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Adaptation to temperature stress by *Vibrio fischeri* facilitates this microbe's symbiosis with the Hawaiian bobtail squid (*Euprymna scolopes*)

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For microorganisms cycling between free-living and host-associated stages, where reproduction occurs in both of these lifestyles, an interesting inquiry is whether adaptation to stress during the free-living stage can impact microbial fitness in the host. To address this topic, the mutualism between the Hawaiian bobtail squid (*Euprymna scolopes*) and the marine bioluminescent bacterium *Vibrio fischeri* was utilized. Using microbial experimental evolution, *V. fischeri* was selected to low (8°C), high (34°C), and fluctuating temperature stress (8°C/34°C) for 2000 generations. The temperatures 8°C and 34°C were the lower and upper growth limits, respectively. *V. fischeri* was also selected to benign temperatures (21°C and 28°C) for 2000 generations, which served as controls. *V. fischeri* demonstrated significant adaptation to low, high, and fluctuating temperature stress. *V. fischeri* did not display significant adaptation to the benign temperatures. Adaptation to stressful temperatures facilitated *V. fischeri*'s ability to colonize the squid host relative to the ancestral lines. Bioluminescence levels also increased. Evolution to benign temperatures did not manifest these results. In summary, microbial adaptation to stress during the free-living stage can promote coevolution between hosts and microorganisms.

KEY WORDS: Adaptation, coevolution, selection-experimental, symbiosis.

Temperature Stress and Vibrionaceae

From the polar regions to the tropical equator, annual mean temperatures of the ocean surface typically range from −2°C to ≥ 30°C (National Oceanic and Atmospheric Administration, www.noaa.gov). These temperatures are expected to increase due to anthropogenic activities (e.g., burning of fossil fuels) that are elevating carbon dioxide levels on the planet and contributing to climate change (Watts et al. 2017; Bonan and Doney 2018). Forecasts of the exact magnitude that ocean temperatures will increase by vary and are dependent on the assumptions made with many different parameters, along with their complex interactions. However, some climate models project that a 2–5°C increase in ocean temperature is possible within the next one to two centuries, if atmospheric carbon dioxide continues to rise at the present rate (Fürst et al. 2014; Nicholls et al. 2018). Warmer ocean temperatures will have drastic effects on the ecology and evolution of marine microorganisms, including the Vibrionaceae. The Vibrionaceae are especially interesting within the context of climate change (Vezzulli et al. 2015). This taxonomic group is sensitive to temperature changes, has excellent starvation survival strategies, and can exploit sudden nutrient surges with short generation times (McDougald and Kjelleberg 2006; Baker-Austin et al. 2017). The Vibrionaceae also possess a notable capacity in being able to invade new harsh environments (Banerjee et al. 2018). Although the Vibrionaceae can freely adopt a planktonic lifestyle, they also are highly proficient at forming biofilms, which permits microbial dispersal via rafting (Theil and Gutow 2005; Soto and Nishiguchi 2014). These characteristics greatly facilitate the Vibrionaceae's ability to disseminate throughout the world's oceans, a feature that

will be immensely heightened with climate change (Martinez-Urtaza et al. 2016; Racault et al. 2016). Even single strains have been able to cross entire ocean basins (González-Escalona et al. 2015; Martinez-Urtaza et al. 2017).

The Vibrionaceae are renowned for being able to initiate host–microbe interactions with animals, including pathogeneses (parasitisms), commensalisms, and mutualisms (Soto et al. 2010). *Vibrio* and *Photobacterium* are the largest genera within the Vibrionaceae (Farmer and Janda 2005; Gomez-Gil et al. 2014). There is ample evidence that extreme temperatures impact the regulation of gene expression in the Vibrionaceae in a manner that affects the capacity of this family to engage with animal hosts. Elevated temperatures prompt the expression of several virulence factors in the coral pathogen *Vibrio coralliilyticus* (Kimes et al. 2012). Higher temperatures are also known to stimulate virulence in *Vibrio alginolyticus*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus* (Mahoney et al. 2010; Gu et al. 2016; Kim et al. 2016). Lower temperatures can also trigger virulence, as is the case in *Vibrio salmonicida* (Colquhoun and Sørum 2001). Pathogenicity is greatly increased by low temperatures in *Vibrio splendidus*, which causes high mortality in many aquaculture animals, due to a drastic augmentation in gene expression (Liu et al. 2016). As a result, examining how extreme temperatures can shape the evolution of host–microbe interactions is valuable. In this study, the hypothesis was tested that microbial evolution to temperature stress during the free-living stage affects symbiosis for microorganisms engaged in host–microbe interactions. To test this hypothesis, the sepiolid squid-*Vibrio* mutualism was utilized.

The Sepiolid Squid-Vibrio fischeri Mutualism

V. fischeri is a cosmopolitan microbe with a ubiquitous distribution (Soto and Nishiguchi 2014). *V. fischeri* has been isolated from oceans, estuaries, brackish waters, and marine sediments throughout the world. *V. fischeri* can exist as either free-living bacterioplankton or as biofilms. As biofilms, bacterial cells may either be attached to abiotic surfaces (suspended particulate matter) or adhered to the external surfaces of animals (Soto et al. 2009; Soto and Nishiguchi 2014). Although attached to animal skin, invertebrate chitin, or as part of the gut microflora, *V. fischeri* can also persist as a commensal associated with marine animals (Soto and Nishiguchi 2014). Thus, due to its widespread nature, *V. fischeri* experiences extensive temperature variation in the various aquatic environments it occupies. Consequently, *V. fischeri* may be quite prone to substantial selection pressures imposed by low and high temperature stress. How selection pressure from extreme temperature affects *V. fischeri*'s capacity to interact with its light organ animal hosts throughout the world is an interesting research question (Soto et al. 2009). *V. fischeri* is able to form light organ symbioses with sepiolid squids (*Sepiola* and *Euprymna*) and monocentrid fishes (*Monocentris* and *Cleidopus*; Ruby 1996). Sepiolid squids are distributed throughout the Indo-West Pacific, Asia-Pacific, the Atlantic, and the Mediterranean Sea (Jereb and Roper 2005). Monocentrid fishes are found in the Indo-West Pacific, Asia-Pacific, and South Pacific (Soto et al. 2009; Soto and Nishiguchi 2014).

Interestingly, the mutualisms between sepiolid squids and the marine bioluminescent bacterium *V. fischeri* is a model system for studying associations between bacteria and animal hosts (Soto et al. 2012). *V. fischeri* can be grown in pure culture, whereas sepiolid squids can be raised gnotobiotically (Nishiguchi et al. 1998). For this particular study, the sepiolid squid species utilized was *Euprymna scolopes* (Cephalopoda: Sepiolidae). In nature, *E. scolopes* inhabits the shallow coastal waters of Hawaii (Jereb and Roper 2005). Climate change is likely to affect the ecology of *E. scolopes*, as Hawaii will undoubtedly experience increased sea level rise, elevated temperatures, ocean acidification, and novel water–sediment interactions (Watts et al. 2017). Other sepiolid squid species have different biogeographical distributions. For instance, *Euprymna tasmanica* and *Euprymna morsei* are found in the coastal waters of Australia and Japan, respectively (Soto et al. 2009). Within the squid host, *V. fischeri* cells reside in a specialized morphological structure called the light organ, where the bacteria benefit from being in a nutrient-rich microenvironment relative to the oceanic water column (Soto et al. 2012). The squid utilize light produced by the bacteria for a cryptic behavior called counterillumination, which permits a squid to move with stealth at nighttime amid bright light from celestial sources such as the moon, stars, and nightglow (Jones and Nishiguchi 2004). Counterillumination functions analogously to countershading (Latz 1995). Squid hatchlings emanating from their eggs possess axenic light organs, which are colonized within hours by free-living *V. fischeri*. These free-living cells of *V. fischeri* originate from bacterioplankton communities or the microbiota in marine sediments (Soto et al. 2012).

Every day at dawn, the squid eject or vent 90–95 % of the light organ bacteria to the surrounding ocean environment (McFall-Ngai 2014). Bacteria remaining in the squid after venting undergo rapid cell division with a 30-minute generation time and repopulate the light organ to full capacity by the following evening (Soto et al. 2009). During the day, the squid sleep while remaining buried in the sand. At dusk, the squid emerges from the sand to engage in its nocturnal activity (e.g., foraging and searching for mates) (Jereb and Roper 2005). *V. fischeri* cells expelled via venting can survive and undergo binary fission (reproduction) in the ocean, albeit with a much slower generation time (from hours to weeks) than in the squid (Soto et al. 2009). Vented *V. fischeri* cells retain the ability to colonize future generations of squid hatchlings. As alluded to earlier, *V. fischeri* can be isolated

from oceans worldwide, including where the microorganism's animal hosts are not present (Soto et al. 2009). Furthermore, *V. fischeri* strains have been isolated from the ocean that are unable to develop bioluminescent mutualisms with either sepiolid squids or monocentrid fishes, apparently forced to subsist as free-living members of the bacterioplankton or as saprophytes/biofilms attached to suspended debris, detritus, sediment, or other living organisms (Soto et al. 2009; Wollenberg et al. 2012).

Materials and Methods

STRAINS, NEUTRAL MARKERS, AND MICROBIOLOGICAL MEDIA

V. fischeri strain ET00-7-1 was the strain used for this study (Soto et al. 2009). This strain was originally isolated from the sepiolid squid *E. tasmanica*, which inhabits ocean waters near Australia. For microbial selection experiments, one needs to distinguish the evolving bacteria from the ancestral strain (Lenski et al. 1991). This is done with the use of "neutral markers." Neutral markers are traits that permit the ancestral and derived (descendent) bacteria to be delineated from one another, but otherwise these traits have no effect on relative fitness for any assays conducted in the study (Soto et al. 2012). *V. fischeri* ET00-7-1 is sensitive to the antibiotic chloramphenicol (CAMS). Chloramphenicol resistance (CAMR) was used as a neutral marker in this investigation (McCann et al. 2003; Soto et al. 2012). An isogen of *V. fischeri* ET00-7-1 that is resistant to chloramphenicol was constructed using the mini-Tn7 transposon and named *V. fischeri* ET00-7-1Tn7. The routine insertion of the chloramphenicol resistance gene as a neutral marker, into the *V. fischeri* chromosome, using the mini-Tn7 transposon has already been described in detail elsewhere (McCann et al. 2003; Soto et al. 2014). See Supporting Information for additional details on strain maintenance, media recipes, aseptic techniques used, and for the details on how *V. fischeri* was evolved to establish a baseline "ancestral" environment. Herein, the term "growth" is used generally to refer to total cell yield (as CFUs) at a single endpoint, namely after incubation for 12 hours in liquid culture or 48 hours in the squid.

DETERMINING THE LOWER AND UPPER TEMPERATURE LIMITS OF GROWTH FOR *V. fischeri*

The temperature growth limits of *V. fischeri* ET (ET00-7-1 ancestral baseline) and TN (ET00-7-1Tn7 ancestral baseline) were examined in FLS (fortified Luria–Bertani salt) media using 2°C increments with 4°C serving as the starting low end. See Supporting Information for the methodology of how the ancestral baselines were established. The goal was to find the lower and upper temperatures that completely stopped *V. fischeri* ET and TN growth. To generate starter cultures, single colonies of the *V. fischeri* ET and TN lines (20 lines each) were separately inoculated into test tubes containing 10.0 mL FLS. Liquid cultures were incubated for 12 hours at 28°C and 200 rpm. To generate subcultures, the *V. fischeri* starter cultures (100.0 μL) were then used to inoculate test tubes containing 9.9 mL FLS. Broth cultures were incubated for 3 hours at 28°C and 200 rpm to generate log phase subcultures. Subcultures (100 μL) of all *V. fischeri* ET and TN lines ($n = 20$ each) were used to inoculate replicate test tubes containing 9.9 mL FLS for each temperature from 4°C to 40°C in 2°C increments. Most *V. fischeri* strains cannot grow at 4°C or 40°C (Farmer and Janda 2005). The starting cell density for each strain was 5.0×10^5 CFUs/mL. These broth cultures were then incubated for 12 hours and 200 rpm at their respective temperatures (4–40°C, 2°C increments, and $n = 20$ each for ET and TN per temperature).

To determine cell density (CFUs/mL), the test tube cultures were then spread onto FLS agar plates and incubated at 28°C for 24–48 hours. For the microbial selection studies, the incubation period for *V. fischeri* was restricted to 12 hours before transferring into fresh medium to maximize the amount of microbial evolution that occurred each day (24 hours). As the microbial generation time slows down as a liquid culture enters late log phase and stationary phase, transferring liquid cultures into fresh medium more frequently leads to more evolution in numbers of generations per 24 hours (Gerhardt and Drew 1994; White et al. 2011). *V. fischeri* is a fast reproducing microbe with a 20- to 30-minute generation time in nutrient-rich media such as FLS (Soto et al. 2009). At optimal temperature, *V. fischeri* ET and TN can easily reach a cell density greater than 1.0×10^{10} CFUs/mL in FLS within 12 hours, when the starting cell density is 5.0×10^5 CFUs/mL or higher.

MICROBIAL EXPERIMENTAL EVOLUTION OF *V. fischeri* TO THE LOWER AND UPPER TEMPERATURE LIMITS OF GROWTH FOR 2000 GENERATIONS

To generate starter cultures, single colonies of all the *V. fischeri* ET and TN lines were separately inoculated into test tubes containing 10.0 mL FLS. Broth cultures were incubated for 12 hours at 28°C and 200 rpm. To generate subcultures, the *V. fischeri* starter cultures (100.0 μL) were used to inoculate test tubes containing 9.9 mL FLS. Broth cultures were incubated for 3 hours at 28°C and 200 rpm to generate log phase subcultures. Subcultures (100.0 μL) of all *V. fischeri* ET and TN lines were used to inoculate replicate test tubes ($n = 20$) containing 9.9 mL FLS that were incubated at either 8°C (lower growth limit), 21°C (middle control), 28°C (optimal growth control), or 34°C (upper growth limit). These were the different temperature selection regimes (Table 1). The different derived lines that resulted from each of these selection regimes were called "cold specialists," "centrists," "optimists," and "heat specialists" (Table 1), respectively.

Table 1. The ancestors and derived lines from the current study are listed.

FLM Temp Lineage	ET (CAMS) and TN (CAMR) varieties
Ancestral lines	ET and TN – unevolved line – AKA ancestor
8°C derived lines	ET2000 8°C and TN2000 8°C – evolved line – lower growth limit – AKA cold specialist
21°C derived lines	ET2000 21°C and TN2000 21°C – evolved line – middle control – AKA centrist
28°C derived lines	ET2000 28°C and TN2000 28°C – evolved line – optimal control – AKA optimist
34°C derived lines	ET2000 34°C and TN2000 34°C – evolved line – upper growth limit – AKA heat specialist
8°C/34°C derived lines	ET2000 8°C/34°C and TN2000 8°C/34°C – evolved line – fluctuating lower upper – AKA temp generalist

V. fischeri **ET and TN (see text for explanation) are isogenic strains that are sensitive (CAMS) and resistant (CAMR) to the antibiotic chloramphenicol, respectively. Each was evolved for 2000 generations to the same temperature selection regimes, 8°C, 21°C, 28°C, 34°C, and 8°C/34°C ($n = 20$ each). Hence, two ancestral varieties (ET and TN) underwent 2000 generations of evolution in each temperature selection regime, which led to two varieties of derived lines (ET2000 and TN2000). For example, the two lines of cold specialists are ET2000 8°C (CAMS) and TN2000 8°C (CAMR).**

The starting cell density for each strain was 5.0×10^5 CFUs/mL. These test tube cultures were incubated for 12 hours at 200 rpm.

After 12 hours, each replicate liquid culture was subcultured (100.0 μL) into test tubes containing fresh 9.9 mL FLS ($n = 20$) that were once again incubated at either 8°C, 21°C, 28°C, or 34°C. The temperature of an evolving line never changed. These liquid cultures were incubated for 12 hours and 200 rpm. This procedure continued with 100-fold dilution transfers every 12 hours until the *V. fischeri* ET and TN lines all underwent 2000 generations of evolution at 8°C, 21°C, 28°C, or 34°C. The number of generations was determined through growth curve kinetics (Gerhardt and Drew 1994; White et al. 2011). In this serial passage experiment, 1.0 mL of liquid culture from each transfer was mixed with 1.0 mL of cryoprotectant solution in 2-mL cryovial tubes. Cryovial tubes were vortexed and placed in the –80°C freezer (Soto et al. 2012). The *V. fischeri* ET lines resulting from these selection regimes were called *V. fischeri* ET2000 8°C, ET2000 21°C, ET2000 28°C, and ET2000 34°C. Similarly, the *V. fischeri* TN lines were named *V. fischeri* TN2000 8°C, TN2000 21°C, TN2000 28°C, and TN2000 34°C.

MICROBIAL EXPERIMENTAL EVOLUTION OF *V. fischeri* TO TEMPORALLY FLUCTUATING LOWER AND UPPER TEMPERATURE LIMITS OF GROWTH FOR 2000 GENERATIONS

To generate starter cultures, single colonies of all the *V. fischeri* ET and TN lines were separately inoculated into test tubes containing 10.0 mL FLS. Liquid cultures were incubated for 12 hours at 28°C and 200 rpm. To generate subcultures, the *V. fischeri* starter cultures (100.0 μL) were used to inoculate test tubes containing 9.9 mL FLS. Broth cultures were incubated for 3 hours at 28°C and 200 rpm to generate log phase subcultures. Subcultures (100.0 μL) for each strain were used to inoculate replicate test tubes ($n = 20$) containing 9.9 mL FLS. The starting cell density for each strain was 5.0×10^5 CFUs/mL. These test tube cultures were incubated for 12 hours at 8°C (lower growth limit) and 200 rpm.

Each replicate liquid culture for each strain was subcultured (100.0 μL) into test tubes ($n = 20$) with fresh 9.9 mL FLS. These test tube cultures were incubated for 12 hours at 34°C (upper growth limit) and 200 rpm. Afterward, each replicate liquid culture for each strain was subcultured (100.0 μL) into test tubes ($n = 20$) with fresh 9.9 mL FLS and incubated for 12 hours at 8°C and 200 rpm. Next, each replicate liquid culture for each strain (100.0 μL) was transferred to test tubes ($n = 20$) with fresh 9.9 mL FLS and incubated for 12 hours at 34°C and 200 rpm. This procedure continued with 100-fold dilution transfers every 12 hours until the *V. fischeri* ET and TN lines both underwent 2000 generations of microbial evolution to temporally fluctuating 8°C and 34°C (fluctuating lower upper, Table 1, termed "8°C/34°C"). A "frozen fossil" record for this serial passage experiment was prepared as described earlier. The derived lines resulting from this selection regime were called "temp generalists" (Table 1). The *V. fischeri* ET lines resulting from this selection regime were named *V. fischeri* ET2000 8°C/34°C, while the *V. fischeri* TN lines were called *V. fischeri* TN2000 8°C/34°C (Table 1).

MICROBIAL GROWTH OF DERIVED LINES ALONG A TEMPERATURE GRADIENT

The cold specialists, centrists, optimists, heat specialists, and temp generalists were used for this experiment (Table 1). To generate starter cultures, single individual colonies from these derived lines were separately inoculated into test tubes containing 10.0 mL FLS. Liquid cultures were incubated for 12 hours at 28°C and 200 rpm. To generate subcultures, the *V. fischeri* starter

cultures (100.0 μL) were then used to inoculate test tubes containing 9.9 mL FLS. Broth cultures were incubated for 3 hours at 28°C and 200 rpm to generate log phase subcultures. Subcultures (100.0 μL) for each derived line were used to inoculate replicate test tubes ($n = 20$) containing 9.9 mL FLS for each temperature from 4°C to 40°C in 2°C increments. The starting cell density for each strain was 5.0×10^5 CFUs/mL. These broth cultures were then incubated for 12 hours and 200 rpm at their respective temperatures (4–40°C, 2°C increments, and $n = 20$ each for ET and TN per temperature). Each of the 20 derived lines for the cold specialists, centrists, optimists, heat specialists, and temp generalists went into a single test tube. To determine cell density (CFUs/mL), the test tube cultures were then spread onto FLS agar plates and incubated at 28°C for 24–48 hours. The goal for this experiment was to compare how evolution at 8°C, 21°C, 28°C, 34°C, and 8°C/34°C for 2000 generations affected the ability to grow along a temperature gradient relative to the *V. fischeri* ET and TN ancestors.

RELATIVE FITNESS ASSAYS

Competition experiments were set up between the derived lines (bacteria evolved at 8°C, 21°C, 28°C, 34°C, and 8°C/34°C) and the ancestors (*V. fischeri* ET and TN). Competitions were arranged between a derived line (at 400, 800, 1200, 1600, and 2000 generations) and an ancestor that were oppositely marked for sensitivity and resistance to chloramphenicol. Competitions between an ancestor and a derived line took place only in the temperature environment that the derived line evolved in. The aim of these experiments was to examine relative fitness between the derived lines and their ancestors in the temperature environments, where evolution took place for the derived line participating in the competition. Competitions between two ancestors (ET vs. TN) were "neutral marker" controls. In this study, the derived lines did not directly compete against one another. These relative fitness assays are similar to those in previous studies, where microbial adaptation to extreme temperatures has been investigated (Bennett et al. 1990, 1992; Mongold et al. 1999). Further details of the relative fitness assays are in Supporting Information.

ANIMAL EXPERIMENTS

Instant Ocean (SS15-10) was mixed with double-distilled water (34.0 g/L) to make 34.0 ppt artificial seawater (ASW) (Soto et al. 2012). A refractometer was used to confirm the salinity of ASW. Single colonies from FLS agar plates were used to generate starter cultures for the cold specialists, centrists, optimists, heat specialists, temp generalists, and the ancestors in test tubes containing 10.0 mL FLS. Starter cultures were also generated for the derived lines at 400, 800, 1200, and 1600 generations. Broth cultures were incubated for 12 hours at 28°C and 200 rpm. To generate subcultures, the *V. fischeri* starter cultures (100.0 μL) were used to inoculate test tubes containing 9.9 mL FLS. Broth cultures were incubated for 3 hours at 28°C and 200 rpm to generate log phase subcultures. Monoculture experiments were set up, where squid were inoculated with only one lineage of bacteria, either a derived line or an ancestor but not both. For the monoculture experiments in squid, the log phase subcultures for each derived line or ancestor were used to inoculate replicate 10-mL scintillation vials, with 5.0 mL ASW ($n = 20$), at a cell density of 1.0×10^3 CFUs/mL. For the derived lines ($n = 20$), each of the 20 replicates went into a single scintillation vial.

Competition experiments were also set up, where squid were inoculated with a 1:1 ratio between a derived line (at 2000 generations only) and an ancestor. Competitions were arranged between a derived line and an ancestor that were oppositely marked. For the competition experiments in squid, the log phase subcultures for each derived line and ancestor were used to co-inoculate replicate 10-mL scintillation vials with 5.0 mL ASW ($n = 20$). Coinoculations between a derived line and an oppositely marked ancestor (CAMS/CAMR) were at a 1:1 ratio in ASW, and the cell density for each contestant in a competition was 5.0×10^2 CFUs/mL. Hence, the combined cell density with both contestants was 1.0×10^3 CFUs/mL. Analogously, a competition was also set up between ancestors *V. fischeri* ET and *V. fischeri* TN in ASW ($n = 20$), which served as a control. Hence, all competitions performed in squid were between a derived line and an ancestor or between two ancestors. In the current study, no two derived lines were competed directly against one another.

E. scolopes hatchlings just emerging from their eggs and possessing axenic light organs were used for all experiments. *E. scolopes* hatchlings were placed in 10-mL scintillation vials with 5.0 mL ASW that were previously inoculated with bacteria as outlined earlier. With an initial cell density of 1.0×10^3 CFUs/mL in the scintillation vials, squid hatchlings are guaranteed to be colonized by *V. fischeri* (Soto et al. 2012). After a 3-hour incubation with *V. fischeri*, animals were rinsed three times with ASW to synchronize symbiont colonization within this time window. Rinsed animals were then placed back into new 10-mL scintillation vials with fresh 5.0 mL ASW. To create negative controls ($n = 20$), animals were also placed in 10-mL scintillation vials with 5.0 mL ASW, which contained no *V. fischeri*. Negative control animals were incubated in scintillation vials for 3 hours before being rinsed. Negative control animals were then put back into new 10-mL scintillation vials with fresh 5.0 mL ASW. As a result, negative control squid were never exposed to *V. fischeri*. In summary, $n = 20$ for all treatments and controls. Each replicate of the derived lines was inoculated into one squid animal for the monoculture experiments. Similarly for the competition experiments, each replicate of the derived lines was competed against an ancestor in one squid individual.

All animals were maintained in a 12-hour:12-hour dark–light cycle at 25°C. The focus of the current study was to conduct animal experiments at a temperature that was in the normal tolerance range for the Hawaiian bobtail squid, which is approximately 22–26°C (Soto et al. 2009). This temperature range is not stressful to *E. scolopes*. Experiments conducted at temperatures that were stressful to *E. scolopes* were not the scope of the current study. Water changes occurred every 12 hours with fresh ASW (Soto et al. 2012). At timepoint 48 hours, squid hatchlings were placed in a Promega GloMax 20/20 luminometer to measure bioluminescence (as relative light units). After this, animals were rinsed three times with ASW. Squid were then sacrificed and their light organs homogenized in 1.5-mL microfuge tubes containing ASW (Soto et al. 2012). Teflon microfuge pestles were used to homogenize light organs. Light organ homogenates were serially diluted with ASW. The serial dilutions were spread onto agar plates with and without chloramphenicol. These plate counts were used to determine *V. fischeri* colonization levels in the squid host (CFUs/squid). Plate cultures were incubated for 24–48 hours at 28°C (Soto et al. 2012).

For the animal experiments, the least significant difference (LSD) with a modified Bonferroni correction using the Dunn–Sidak method (experiment-wise Type 1 α error = 0.05) for all possible pairwise comparisons (k) was calculated (Sokal and Rohlf 1995; Soto et al. 2014). All possible pairwise comparisons $k = (a)(a-1)/2$, where "a" is the number of groups or classes (means). LSD values for k comparisons were implemented at the level of $\alpha' = 1 - (1 - \text{experiment-wise Type 1 } \alpha \text{ error})^{1/k}$ (Sokal and Rohlf 1995). This design of statistical analysis was chosen, because significant differences can easily be determined by visual inspection of the figures that present the data (Soto et al. 2014). This is helpful when many pairwise comparisons are being made. LSDs are represented as error bars. If the LSD error bars do not overlap, then the pairwise comparisons are significantly different. Overlapping LSD error bars are not significantly different. LSD error bars in figures function analogously to confidence intervals (Sokal and Rohlf 1995). This approach is acceptable when sufficiently large sample sizes are available. With large sample sizes, enough statistical power can be maintained to conduct many pairwise comparisons after a Bonferroni correction has been applied (Soto et al. 2014). In the present study, there is one animal per each *V. fischeri* line for each temperature selection regime. As a result, the present experimental design does not permit nested statistical analysis (Sokal and Rohlf 1995). Additionally, the variation of subsampling cannot be determined.

CORRELATIONAL STUDIES

Linear regression studies were implemented to demonstrate a positive correlation between *V. fischeri* adaptation to temperature stress and symbiosis with the sepiolid squid host. To this end, "Squid host colonization" and "bioluminescence" were each separately regressed onto relative fitness to temperature stress (i.e., "adaptation to temperature stress") at 0, 400, 800, 1200, 1600, and 2000 generations. (See "Relative Fitness Assays" and "Animal Experiments" sections for methodological details.) Separate linear regressions were done for the *V. fischeri* ET and TN varieties at each temperature stress, namely 8°C, 34°C, and 8°C/34°C. These correlations were then statistically analyzed as model I linear regressions (Sokal and Rohlf 1995).

Results

LOWER AND UPPER TEMPERATURE LIMITS OF GROWTH FOR *V. fischeri* ET AND TN

To determine its thermal niche space, *V. fischeri* was incubated over the entire temperature range it was capable of growing. This permitted a rigorous assessment of what temperatures were benign, optimal, and stressful to *V. fischeri*. This approach revealed what temperatures were appropriate as "stressful challenges" and benign controls for microbial experimental evolution with *V. fischeri*. Figure S1A shows the growth of the ancestors (*V. fischeri* ET and TN) along a temperature gradient (4–40°C). Results above 38°C are not shown in Fig. S1A, because no net growth occurred. *V. fischeri* ET and TN displayed highly similar results. The lower and upper growth limits were 8°C and 34°C, respectively. No reproducible microbial growth occurred below or above these temperatures. Within the range 8–34°C, *V. fischeri* ET and TN were always able to at least grow to a cell density of $\sim 10^7$ CFUs/mL (Lenski et al. 1991; Bennett and Lenski 1993). These results show that 8°C and 34°C were the appropriate regimes to evolutionarily select for increased tolerance to extreme temperature for 2000 generations in *V. fischeri*. Selection regimes conducted at the benign temperatures 21°C and 28°C served as "nonstress" controls, which were the midpoint (for the range 8–34°C) and optimal temperatures for *V. fischeri*, respectively. Please refer to Supporting Information to see the growth results of the derived lines along a temperature gradient.

RELATIVE FITNESS VALUES BETWEEN ANCESTRAL AND DERIVED LINES AT EVOLVED TEMPERATURES

Using the "frozen fossil record" constructed during the course of microbial experimental evolution to the different temperature regimes, the ancestral and derived lines were compared against one another at different evolutionary time points to assess relative fitness. The main aim here was to determine whether microbial adaptation occurred to benign, optimal, and stressful temperatures. Adaptation to benign and optimal temperatures were controls utilized to assess adaptation to temperatures that were taxing to microbial growth. The ultimate goal was to determine whether

Table 2. Relative fitness values were calculated with one-sample *t*-tests (two-tailed, Type 1 α error = 0.05) and compared to H_0 = 1.00 (n = 20).

Independent comparison	Relative fitness mean (± SE)
ET v TN 8°C	0.97 (±0.063) ns
ET v TN 34°C	1.00 (±0.065) ns
ET v TN 8°C/34°C (8°C→34°C→8°C)	1.02 (±0.070) ns
ET v TN 8°C/34°C (34°C→8°C→34°C)	1.01 (±0.060) ns
ET v TN 21°C	1.00 (±0.059) ns
ET v TN 28°C	0.99 (±0.062) ns
ET2000 v TN 8°C	**1.98 (±0.062)*****
TN2000 v ET 8°C	**1.96 (±0.049)*****
ET2000 v TN 34°C	**1.94 (±0.058)*****
TN2000 v ET 34°C	**1.99 (±0.064)*****
ET2000 v TN 8°C/34°C (8°C→34°C→8°C)	**1.77 (±0.063)*****
ET2000 v TN 8°C/34°C (34°C→8°C→34°C)	**1.83 (±0.075)*****
TN2000 v ET 8°C/34°C (8°C→34°C→8°C)	**1.76 (±0.067)*****
TN2000 v ET 8°C/34°C (34°C→8°C→34°C)	**1.81 (±0.041)*****
ET2000 v TN 21°C	1.02 (±0.040) ns
TN2000 v ET 21°C	1.05 (±0.052) ns
ET2000 v TN 28°C	1.04 (±0.049) ns
TN2000 v ET 28°C	1.02 (±0.059) ns

ns = $P > 0.05$ (not significant), ***$P \leq 0.001$.

Examples of how to read the notation are provided. ET versus TN 8°C means the two ancestors competing at 8°C. ET versus TN 8°C/34°C (34°C→8°C→34°C) indicates the two ancestors competing in temporally fluctuating 8°C and 34°C in a 34°C→8°C→34°C scheme. TN2000 versus ET 8°C/34°C (8°C→34°C→8°C) denotes *V. fischeri* TN evolved for 2000 generations at 8°C/34°C competing against ancestor *V. fischeri* ET at fluctuating 8°C and 34°C in an 8°C→34°C→8°C scheme. ET versus TN 8°C denotes both ancestors competing at 8°C. Key: ET = ancestral/unevolved *V. fischeri* ET, TN = ancestral/unevolved *V. fischeri* TN, ET2000 = *V. fischeri* ET evolved for 2000 generations, and TN2000 = *V. fischeri* TN evolved for 2000 generations.

microbial adaptation to temperature stress, when compared to microbial evolution at benign and optimal temperatures, significantly affected *V. fischeri*'s symbiosis with the squid host (see "Animal Experiments" section).

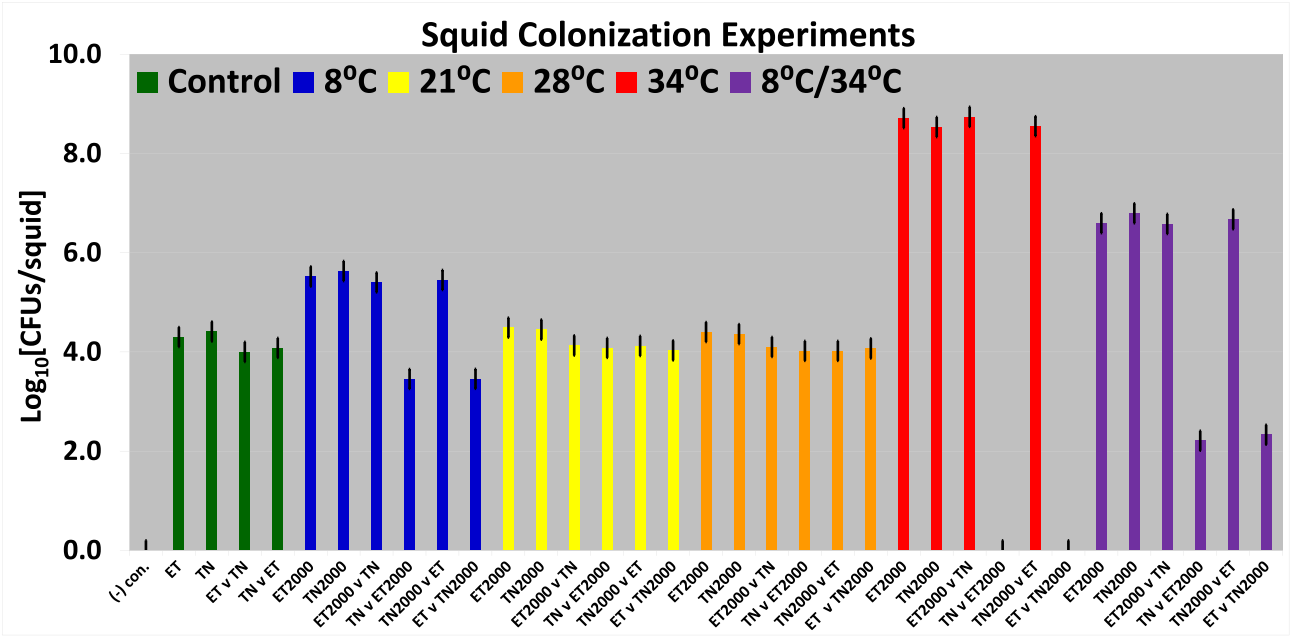
Table 2 shows the data for the relative fitness assays between the ancestral and derived lines. The derived line is always listed first in competitions between the ancestral and derived lines. None of the relative fitness values for the "ancestor versus ancestor" (ET vs. TN) experiments were significantly different from 1.00 at 8°C, 21°C, 28°C, 34°C, and 8°C/34°C (Table 2). *V. fischeri* successfully adapted to 8°C, 34°C, and 8°C/34°C, as all of the relative fitness values for these selection regimes are significantly larger than 1.00. There was at least a ∼90 % increase in relative fitness at 8°C and 34°C, whereas there was minimally a ∼70–80 % increase at 8°C/34°C. *V. fischeri* evolution at 21°C and 28°C displayed a trend of being 2–5 % greater in fitness relative to the ancestors. Nonetheless, these relative fitness increases at 21°C and 28°C were not significantly different from 1.00. Apparently, there was no appreciable selection pressure for *V. fischeri* to adapt to benign temperatures within 2000 generations.

ANIMAL EXPERIMENTS

The hypothesis being tested here was whether a microorganism (*V. fischeri*) experiencing evolution to an environmental stressor (temperature) during the free-living stage affected the microbe's ability to engage in symbiosis with its native host (the Hawaiian bobtail squid *E. scolopes*). Herein, there were two parameters that represented measures of "symbiosis." The first was squid host colonization. The second was bioluminescence induced in the squid host. Previous work with microbial experimental evolution in the squid-*Vibrio* mutualism has shown that these parameters are appropriate measures or readouts of "symbiosis" (Soto et al. 2012; Soto and Nishiguchi 2014). In this study, *E. scolopes* were inoculated with the ancestral and derived *V. fischeri* lines in monoculture and in 50:50 competitions. Host colonization and squid bioluminescence were then monitored. *V. fischeri* lines evolved in benign and optimal temperatures served as controls for the "stress" treatments. Animals never inoculated with any *V. fischeri* (i.e., light organs remaining in the axenic or gnotobiotic condition) were used as negative controls.

Figure 1A,B shows the squid colonization and bioluminescence data, respectively. Error bars in Fig. 1A,B signify the LSD for each data set. Means with overlapping LSD error bars are not significantly different (Sokal and Rohlf 1995). For the monocultural squid colonization experiments (hatchlings inoculated in pure culture), only a single participating strain is listed at the bottom of Fig. 1A. The competition studies in the squid are also shown in Fig. 1A. Participants in each competition are always oppositely marked (CAMS/CAMR). In the squid, competitions were either "ancestor versus ancestor" or "ancestor versus derived line." There were no "derived line versus derived line" competitions. At the bottom of Fig. 1A, for the squid competition experiments, both of the contestants are listed that were co-inoculated in a 1:1 ratio with the animals. For these competitions in the animal hosts, the amount of squid colonization is listed for the first member of a pair of contestants. The second member of a pair represents the opponent for that particular competition. Negative control animals were not colonized.

There were no significant differences in the squid colonization levels between the ancestors *V. fischeri* ET and TN in



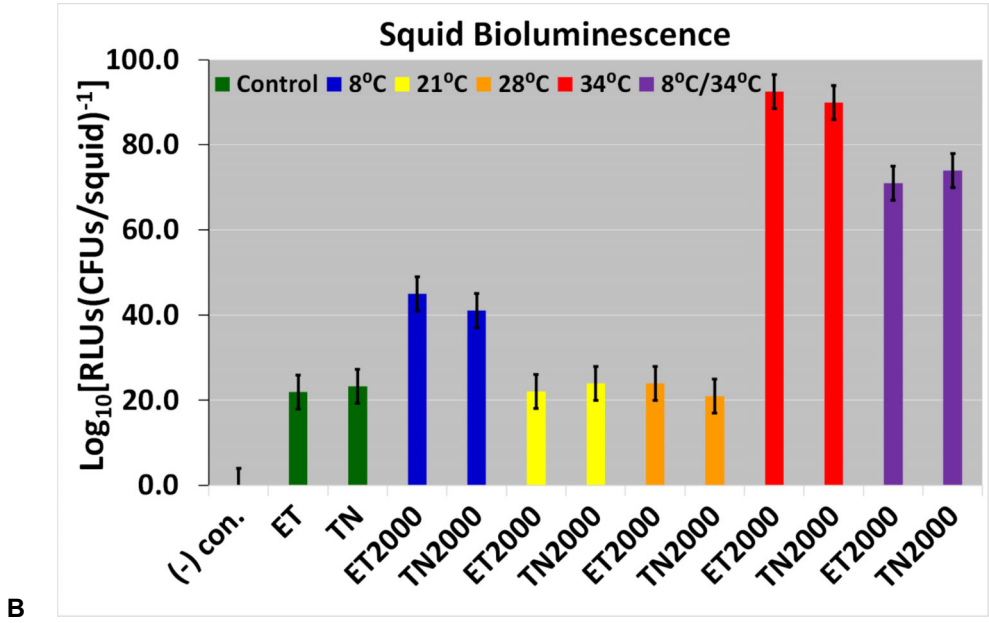


Figure 1. (A) The squid monocultural and competition data with the ancestral and derived lines. Monocultures only list one participant at the bottom of the diagram, whereas the competitions list both contestants. For the competitions, the colonization level (log_{10}[CFUs/squid]) shown is for the first contestant of a pair. The second contestant of a pair is the opponent for that particular competition. (B) Bioluminescence data of squid monocultures (pure cultures). For (A) and (B), the different colors represent either axenic animals (negative control), ancestors (positive controls), or the derived lines (in monoculture and competition). Key: (-) neg. = negative control, v = versus, ET = ancestral/unevolved *V. fischeri* ET, TN = ancestral/unevolved *V. fischeri* TN, ET2000 = *V. fischeri* ET evolved for 2000 generations, and TN2000 = *V. fischeri* TN evolved for 2000 generations. There are no competitions in (B). For (A) and (B), error bars represent least significant difference (LSD) of the mean.

monoculture or competition. Both colonize squid equally (Fig. 1A). In Fig. 1B, the bioluminescence data are omitted for the squid competitions, since there is no way to attribute bioluminescence in an animal to a single strain or line. Negative control animals were not bioluminescent. Moreover, the squid hosts colonized by the two ancestors (ET and TN) were not significantly different from each other in bioluminescence levels (Fig. 1B). In summary, the monocultural colonization levels in

Table 3. Rankings of squid colonization and bioluminescence levels in the various ancestral and temperature-adapted lines of *V. fischeri*.

Rank of squid colonization levels	Rank of squid bioluminescence levels
1. Heat specialists	1. Heat specialists
2. Temperature generalists	2. Temperature generalists
3. Cold specialists	3. Cold specialists
4. Ancestors = centrists = optimists	4. Ancestors = centrists = optimists
5. Axenic animals	5. Axenic animals

The axenic animals had no *V. fischeri* colonization and were not bioluminescent. The rankings are listed in descending order. Thus, squid colonized by the heat specialists contained the most bacteria and were the brightest.

the squid possessed a transitive relationship: heat specialists > temp generalists > cold specialists > ancestors = centrists = optimists > axenic animals (Fig. 1A). Squid bioluminescence levels also mirrored these relationships. These rankings are shown in Table 3 for clarity. These relationships were significantly different where equalities were lacking (no "equal" signs). Thus, the squid colonization and bioluminescence data were correspondent.

CORRELATIONAL STUDIES

Host colonization (green) and squid bioluminescence (blue) were plotted against adaptation to temperature stress (Figs. 2–4). All correlations had positive slopes and were statistically significant using model I linear regression analysis (Sokal and Rohlf 1995). Thus, direct positive correlations were demonstrated between both symbiosis parameters and adaptation to temperature stress over the course of 2000 generations for the cold specialists, the heat specialists, and the temp generalists. Host colonization and squid bioluminescence steadily improve for *V. fischeri* over the entire duration of adaptation to temperature stress. Results were similar for both the ET2000 and TN2000 varieties. Hence, antibiotic resistance did not greatly impact the evolutionary trajectories of the cold specialists, the heat specialists, and the temp generalists.

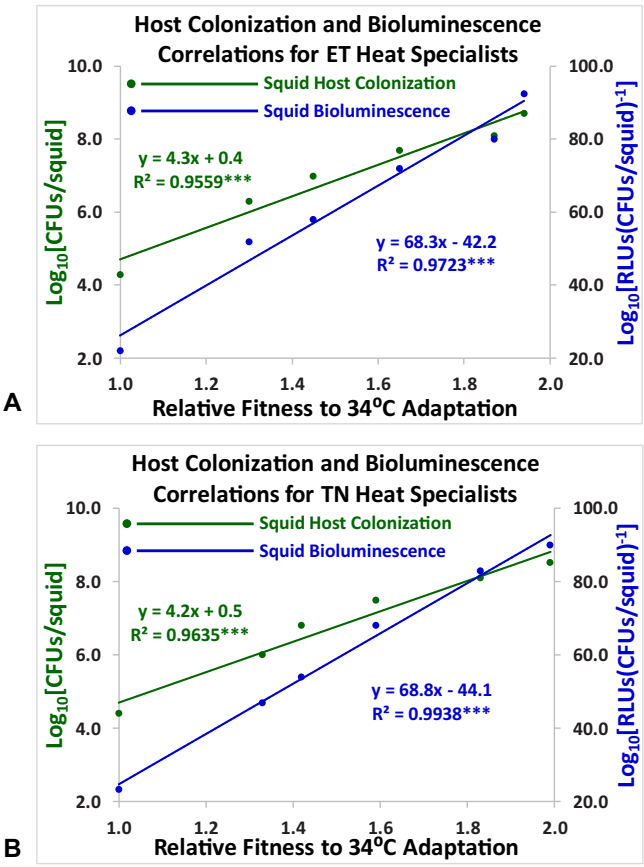


Figure 2. Linear correlations for the ET2000 (A) and TN2000 (B) varieties of the heat specialists. Host colonization (green) and squid bioluminescence (blue) for *V. fischeri* are regressed against adaptation to the upper temperature growth limit (34°C). Each data point represents the arithmetic mean of 20 lines ($n = 20$) at 0, 400, 800, 1200, 1600, and 2000 generations. The slopes are positive and statistically significant.

Discussion

In this study, the lower and upper growth limits due to temperature stress were 8°C and 34°C for *V. fischeri* (Fig. S1A). *V. fischeri* successfully adapted to these extreme temperatures and their fluctuations (Table 2). Moreover, there was no significant adaptation to the benign temperatures 21°C and 28°C. Relative to the ancestors, the ability of *V. fischeri* to grow along a temperature gradient was greatly affected as a result of adaptation to stressful temperatures (Fig. S1A–D). For example, the relative symmetry, skewness, and kurtosis of *V. fischeri*'s growth distribution along a temperature gradient changed as an evolutionary response to temperature stress. This was not true for evolution to the benign temperatures 21°C and 28°C (Fig. S2A,B). The growth distribution of the heat specialists is especially negatively skewed (Fig. S1C). *V. fischeri* was able to increase its relative fitness similarly to both low temperature and high temperature stress, at least 90 % for both (Table 2). Adaptation to temporally fluctuating 8°C and 34°C was somewhat less (70–80 %) relative to the ancestors.

Interestingly, the present data are consistent with taxonomic and biological systematics information reported earlier about *V. fischeri*. Most *V. fischeri* strains fail to grow at 4°C and 40°C, and nearly all strains grow at 30°C (Farmer and Janda 2005). *V. fischeri* never manifested the ability to grow at 4°C and 40°C as a result of evolution to temperature stress. The ancestors were also unable to grow at 4°C and 40°C. Moreover, the ancestors and all the derived lines were always able to grow at 30°C. Intriguingly,

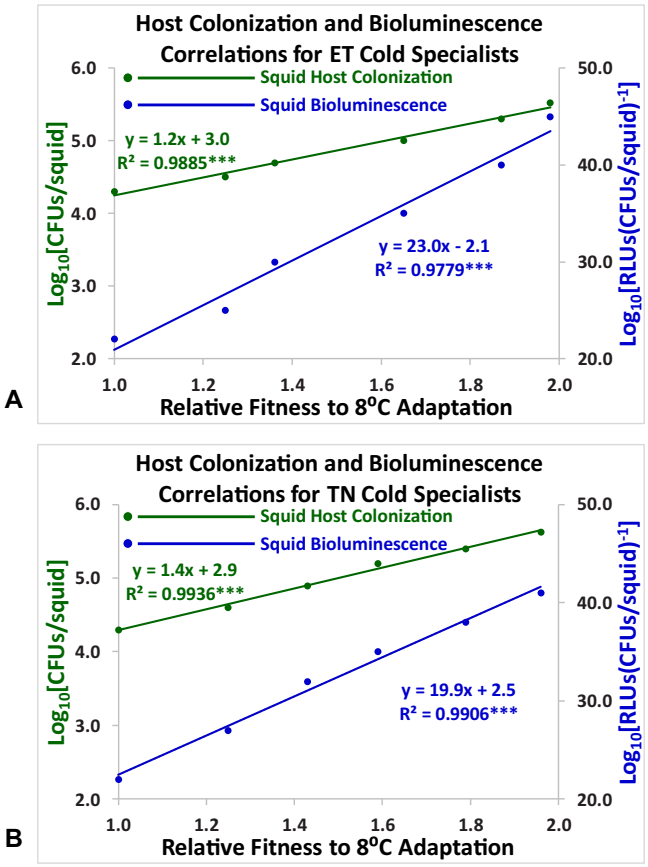


Figure 3. Linear correlations for the ET2000 (A) and TN2000 (B) varieties of the cold specialists. Host colonization (green) and squid bioluminescence (blue) for *V. fischeri* are regressed against adaptation to the lower temperature growth limit (8°C). Each data point represents the arithmetic mean of 20 lines ($n = 20$) at 0, 400, 800, 1200, 1600, and 2000 generations. The slopes are positive and statistically significant.

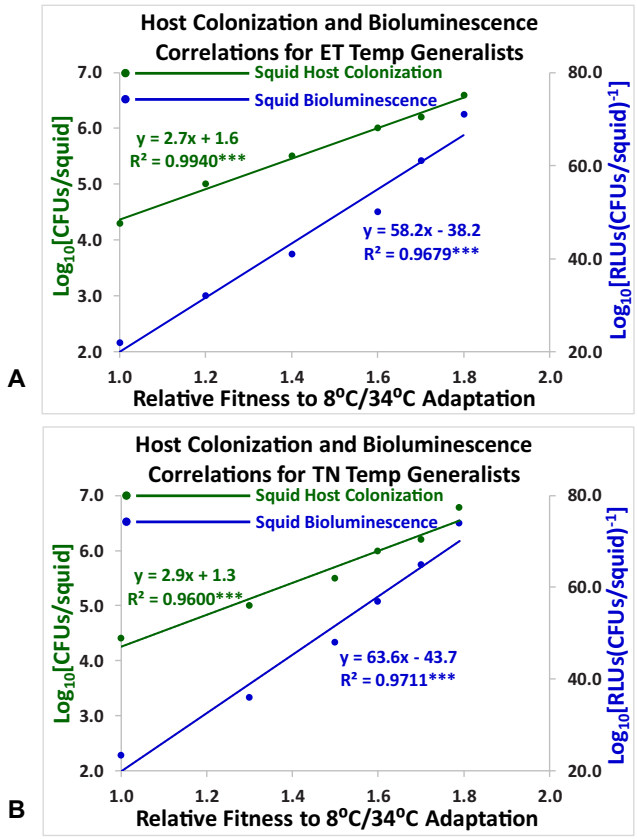


Figure 4. Linear correlations for the ET2000 (A) and TN2000 (B) varieties of the temp generalists. Host colonization (green) and squid bioluminescence (blue) for *V. fischeri* are regressed against adaptation to fluctuation between the lower and upper temperature growth limits (8°C/34°C). Each data point represents the arithmetic mean of 20 lines ($n = 20$) at 0, 400, 800, 1200, 1600, and 2000 generations. The slopes are positive and statistically significant.

the ability to grow at 35°C is polymorphic (Farmer and Janda 2005). The ancestors were unable to grow at 36°C (Fig. S1A), neither were the cold specialists, centrists, optimists, and temp generalists (Figs. S1B,D and S2A,B). Nonetheless, the heat specialists were able to grow at 36°C (Fig. S1C). The cold specialists did not grow at 34°C (Fig. S1B). The ancestors and all the other derived lines were able to grow at 34°C. Thus, temperature evolution may play a role in defining thermal niche space in the Vibrionaceae. Indeed, the temperature range 34–36°C may be an "evolutionary thermocline" for *V. fischeri*.

Within the Vibrionaceae, there is some evidence adaptation to extreme temperatures leads to tradeoffs (Urakawa and Rivera 2006). Explanations for tradeoffs include adaptive specializations in cell membrane fluidity, molecular chaperones, and melting of nucleic acids but many other possibilities exist (White et al. 2011). Few species can simultaneously grow ≥ 40°C and ≤ 4°C. There are some exceptions, *V. hispanicus* for example (Farmer and Janda 2005). These tradeoffs can affect host–microbe relationships (Nishiguchi 2000; Bronikowski et al. 2007). *Photobacterium* is hypothesized to be more tolerant than *Vibrio* to especially low temperatures (< 4°C) for prolonged periods, particularly at great depths where the atmospheric pressure is considerable (Urakawa and Rivera 2006; Gomez-Gil et al. 2014). As a result, *Photobacterium* is the mutualist more likely to be associated with animal hosts in light organ symbioses within the deep sea (Gomez-Gil et al. 2014). However, numerous *Vibrio* species exist that are well adapted to low temperatures, including *V. logei*, *V. wodanis*, and *V. salmonicida* (Urakawa and Rivera 2006). Of these, *V. logei* (a close relative of *V. fischeri*) is known to be a bioluminescent symbiont of deep sea animals, whereas *V. salmonicida* can cause "cold-water disease" in fish (Farmer and Janda 2005; Gomez-Gil et al. 2014).

Selection pressure to temperature stress clearly impacted *V. fischeri*'s ability to colonize the squid host (Fig. 1A). Bioluminescence was also affected (Fig. 1B). These results were true for low (8°C) and high temperature stress (34°C), along with oscillation

between the two. Evolution to benign temperatures (21°C and 28°C) did not have these same effects. Additionally, *V. fischeri* was subjected to these selection pressures while outside the squid host. Consequently, microbial evolution to an environmental stressor during the free-living phase can greatly influence host–microbe interactions. Notably, microbial adaptation to stress during the free-living phase can actually improve or facilitate the fitness of the microorganism, when it subsequently associates with its natural host again. That is, microbial evolution to an environmental stressor during the free-living phase can introduce exaptations for commensalisms, pathogeneses, and mutualisms (Gould and Vrba 1982). Figures 2–4 show statistically significant positive correlations between symbiosis and adaptation to temperature stress. This is a counterintuitive conclusion. Instead, a decrease in microbial fitness (attenuation) within the host might normally be the expected result. This decrease in microbial fitness commonly results from antagonistic pleiotropy, mutation accumulation, and the absence of purifying selection (Dougan et al. 2002; Elena and Lenski 2003; Frey 2007).

In recent years, there has been a growing awareness that distinct genetic regulatory networks, diverse biochemical pathways, and different signal transduction cascades can be cross-connected in complex ways (McAdams et al. 2004). Researchers now possess a better understanding of the crossroads between the bacterial stress responses and host colonization factors for microorganisms involved in host–microbe relationships. Additionally, stressful environments and toxic chemicals in the host may serve as important cues for microbial symbionts and pathogens to initiate specific physiological responses, commencing certain patterns of gene expression for example (Schwartzman and Ruby 2016a,b). Local pH environment is a common cue in many host–microbe associations (Gahan and Hill 1999). For instance, when *Helicobacter pylori* (a human pathogen) encounters the acidic environment of the stomach, this microbe begins expressing virulence factors that promote gastric proliferation and homeostasis against low pH stress (Ang et al. 2001).

In conclusion, microorganisms cycling between a free-living stage and a host-associated phase will likely encounter stress in each of these environments (Atlas and Bartha 1998; Schwartzman and Ruby 2016b). Frequently, microbial evolution in each of these life cycles is studied separately or with a "tradeoff" paradigm (Soto and Nishiguchi 2014; Hoang et al. 2016). Perhaps a better perspective is one that envisions these alternate life cycles as different stages in a single microbial life history, where natural selection can produce adaptations to stress at each stage that synergistically benefits the entire life history. Positive pleiotropy is known to serve this role in the ontogeny of multicellular organisms (Govindaraju 2015). Positive pleiotropy may be expected for genes affecting fitness traits that need to be maximized or maintained efficiently across varying life histories (e.g., free-living versus host environments). For the current study, microbial evolution to temperature stress—cold, heat, and fluctuating—during the free-living stage was positively pleiotropic to animal host colonization and bioluminescence.

AUTHOR CONTRIBUTIONS

MLC, EVM, and NMR participated in conducting experiments, revising the manuscript critically for intellectual content, collecting, interpreting, and analyzing data. They also repeated experiments to increase statistical power and did most work with temperature gradients, media preparation, solutions, daily transfers of bacteria, microbiology, and helped to create tables. EVM and NMR did most of the squid aquaculture. NMR helped with the figures. WS is the corresponding author and principal investigator. He paid for the research materials and was responsible for the experimental design. He did the squid experiments and sacrificed the squid. WS also did squid aquaculture. WS mentored and trained the first three coauthors on how to do work, data analyses, tables, and figures. WS wrote most of the paper. He also participated in revising the manuscript critically for intellectual content, collecting, interpreting, and analyzing data.

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DATA ARCHIVING

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.d12k64m

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. The ancestors **(A)**, cold specialists **(B)**, heat specialists **(C)**, and temp generalists **(D)** were grown along a temperature gradient (4–40°C, $n = 20$ for each variety per temperature). The broken line represents the starting cell density 5×10^5 CFUs/mL. Cell densities below this line failed to display net microbial growth. Error bars represent standard error of the mean.

Figure S2. The centrists **(A)** and optimists **(B)** were grown along a temperature gradient (4–40°C, $n = 20$ each for each variety per temperature). The broken line represents the starting cell density 5×10^5 CFUs/mL. Cell densities below this line failed to display net microbial growth. Error bars represent standard error of the mean.