbial plankton. Nature Rev Genet 2005;437:343–348.
53. Odic D, Turk V, Stopar S. Environmental stress determines the quality of bacterial lysate and its utilization efficiency in a simple microbial loop. Microbiol Ecol 2007;53:639–649.
54. Vattanaviboon P, Mongkolsuk S. Unusual adaptive, cross-protection response and growth phase resistance against peroxide killing in a bacterial shrimp pathogen, *Vibrio harveyi*. FEMS Microbiol Lett 2001;47:111–116. [PubMed: 11410358]
55. Okutani T, Horita E. Identity of *Euprymna berryi* Sasaki, 1929 (Cephalopoda: Sepiolidae). Venus 1987;46:91–107.

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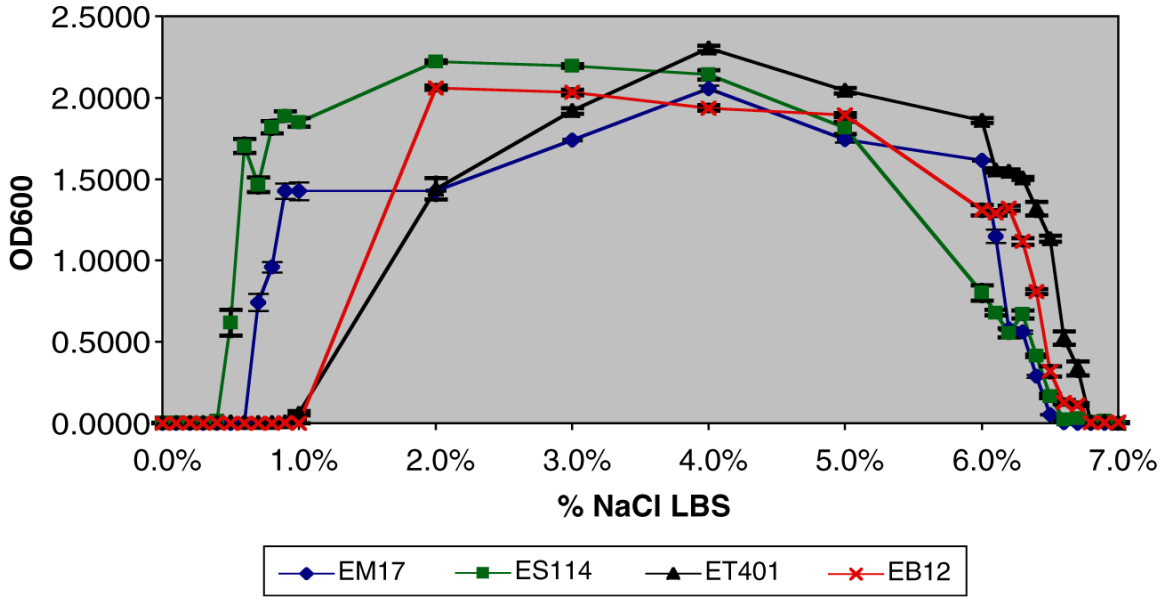

Figure 1.
Salinity effects on growth (±standard error) of host specialist *V. fischeri* isolated from *Euprymna. Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain designations

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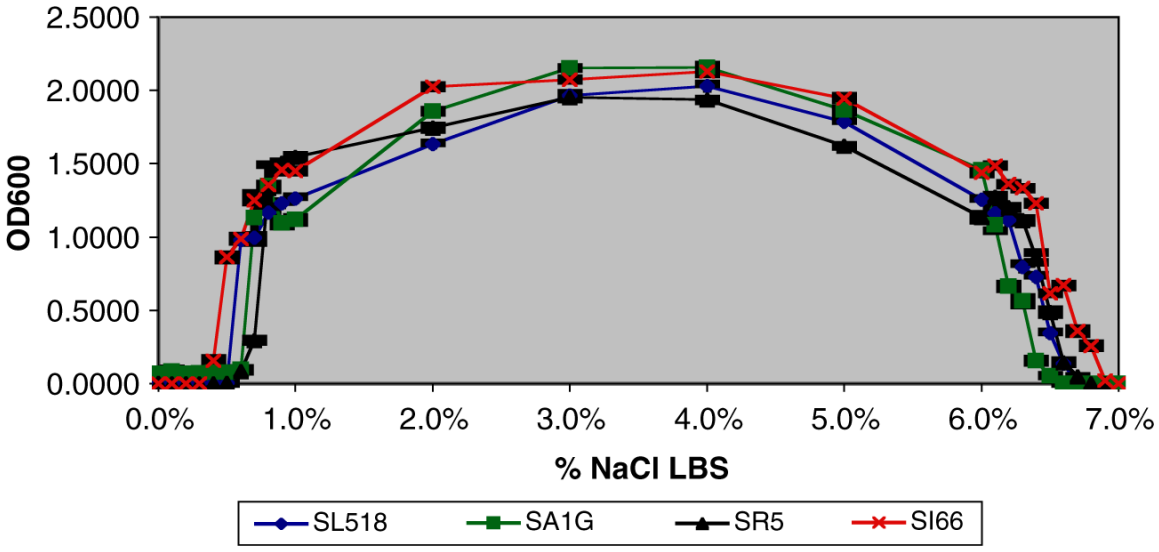

Figure 2.
Salinity effects on growth (±standard error) of host generalist *V. fischeri* isolated from *Sepiola* squid. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain designations

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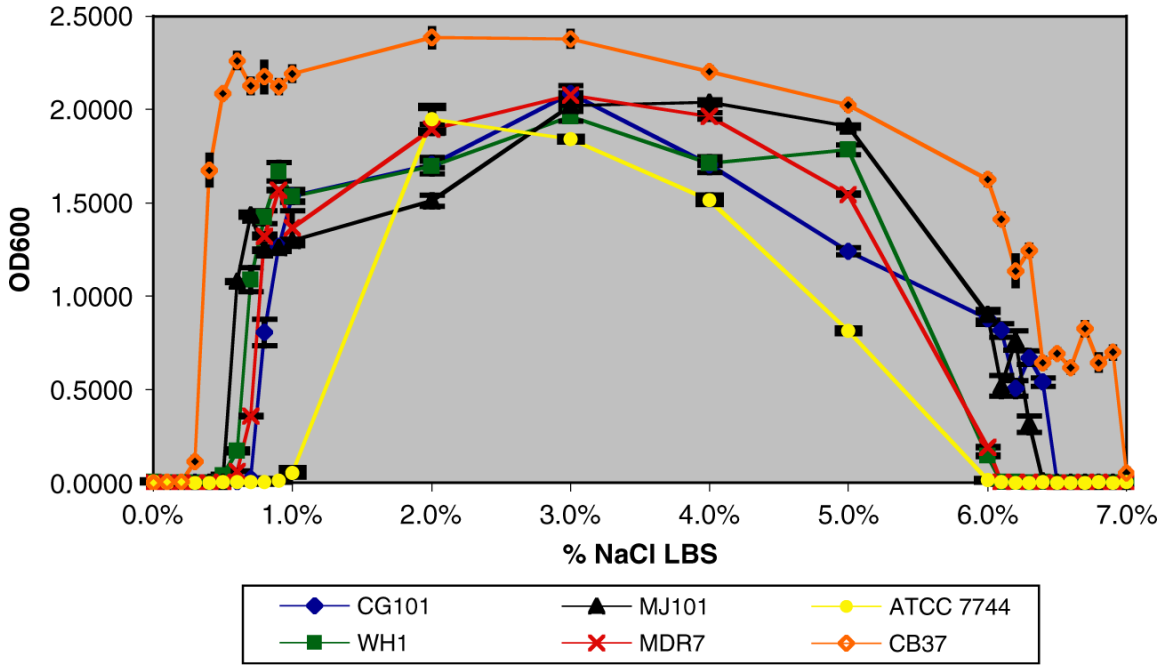

Figure 3.
Salinity effects on growth (±standard error) of fish symbionts and free-living *V. fischeri*. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain designations

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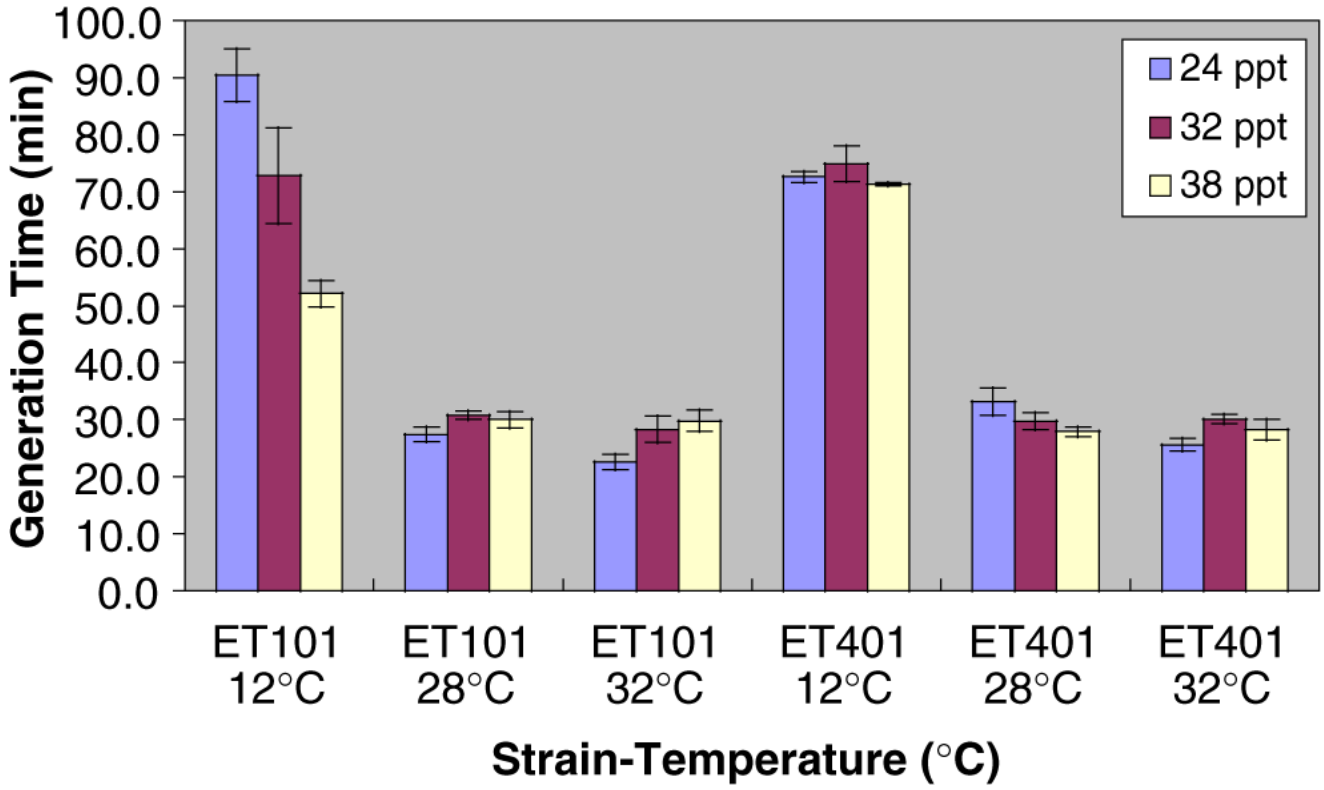

Figure 4.
Mean generation times (±standard error) of *V. fischeri* ET101 (Melbourne, VIC) and ET401 (Townsville, QLD) isolated from *E. tasmanica* measured at three different temperature and salinity conditions. *Standard error bars* were calculated using the unbiased estimator for the mean

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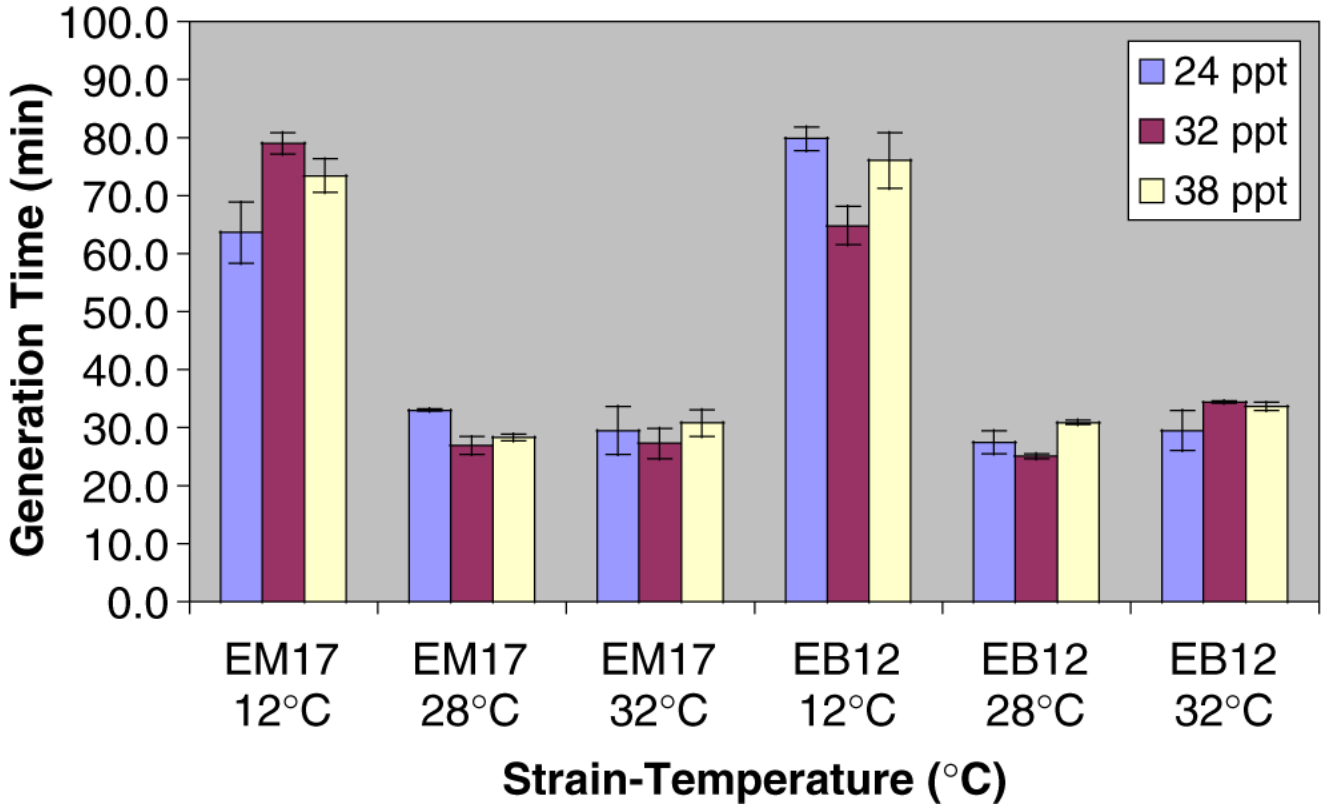

Figure 5.
Mean generation times (±standard error) of *V. fischeri* EM17 and EB12 obtained from *Euprymna morsei* (EM17) or *Euprymna berryi* (EB12) measured at three different temperature and salinity conditions. *Standard error bars* were calculated using the unbiased estimator for the mean. See Table 1 for strain and host designation

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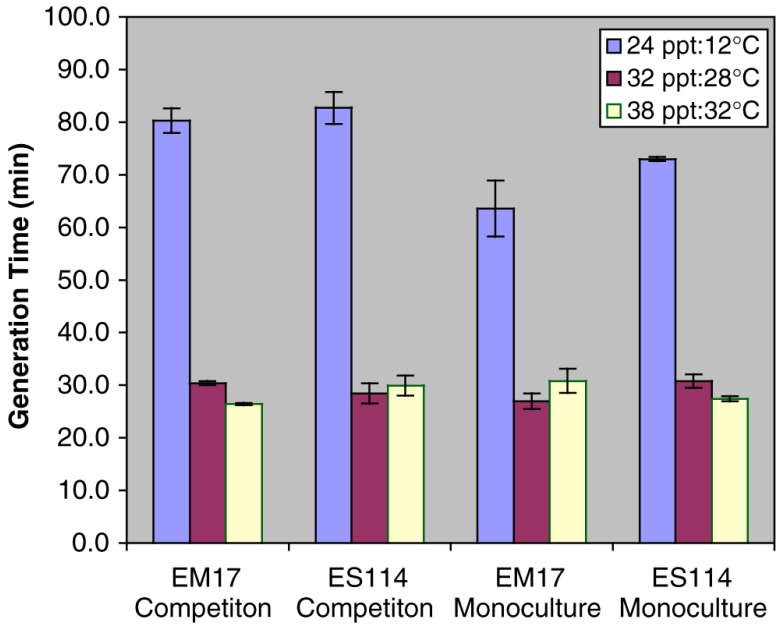

Figure 6.

Mean generation times (±standard error) of *V. fischeri* ES114 and EM17 grown together at different temperature and salinity combinations (competition). Monoculture mean generation times are included in parentheses for comparison. Refer to Table 1 for location of hosts and isolates. *Standard error bars* were calculated using the unbiased estimator for the mean

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Table 1

Strains of *Vibrio fischeri* used in this study

Strain	Host	Location
WH1	Free-living	USA (Massachusetts, Woods Hole)
MDR7	Free-living	USA (California, Marina del Rey)
CB37	Free-living	Australia (Coogee Bay, New South Wales)
ATCC 7744	Free-living	American Type Culture Collection
MJ101	*Monocentris japonicus*	Japan (Tokyo Bay)
CG101	*Cleidopus gloriamaris*	Australia (Townsville, Queensland)
SR5	*Sepiola robusta*	France (Banyuls sur Mer)
SL518	*Sepiola ligulata*	France (Banyuls sur Mer)
SA1G	*Sepiola affinis*	France (Banyuls sur Mer)
SI66	*Sepiola intermedia*	Italy (Bari)
EM17	*Euprymna morsei*	Japan (Tokyo Bay)
ET101	*Euprymna tasmanica*	Australia (Melbourne, Victoria)
ET401	*Euprymna tasmanica*	Australia (Townsville, Queensland)
EB12	*Euprymna berryi*	Japan (Tosa Bay)
ES114	*Euprymna scolopes*	USA (Kaneohe Bay, Hawaii)

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Table 2

Mean generation times of *Vibrio fischeri* strains grown in monoculture at each salinity and temperature condition (n=3)

Salinity-temp (ppt °C)	EB12[a] (min)	EM17[a] (min)	ES114[a,b, c, d] (min)	ET101[a, c, d] (min)	ET401[a] (min)
24-12	77.8a	57.6c	73.0a	90.4e	63.5c
24-28	25.4b	33.0b	29.3b	27.4b	31.8b
24-32	29.5b	29.5b	25.9b	22.6b	25.6b
32-12	68.2c	72.2a	79.1a	72.8a	74.9a
32-28	25.1b	26.9b	29.2b	25.8b	29.8b
32-32	34.4b	27.3b	26.8b	28.3b	30.1b
38-12	76.0a	72.0a	63.1c	52.1d	71.3a
38-28	30.9b	27.4b	28.3b	30.0b	27.9b
38-32	33.7b	30.8b	27.4b	29.8b	28.2b

Generation times with different letters denote values significantly different from each other. For example, all values with the letter “a” are equal to one another, and all values with the letter “b” are also equivalent. However, all generation times with the letter “a” are statistically different from those with the letter “b”

[a] Significant temperature effect (p<0.0001)

[b] Significant salinity effect (p<0.01)

[c] Significant salinity effect (p<0.05)

[d] Significant salinity-temperature interaction (p<0.01)

[e] Significant salinity-temperature interaction (p<0.0001)

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Table 3

Mean generation times (min) for *Vibrio fischeri* ES114 and *V. fischeri* EM17 from competition and minimal media growth studies (monoculture generation times in SWT are included in parentheses for comparison)

Strain	24 ppt 12°C	32 ppt 28°C	38 ppt 32°C	Minimal ribose 28°C
EM17	80.3d (63.6c)	30.4b (26.9b)	26.4b (30.8b)	89.8d (30.0b[a])
ES114	82.7d (73.0a)	28.4b (30.8b)	29.9b (27.4b)	114.5e (28.9b[a])

Generation times with different letters and colors denote values significantly different from each other. For example, all values with the letter “a” are equal to one another, and all values with the letter “b” are also equivalent. However, all generation times with the letter “a” are statistically different from those with the letter “b”

[a]Mean generation times obtained from averaging different growth studies completed at 28°C

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Table 4

Annual mean temperatures and mean salinities for *Euprymna* species (www.nodc.noaa.gov, www.cephbase.utmb.edu)

Host squid	Distribution	0-100 m usual temperature range	0-100 m usual salinity range
E. tasmanica	Australia/Tasmania	12-25°C	20.0-35.8 ppt
E. morsei	Japan (cool temperate waters)	2-17°C	32.2-34.0 ppt
E. berryi	Japan (warm temperate waters)	17-25°C	34.2-34.8 ppt
E. scolopes	Hawaiian Islands	22-26°C	34.6-35.2 ppt
E. hyllebergi	Thailand	21-28°C	31.4-34.4 ppt
E. hoylei	Philippines/North Western Australia/ Marshall Islands	21-29°C	34.0-35.0 ppt

Biogeographic data was obtained from the National Oceanographic and Atmospheric Administration and published literature [46,47,55]