

Ecological Diversification of *Vibrio fischeri* Serially Passaged for 500 Generations in Novel Squid Host *Euprymna tasmanica*

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Abstract *Vibrio fischeri* isolated from *Euprymna scolopes* (Cephalopoda: Sepiolidae) was used to create 24 lines that were serially passaged through the non-native host *Euprymna tasmanica* for 500 generations. These derived lines were characterized for biofilm formation, swarming motility, carbon source utilization, and in vitro bioluminescence. Phenotypic assays were compared between “ES” (*E. scolopes*) and “ET” (*E. tasmanica*) *V. fischeri* wild isolates to determine if convergent evolution was apparent between *E. tasmanica* evolved lines and ET *V. fischeri*. Ecological diversification was observed in utilization of most carbon sources examined. Convergent evolution was evident in motility, biofilm formation, and select carbon sources displaying hyperpolymorphic usage in *V. fischeri*. Convergence in bioluminescence (a 2.5-fold increase in brightness) was collectively evident in the derived lines relative to the ancestor. However, dramatic changes in other properties—time points and cell densities of first light emission and maximal light output and emergence of a lag phase in growth curves of derived lines—suggest that increased light intensity per se was not the only important factor. Convergent evolution implies that gnotobiotic squid light organs subject colonizing *V. fischeri* to similar selection pressures. Adaptation to novel hosts appears to involve flexible microbial metabolism, establishment of biofilm and swarmer

V. fischeri ecotypes, and complex changes in bioluminescence. Our data demonstrate that numerous alternate fitness optima or peaks are available to *V. fischeri* in host adaptive landscapes, where novel host squids serve as habitat islands. Thus, *V. fischeri* founder flushes occur during the initiation of light organ colonization that ultimately trigger founder effect diversification.

Introduction

The Sepiolid Squid–*Vibrio* Mutualism

Sepiolid squids in the genera *Sepiolo* and *Euprymna* form light organ mutualisms with marine bioluminescent bacteria from the genera *Vibrio* and *Photobacterium* from the family Vibrionaceae [1]. Sepiolid squids use light produced by their bacterial symbionts for a cryptic behavior termed counterillumination [2], and the light organ bacteria are in turn exposed to a nutrient-rich microcosm in the host [3]. Particularly, the mutualism between *Vibrio fischeri* and *Euprymna* has become a model for studying associations between eukaryotic hosts and bacteria, since both partners can be maintained independently of each other in the laboratory [4, 5]. Axenic juvenile *Euprymna* squids hatch from their eggs with sterile light organs and are quickly colonized by symbiotically competent *V. fischeri* present in bacterioplankton, reaching a light organ carrying capacity (10^4 – 10^7 colony forming units (CFUs)/light organ) within 12–24 h [6, 7]. Sepiolid squids are nocturnal and, seed the surrounding water with symbiotic competent *V. fischeri* at dawn by venting 90–95 % of the symbiont light organ population. By the next evening, the remaining *V. fischeri* inside the animal grow to repopulate the light organ to full capacity [5]. These vented symbionts in the oceanic water column serve as a source population for colonizing the next generation of squid hatchlings.

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Not all strains of *V. fischeri* are capable of colonizing *Euprymna* and *Sepiolo* hosts, as some isolates are restricted to planktonic and commensal lifestyles (non-light organ associations with animals) [6]. *V. fischeri* is also able to initiate light organ mutualisms with monocentrid fishes. These mutualisms are each formed with ecologically and genetically distinct *V. fischeri* [8], and strains indigenous to monocentrid fish hosts do not colonize sepiolid squid to the same population levels (i.e., CFUs/light organ) as “squid” isolates [9]. Additionally, *Euprymna* species are distributed allopatrically throughout the Indo-West Pacific Ocean, and *V. fischeri* colonizing this genus are host specialists, exhibiting competitive dominance, whereas strains forming mutualisms with several sympatric *Sepiolo* species from the Mediterranean Sea [1, 9–11] are host generalists and display no competitive dominance. Prior evidence from experimentally evolved lines of *V. fischeri* ES114 (native to Hawaiian *Euprymna scolopes*) selected through a non-native host (Australian *Euprymna tasmanica*) for 500 generations exhibited a significant increase in mean fitness relative to the ancestral strain in colonizing *E. tasmanica* [7], yet the traits responsible for this host specificity change remain unknown. Therefore, to determine which phenotypes are subject to host selection, both ancestor and evolved clones were characterized for biofilm formation, motility, carbon source utilization, and in vitro bioluminescence. Wild *V. fischeri* isolates from field-caught *E. tasmanica* (“ET” isolates) and *E. scolopes* (“ES” isolates) were also compared to experimentally evolved strains to determine if the derived lines exhibited any convergent evolution relative to indigenous ET *V. fischeri*.

Methods and Materials

Motility Assay

A substantial literature exists on Vibrionaceae motility, and evidence for swarming in *Vibrio* is prolific and well documented [12–19]. The genus *Vibrio* possesses a dual flagellar system [18, 20, 21]. Polar flagella are produced by cells continuously for facile locomotion such as swimming in liquid, while inducible lateral or peritrichous flagella are manufactured for more arduous navigation via swarming on solid surfaces or through viscous milieus, including 0.5 % agar [18]. Polar and lateral flagella in *Vibrio* spp. are encoded and regulated by distinct gene sets [19]. When performing motility assays on 0.5 % agar with *Vibrio*, swarming can be quantified by measuring the diameter of an expanding and growing bacterial mass. Since twitching motility occurs much more slowly than swarming, the diameter of the expanding bacterial mass does not represent the former. Swimming was not measured in these assays, since the locomotion we observed occurred on the surface of 0.5 % agar rather than

within. This was confirmed by examining the motile bacteria under a dissecting microscope. Motility assays were modified from a previously published protocol [22] to monitor swarming. All bacterial strains were grown in 18×150 mm glass test tubes containing 5 mL 70 % 32 ppt seawater tryptone (SWT: 5 g tryptone, 3 g yeast extract, 3.75 mL glycerol, 300 mL double distilled water, and 700 mL 32 ppt artificial seawater made with Instant Ocean®) [23–25] liquid media at 28°C and 225 rpm for 16 h. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of overnight starter cultures was used to inoculate 18×150 mm glass test tubes with 10 mL 70 % 32 ppt SWT and incubated at 28°C at 225 rpm until subcultures reached 0.10 OD₆₀₀ (~1.0×10⁷ CFUs/mL). Bacterial cells were pelleted by centrifugation (10,000 rpm) at 4 °C for 10 min in 15-mL conical tubes, discarding the supernatant afterward. Bacterial pellets were resuspended with 10 mL washes of either filter-sterilized 34 ppt artificial seawater (Instant Ocean®), minimal ribose media, or 34 ppt SWT to remove residual nutrient media from the overnight culture [26]. This centrifugation and wash procedure was performed three times. After the third centrifugation step and resuspension wash, the cell suspension was once again centrifuged for 10 min and pelleted. The bacterial pellet was then resuspended either with filter-sterilized 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT. For each isolate or strain, 10 μL of the resuspended pellet (~10⁵ total cells) was placed directly center onto (0.5 %) swarm motility agar plates [26, 27] containing either 34 ppt artificial seawater, minimal ribose [25], or 34 ppt SWT, representing different nutrient conditions to observe motility (*n*=3). This was also repeated for negative control (5.0 %) agar plates, where the agar concentration is sufficiently high to prevent swarming. The negative control plates also provided a baseline for comparing motility on each of the different media or nutrient conditions. All agar plates utilized in the motility assays received only a single 10 μL resuspension per isolate. Agar volume placed into each Petri dish for motility assays was measured and kept constant (20 mL). Immediately after the swarm agar plates (including negative controls) solidified, they were allowed to air dry at 25°C for 24 h before use. The plates were incubated for 24 h at 28°C, when diameters of swarming bacteria were measured [22, 26]. Fisher least significant differences (LSDs), Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate α =0.05) [28], were calculated separately for the wild isolates and each evolved time point (100, 200, 300, 400, and 500 generations). Chloramphenicol resistance has no effect on motility, as *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were not significantly different from each other under all conditions. Chloramphenicol resistance is thus a neutral marker with respect to motility between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200.

Microtiter Plate Biofilm Assay

Microtiter plate biofilm assay was adapted from a published methodology initially used for staphylococci [29]. Sixteen-hour *V. fischeri* cultures were grown in 18×150 mm glass test tubes containing 5 mL 70 % SWT in a 28°C air shaker at 225 rpm. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of overnight starter cultures were each separately inoculated into 18×150 mm glass test tubes with 10 mL of fresh 70 % 32 ppt SWT and incubated in a 28°C air shaker at 225 rpm. These subcultures were grown to 0.10 OD₆₀₀ (~1.0 × 10⁷ CFUs/mL). All 0.10 OD₆₀₀ subcultures were centrifuged (10,000 rpm) at 4 °C, pelleted, resuspended, and washed three times as previously described with either liquid filter-sterilized 34 ppt artificial seawater, minimal ribose, or 34 ppt SWT to ensure all residual 70 % 32 ppt SWT were removed. After the third centrifugation step and resuspension wash, the cell suspension was once again centrifuged for 10 min and pelleted. The bacterial pellet was then resuspended either with filter-sterilized 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT. Cell suspensions (2 µL) were subsequently inoculated into polystyrene 96-well microtiter plates containing 200 µL of either filter-sterilized 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT (*n*=12). Three liquid media types were used to examine *V. fischeri* biofilm formation in different environments representing varying degrees of nutrient availability. Uninoculated wells remained on each of the three different liquid media microtiter plates to serve as negative controls. Plates were incubated for 18 h at 28 °C without shaking. Media in all wells were then gently removed and washed three times appropriately with either 200 µL filter-sterilized 34 ppt artificial seawater, minimal ribose, or 34 ppt SWT, removing wash liquid each time. Microtiter plates were then incubated at room temperature for 15 min. Two hundred microliters of 0.2 % crystal violet dye was then added to each well and incubated for 30 min at room temperature. After staining, crystal violet was gently removed with a pipet and the wells washed three times with either 200 µL 34 ppt artificial seawater, minimal ribose, or 34 ppt SWT, removing wash liquid after each time. Microtiter plates were allowed to incubate at room temperature for 15 min. Two hundred microliters of 70 % ethanol was added to all the wells in the microtiter plates and incubated at room temperature for 20 min. Absorbance readings were measured for all microtiter plates at 562 nm using a Bio-Tek ELx800™ microplate reader (Winooski, VT). Fisher LSDs, Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate $\alpha=0.05$) [28], were calculated separately for the wild isolates and each evolved time point (100, 200, 300, 400, and 500 generations). Chloramphenicol resistance has no effect on biofilm formation, as *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were

not significantly different from each other under all conditions. Chloramphenicol resistance is thus a neutral marker with respect to biofilms between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200.

Carbon Source Utilization for 95 Substrates

Biolog GN2 microplates (Hayward, CA) for gram-negative bacteria were used, where substrates in each of 95 wells represent different carbon sources, along with one negative control well that contains only distilled water [30, 31]. Utilization of a substrate was recorded by measuring increases in well absorbance relative to the negative control at 590 nm. Manufacturer's instructions (Biolog GN2 MicroPlate™) and protocols were used with modification. All bacterial strains were grown in 18×150 mm glass test tubes containing 5 mL 70 % 32 ppt SWT at 28°C and 225 rpm for 16 h. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of overnight starter cultures was used to inoculate 125-mL triple-baffled culture flasks with 50 mL of fresh 70 % 32 ppt SWT, which were incubated at 28°C and shaken at 225 rpm until the flask subculture reached 0.220 OD₅₉₀ (~2.0×10⁷ CFUs/mL). Bacterial cells from each 0.220 OD₅₉₀ subculture were pelleted by 4 °C centrifugation (10,000 rpm) for 10 min and resuspended with Biolog GN/GP inoculation fluid (product no. 72101) to remove residual nutrient media from overnight cultures. To meet *V. fischeri* osmolar requirements, the salinity of the Biolog inoculation fluid was adjusted to 3.4 % NaCl final concentration. The centrifugation and wash procedure was completed three times. After the third centrifugation step and resuspension wash, cells were once again centrifuged for 10 min and pelleted. The bacterial pellet was then resuspended with Biolog inoculation fluid. Working *V. fischeri* cell suspensions for each strain were dispensed into Biolog GN2 microplates. All wells in Biolog GN2 microplates were inoculated with 150 µL of washed *V. fischeri* cells at 2.0×10⁷ CFUs/mL suspended in Biolog GN/GP inoculation fluid. Uninoculated Biolog GN2 microplate wells containing only Biolog GN/GP inoculation fluid (adjusted to 3.4 % NaCl final concentration) and no bacteria served as negative controls. All negative controls and *V. fischeri* strains were measured in triplicate (*n*=3). Microplates were incubated at 28°C for 24 h. Afterwards, optical densities (590 nm) of the wells were measured by placing GN2 plates in a Bio-Tek ELx800™ microplate reader (Winooski, VT). Fisher LSDs, Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate $\alpha=0.05$) [28], were calculated separately for wild isolates and the 500-generation evolved time point. Chloramphenicol resistance has no effect on carbon source utilization, as *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were not significantly different from each other on any carbon source. Chloramphenicol

resistance is thus a neutral marker with respect to carbon source utilization between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200.

Bioluminescence

Sixteen-hour *V. fischeri* cultures were grown in 18×150 mm glass test tubes containing 5 mL 70 % SWT liquid media in a 28°C air shaker at 225 rpm. These test tubes were initially inoculated with a single colony from an SWT 1.5 % agar plate. Ten microliters of the overnight cultures was each separately inoculated into 18×150 mm glass test tubes with 5 mL of fresh 70 % 32 ppt SWT and incubated in a 28°C air shaker at 225 rpm to obtain exponential growth subcultures at 0.5 OD₆₀₀. The 0.5 OD₆₀₀ subcultures were used to inoculate 125-mL triple-baffled culture flasks with fresh 50 mL 34 ppt SWT to an initial 5×10⁵ CFUs/mL cell density. Culture flasks were incubated at 28°C and shaken at 225 rpm for 8 h with bioluminescence readings and plate count enumeration taken every 30 min (*n*=6). Bioluminescence readings were recorded with a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Since total bacterial luminescence is dependent on cell density and total volume, measurements were reported as relative light units (RLUs) per log₁₀ (CFUs/mL) per milliliter of liquid culture (RLUs [log₁₀ (CFUs/mL)]⁻¹mL⁻¹) over time. Cell density determinations were completed on 34 SWT 1.5 % agar plates, which were incubated at 28 °C for 24 h. During these time interval experiments, *V. fischeri* broth cultures have nearly 100 % plating efficiency [6]. Bioluminescence readings of *Escherichia coli* K12 MG1655 were also monitored as a negative control under the same experimental conditions with the exception that Luria Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 10 g NaCl, and 1 L double distilled water) and LB 1.5 % agar plates were used. There was no difference in bioluminescence between *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 through all time points, showing that chloramphenicol is a neutral marker with this trait. Bioluminescence data were assessed using repeated measures analysis. No assumption was made with sphericity. The quite conservative Extreme Greenhouse-Geisser and Lower Bound corrections were applied separately to statistical main effects [32]. Fisher LSDs, Bonferroni corrected for multiple pairwise comparisons using the Dunn-Sidak method (type I experimentwise error rate $\alpha=0.05$) [28], were calculated separately for wild isolates and the 500-generation evolved time point.

Results

Motility

Ten ET (wild isolates from *E. tasmanica*) and ten ES (wild isolates from *E. scolopes*) *V. fischeri* strains were previously

shown to be genetically distinct from one another [7, 8, 33]. These field isolates were randomly sampled from their *Euprymna* squid hosts and used as a comparison to the evolved clones for motility (Fig. 1a). ES wild strains were more motile than ET on minimal ribose and 34 ppt SWT. *V. fischeri* ES114 is chloramphenicol sensitive, while *V. fischeri* JRM200 is a *V. fischeri* ES114 derivative which is chloramphenicol resistant [24]. *V. fischeri* JRM200 was used to create 24 lines that were serially transferred through novel squid host *E. tasmanica* for 500 generations [7] and used in this study. All 24 evolved lines improved in colonization of *E. tasmanica* relative to ancestor, as no lineage remained the same or became worse. *V. fischeri* lines serially passaged through *E. tasmanica* for 500 generations became less motile relative to ancestor *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 (zero generations through *E. tasmanica*) on minimal ribose and 34 ppt SWT (Fig. 1b–d for 100, 200, and 500 generations; supplementary Figs. S1A and S1B for 300 and 400 generations). Unlike many bacterial species, *V. fischeri* is capable of motility on minimal media without nutritional supplements such as casamino acids [27]. No growth was observed on 34 ppt artificial seawater 0.5 % agar plates as well as on the motility negative control 5.0 % agar plates. Significant decreases in motility were first observed for all lines by 100 generations (Fig. 1b) on motility SWT plates and continued to decline throughout the remainder of the selection experiment. However, by 500 generations, most derived lines still remained motile on SWT plates relative to SWT motility negative controls (Fig. 1d). Although significant decreases in motility on minimal ribose is evident by 100 generations (Fig. 1b), a marked drop was noted by 200 generations (Fig. 1c), as most of the derived lines are no longer significantly more motile than on minimal ribose motility negative control plates. Motility on minimal ribose plates was completely lost by 300 generations (Fig. S1A). Thus, the derived lines converged on ET motility on minimal ribose and 34 ppt SWT, yet significant differences (polymorphisms) were seen among the various lineages in motility as a result of adaptation to novel host *E. tasmanica*.

Biofilm Formation

No bacterial contamination was observed in uninoculated negative control wells for 34 ppt artificial seawater, minimal ribose media, or 34 ppt SWT. Biofilm formation is higher for the ET wild isolates than the ES ones in artificial seawater, minimal ribose, and 34 ppt SWT (Fig. 2a). *V. fischeri* passaged through *E. tasmanica* increased their biofilm formation capacity (Fig. 2b–d for 100, 400, and 500 generations; supplementary Figs. S2A and S2B for 200 and 300 generations). Relative to the loss of motility, the evolution of elevated biofilm formation was delayed, not appearing in great levels until 400 generations (Fig. 2c), when it simultaneously appeared

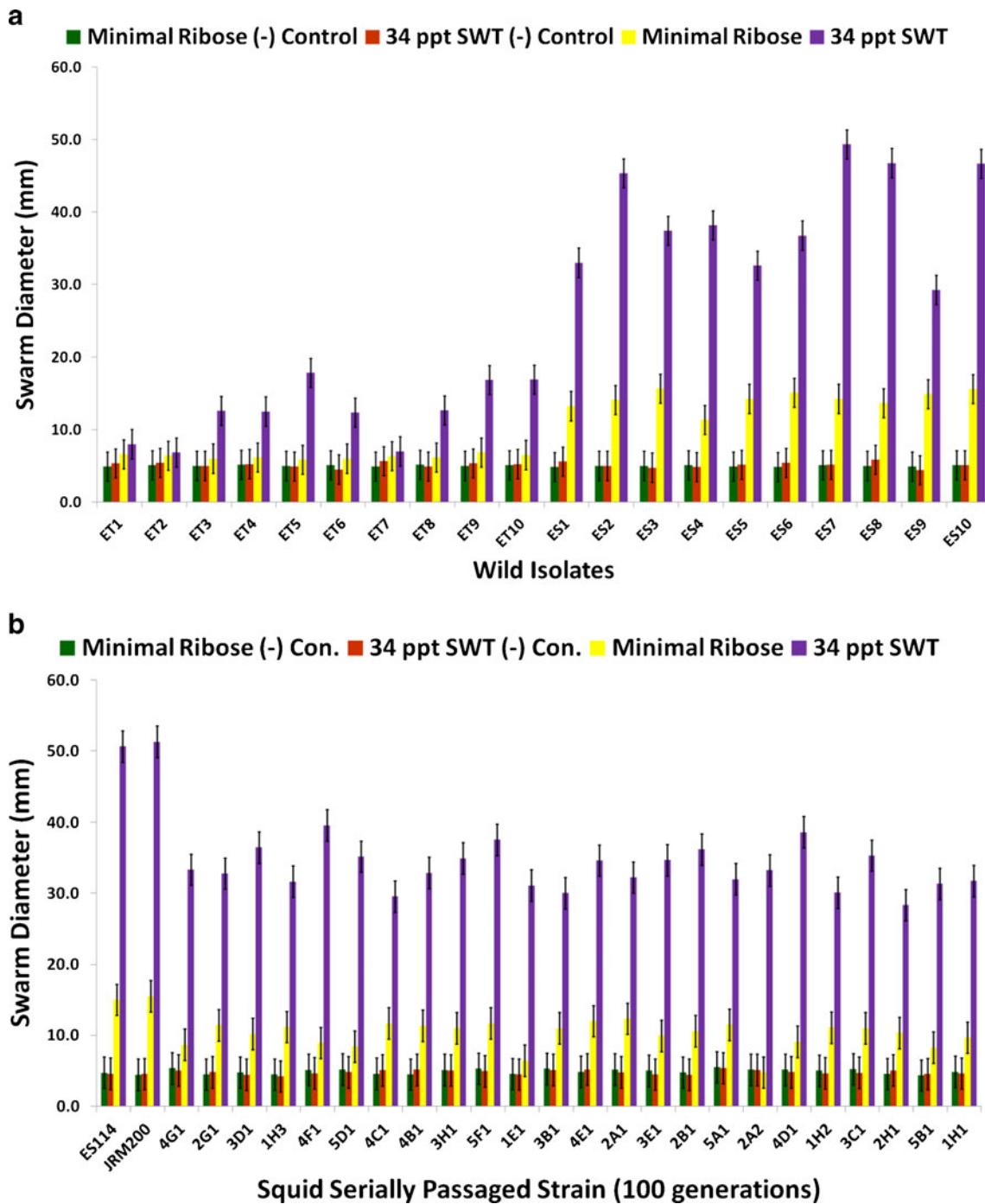


Fig. 1 **a** Motility assays for natural *V. fischeri* isolates extracted from *E. tasmanica* (“ET”) and *E. scolopes* (“ES”) squid hosts ($n=3$). Motility assays for *V. fischeri* JRM200 lines serially passed for **b** 100, **c** 200, and **d** 500 generations through *E. tasmanica* ($n=3$). Error bars represent

Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

dramatically in minimal ribose and SWT. Heightened biofilm formation in artificial seawater arose by 400 generations (Fig. 2c), as some derived lines were significantly higher than the ancestor in this medium. Interestingly, *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 were initially incapable of biofilm growth in artificial seawater. Significant dissimilarity

from the ancestral state in biofilm construction did not manifest in the evolved lines in 34 ppt SWT and minimal ribose until 200 and 300 generations, respectively (Figs. S2A and S2B). Thus, like motility, the relative evolutionary change in biofilm formation is nutrient dependent and convergent to ET wild variation, as “domesticated” variation created by artificial

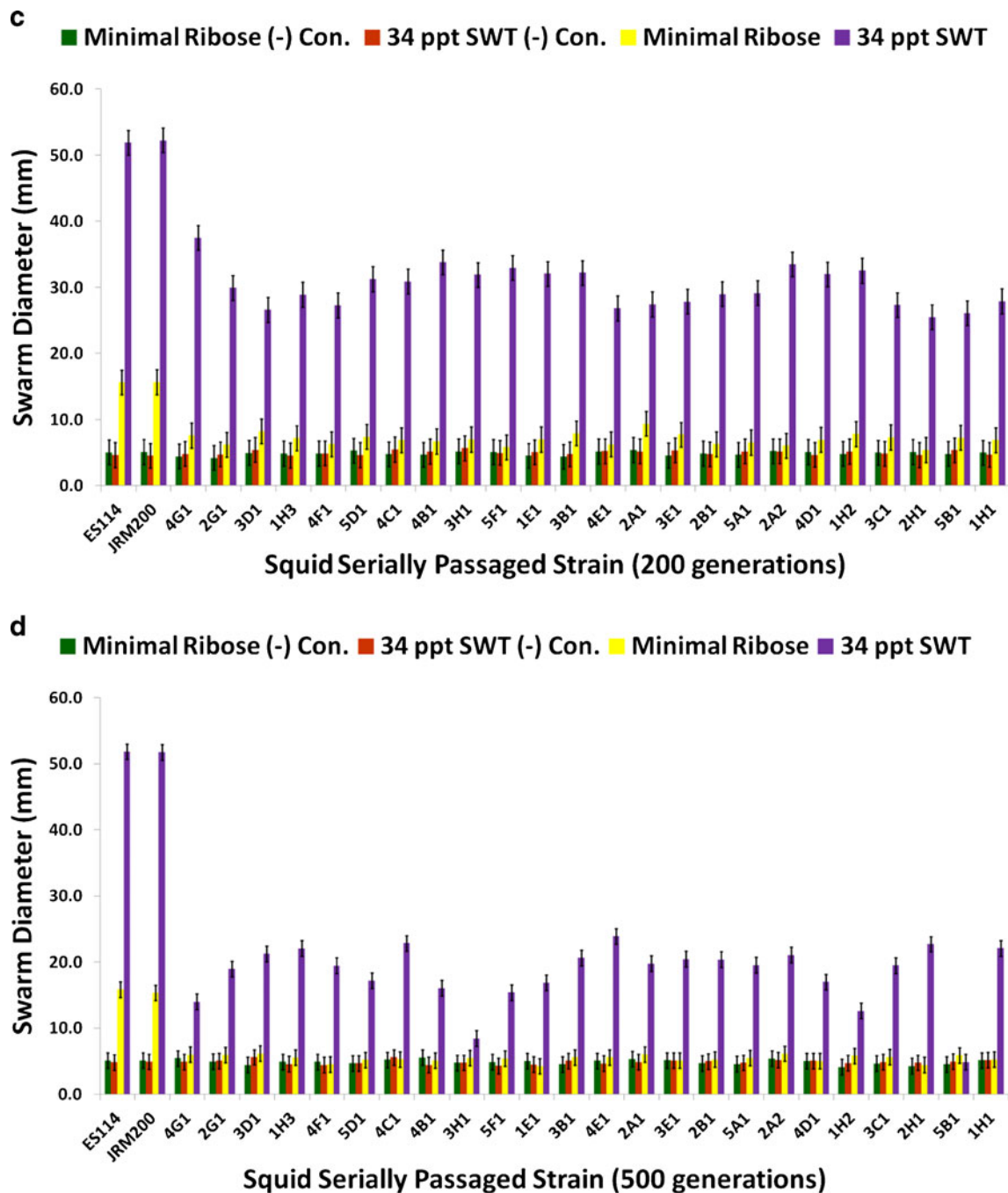


Fig. 1 (continued)

selection through novel host *E. tasmanica* showed a parallel biofilm response in all media types. The evolved lines have also diverged from one another in biofilm formation.

Carbon Source Utilization for 95 Substrates

The goal of measuring carbon source utilization in this study was to identify metabolic or physiological changes in the 24 *V. fischeri* lines as results of adapting to a novel squid host

environment in the light organ. Some of these changes may have been directly responsible for adaptation to the new host animal; others may have been the result of tradeoffs, epistasis, pleiotropy, or mutation accumulation [34]. No bacterial contamination was observed on the Biolog GN2 negative control plates. The Biolog GN2 microplate data for *V. fischeri* ES114 and *V. fischeri* unevolved JRM200 (supplementary Table S1) is congruent with what has been previously reported as accessible carbon sources for *V. fischeri*, supporting the validity of

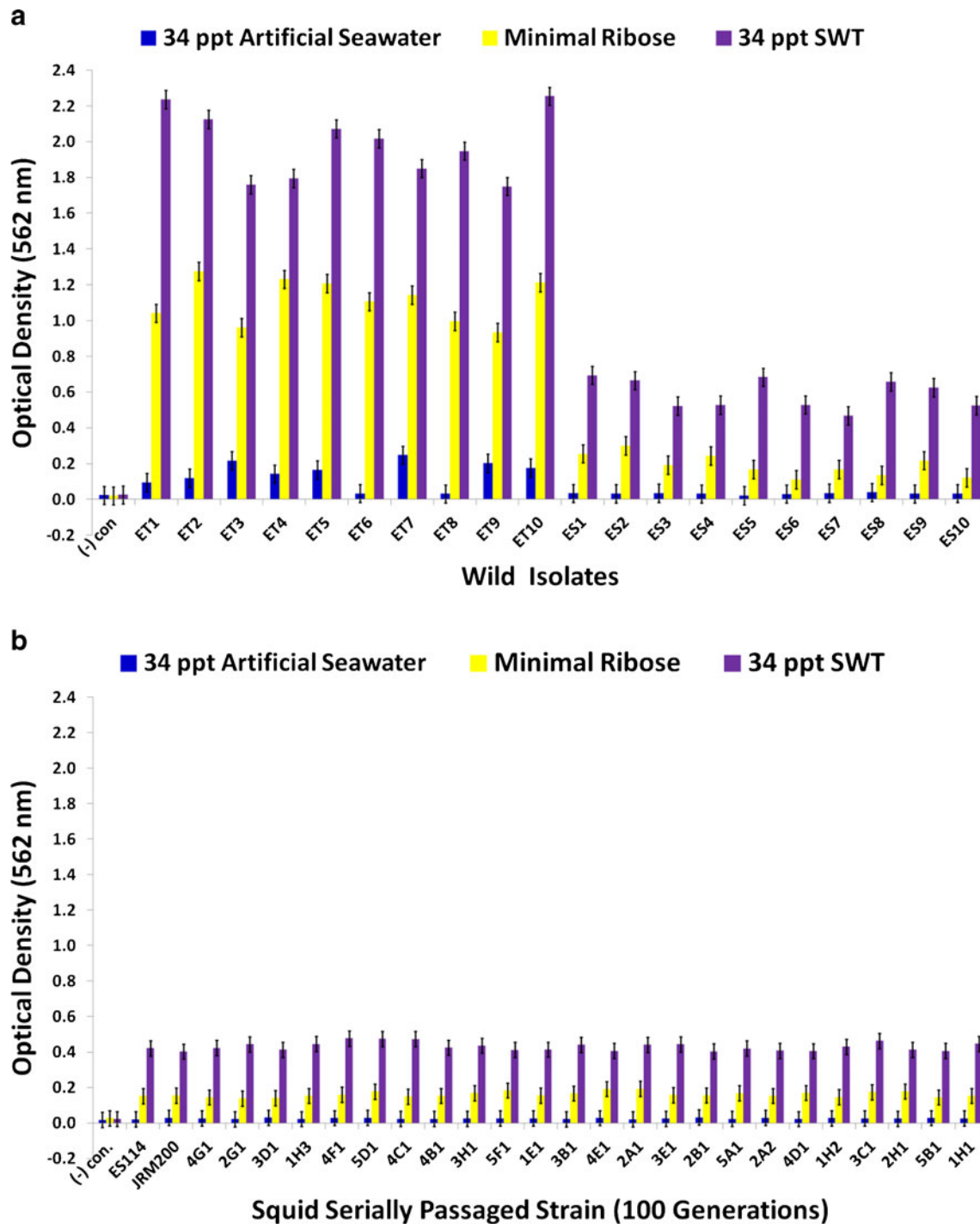


Fig. 2 **a** Biofilm assays for wild *V. fischeri* strains from *E. tasmanica* (“ET”) and *E. scolopes* (“ES”) specimens collected from the field ($n=12$). Biofilm assays for *V. fischeri* JRM200 lines serially passed for **b** 100, **c** 400, and **d** 500 generations through *E. tasmanica* ($n=12$). Error bars

represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

the results [35–40]. ET and ES wild isolates were heterogeneous for carbon substrate utilization (Figs. 3a–4b). Relative to ancestral metabolism, *V. fischeri* lines serially passed through *E. tasmanica* diversified in their utilization in every substrate in the Biolog microplate initially physiologically procurable (Figs. 5a–6b). As is wild ET variation

(Fig. 3a–c), “domesticated” variation (Fig. 5a–c) in carbon utilization from experimentally evolved *V. fischeri* is quite polymorphic. The precise manner of change between the derived lines and the ancestral state was carbon source dependent. For example, the various lineages remained the same or decreased on *N*-acetyl-D-glucosamine, while the evolutionary

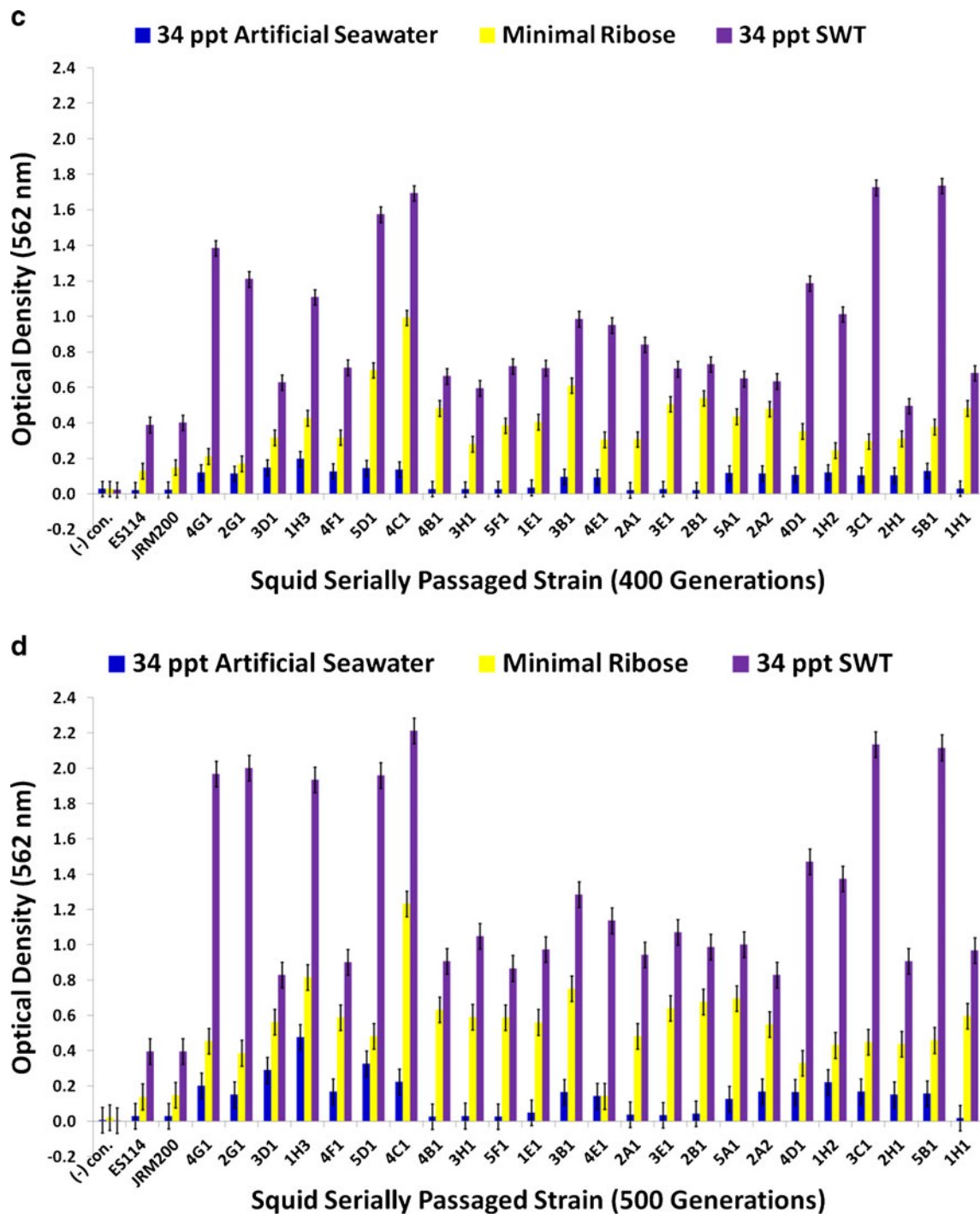


Fig. 2 (continued)

response with α -cyclodextrin was to increase or stay the same (Fig. 5a). On D-psicose (a rare sugar in nature [41]), the evolved lines either gained, lost, or were unaltered (Fig. 5b). However, no compelling convergent evolution was evident relative to wild isolates in most carbon substrates (representative examples shown in Figs. 3a–c and 5a–c).

Genetic evidence exists that these carbon sources play an important role for *V. fischeri* metabolism in its association with

sepiolid squid hosts. [3, 42] L-asparaginase, chitinases (liberate *N*-acetyl-D-glucosamine monomers), and dextrinases are produced in the symbiont while in symbiosis with the host squid. Protein importers and permeases for L-serine, D-galactose, and D-fructose have also been found in symbiotic *V. fischeri* [42]. L-Threonine is supplied by *Euprymna* squid to *V. fischeri* within the light organ in nutrient limiting amounts relative to other amino acids [3]. Moreover, a

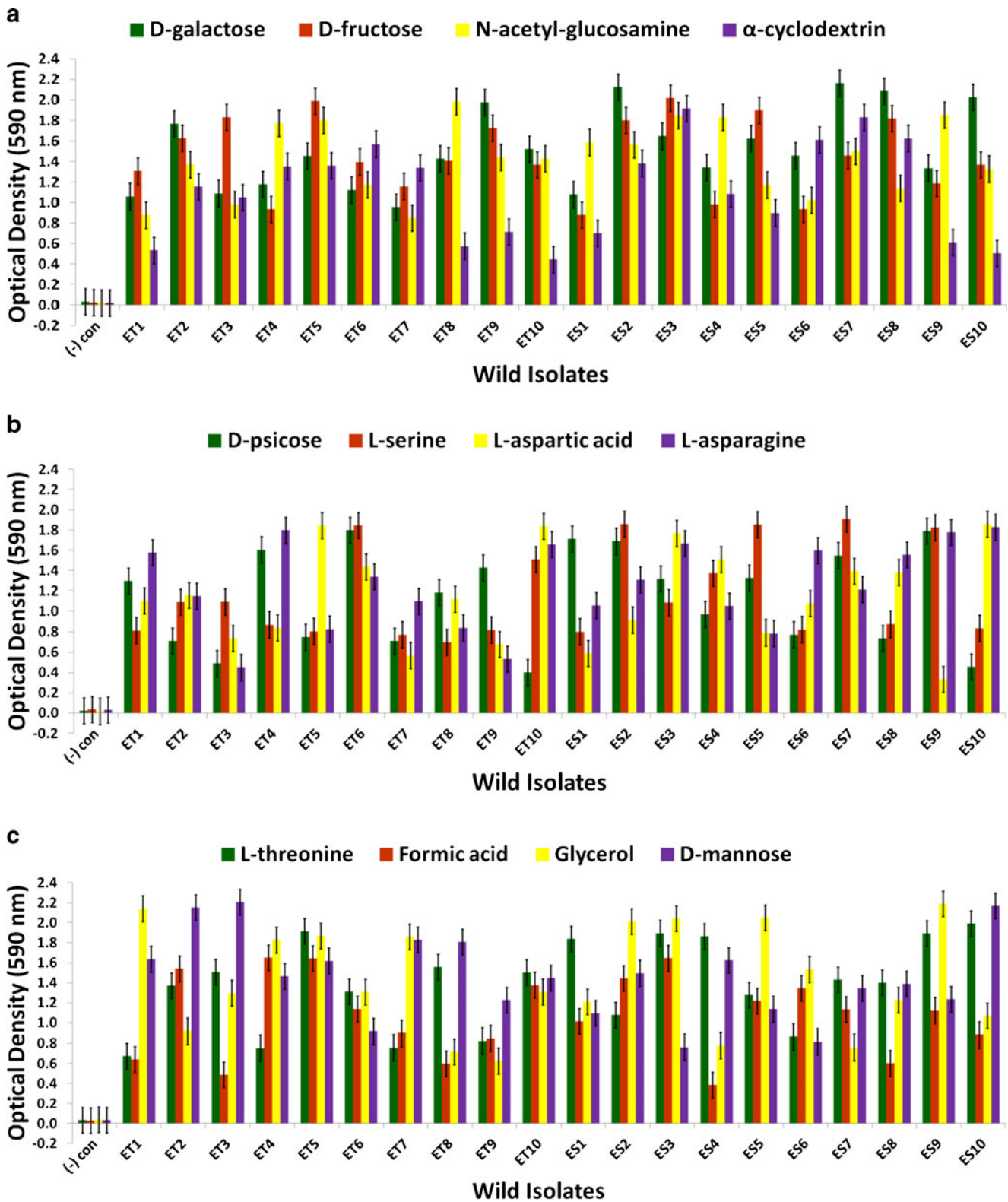


Fig. 3 Optical density (590 nm) measurements in **a** D-galactose, D-fructose, N-acetyl-D-glucosamine, and α -cyclodextrin; **b** D-psicose, L-serine, L-aspartic acid, and L-asparagine; and **c** L-threonine, formic acid, glycerol, and D-mannose of wild *V. fischeri* isolates from *E. tasmanica* (“ET”) and *E. scolopes* (“ES”) specimens collected from the field ($n=3$).

Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

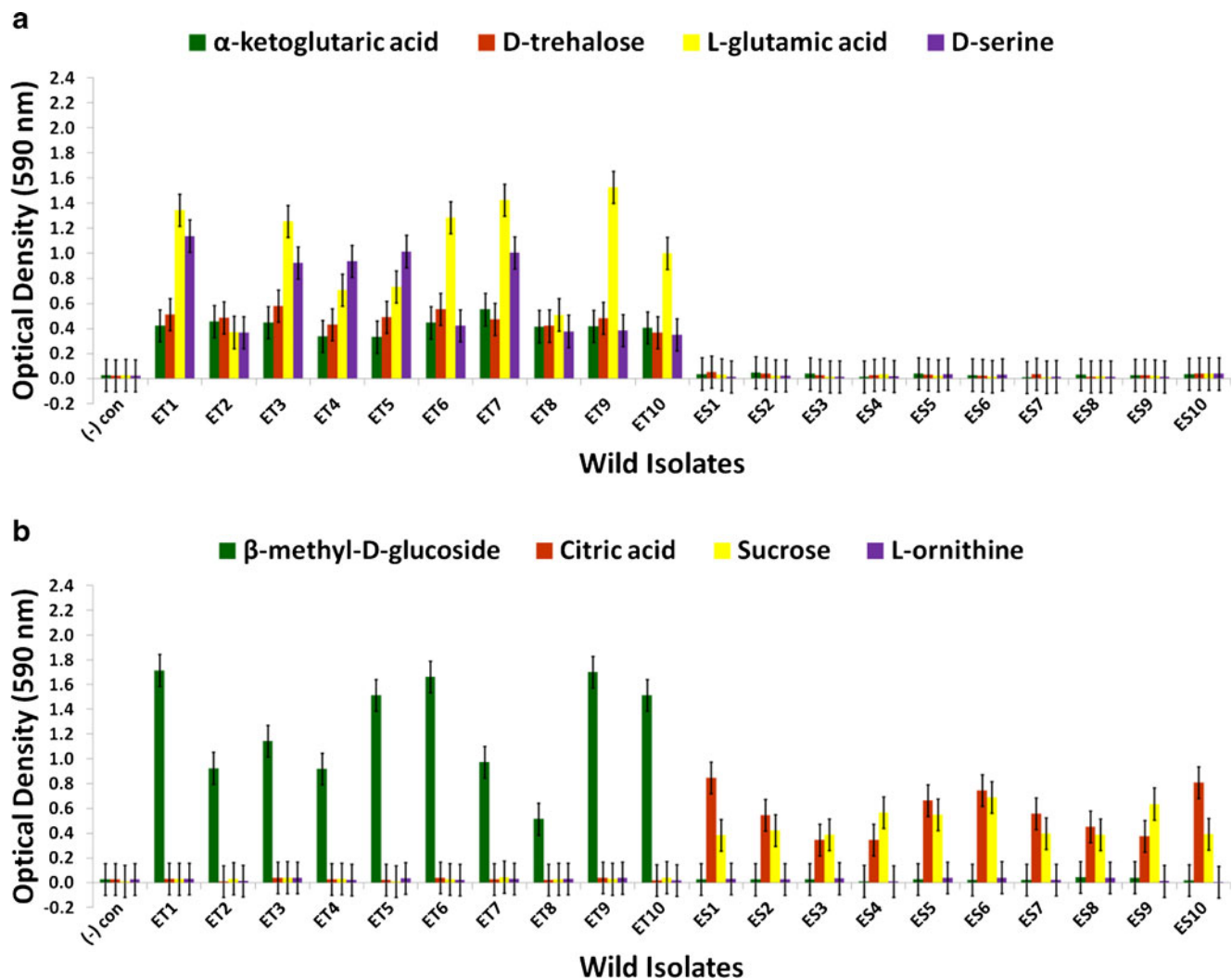


Fig. 4 Optical density (590 nm) measurements in **a** α -ketoglutaric acid, D-trehalose, L-glutamic acid, and D-serine, and **b** β -methyl-D-glucoside, citric acid, sucrose, and L-ornithine of wild *V. fischeri* isolates from Australian *E. tasmanica* (“ET”) and Hawaiian *E. scolopes* (“ES”) specimens collected from the field ($n=3$). Error bars represent Fisher LSDs

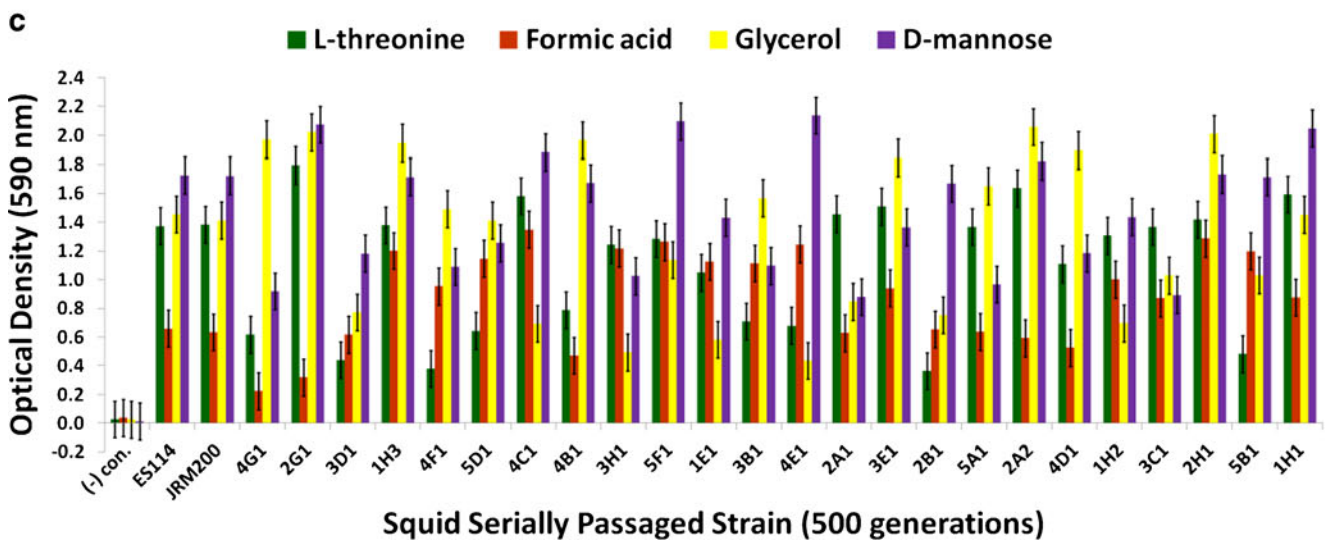
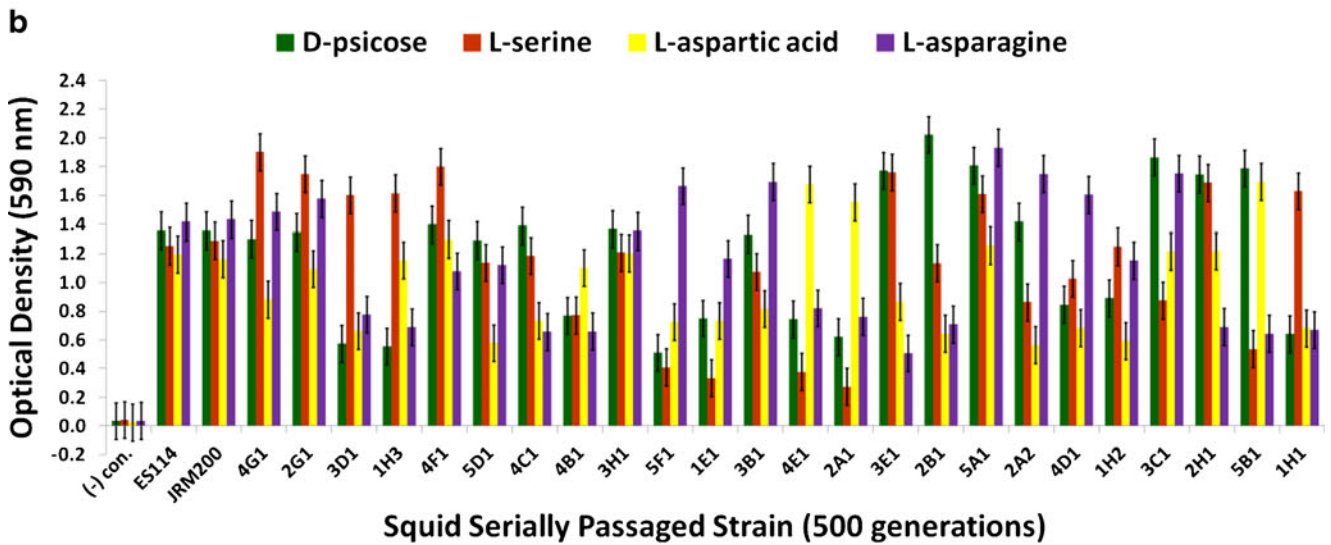
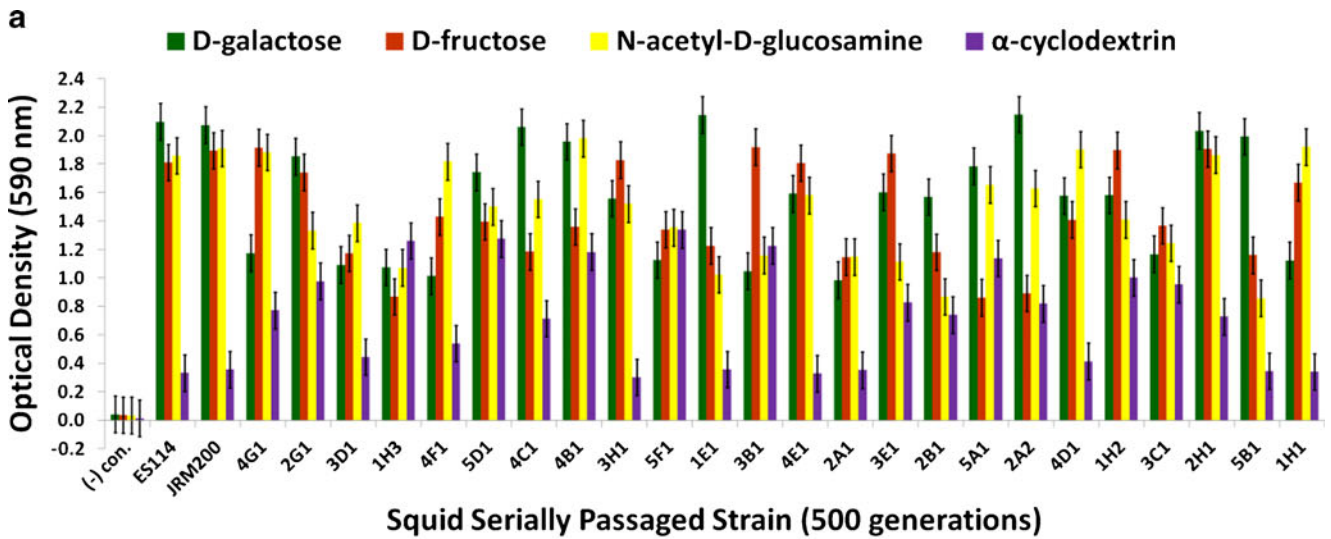
with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

multitude of genes are dedicated to the *V. fischeri* catabolism of glycerol, formic acid, chitin, and fumaric acid while in the light organ. Fumaric acid can be made by the deamination of L-aspartic acid, a reaction carried out by aspartate ammonia-lyase, an enzyme expressed by symbiotic *V. fischeri* [42]. D-Mannose residues coat the epithelial linings of light organs within sepiolid squid hatchlings and play a fundamental role in *V. fischeri* attachment to squid host eukaryotic cells through mannose-recognizing adhesins on the bacterial surface [22, 43], which potentially serve as a source for “mannose” grazing. Prolific variation in catabolism of D-mannose among the derived lines relative to precursor state was evident (Fig. 5c).

For *V. fischeri*, some carbon sources in the Biolog GN2 microplate exhibit the most substantial metabolic diversity (hyperpolymorphic) known for any particular substrate. They

include *N*-acetyl-D-galactosamine, L-fucose, α -ketoglutaric acid, D-trehalose, L-glutamic acid, D-serine, β -methyl-D-glucoside, citric acid, sucrose, and L-ornithine [37–40, 44, 45]. The Biolog data for the last eight of these hyperpolymorphic carbon sources are shown for the wild isolates (Fig. 4a, b).

Fig. 5 Optical density (590 nm) measurements in **a** D-galactose, D-fructose, *N*-acetyl-D-glucosamine, and α -cyclodextrin; **b** D-psicose, L-serine, L-aspartic acid, and L-asparagine; and **c** L-threonine, formic acid, glycerol, and D-mannose of ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations ($n=3$). Error bars represent Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]



All ET and no ES strains utilize α -ketoglutaric acid, D-trehalose, L-glutamic acid, and D-serine, while no ET and all ES isolates metabolize citric acid and sucrose. The ability to utilize seven new carbon sources was gained in some *V. fischeri* lines serially passed through *E. tasmanica* for 500 generations—*N*-acetyl-D-galactosamine, L-fucose, α -ketoglutaric acid, D-trehalose, L-glutamic acid, D-serine, and β -methyl-D-glucoside, relative to forerunners *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 (last five shown in Fig. 6a, b). Conversely, substrates such as citric acid, sucrose, and L-ornithine were lost in some lines (Fig. 6b). *N*-acetyl-D-galactosamine has been found to participate in cell–cell attachment through lectins, carbohydrate-binding proteins on

extracellular surfaces, including bioluminescent bacteria [46]. Lectins may be involved in biofilm formation and bacterial microcolony aggregations. Previous research has hypothesized that lectins with *N*-acetyl-D-galactosamine specificity govern symbiosis initiation in bioluminescent bacteria with marine animals, *V. fischeri* included [47]. Furthermore, D-serine, citric acid, α -ketoglutaric acid, β -methyl-D-glucoside, and *N*-acetyl-D-galactosamine have all been listed as carbon substrates dispensable for *Vibrio cholerae*, which are evolutionarily discarded and reacquired as necessary for certain niche environments [48]. Metabolic pathways can evolve modularly in function, some being especially malleable, to accommodate the niche breadth ecologically necessary for

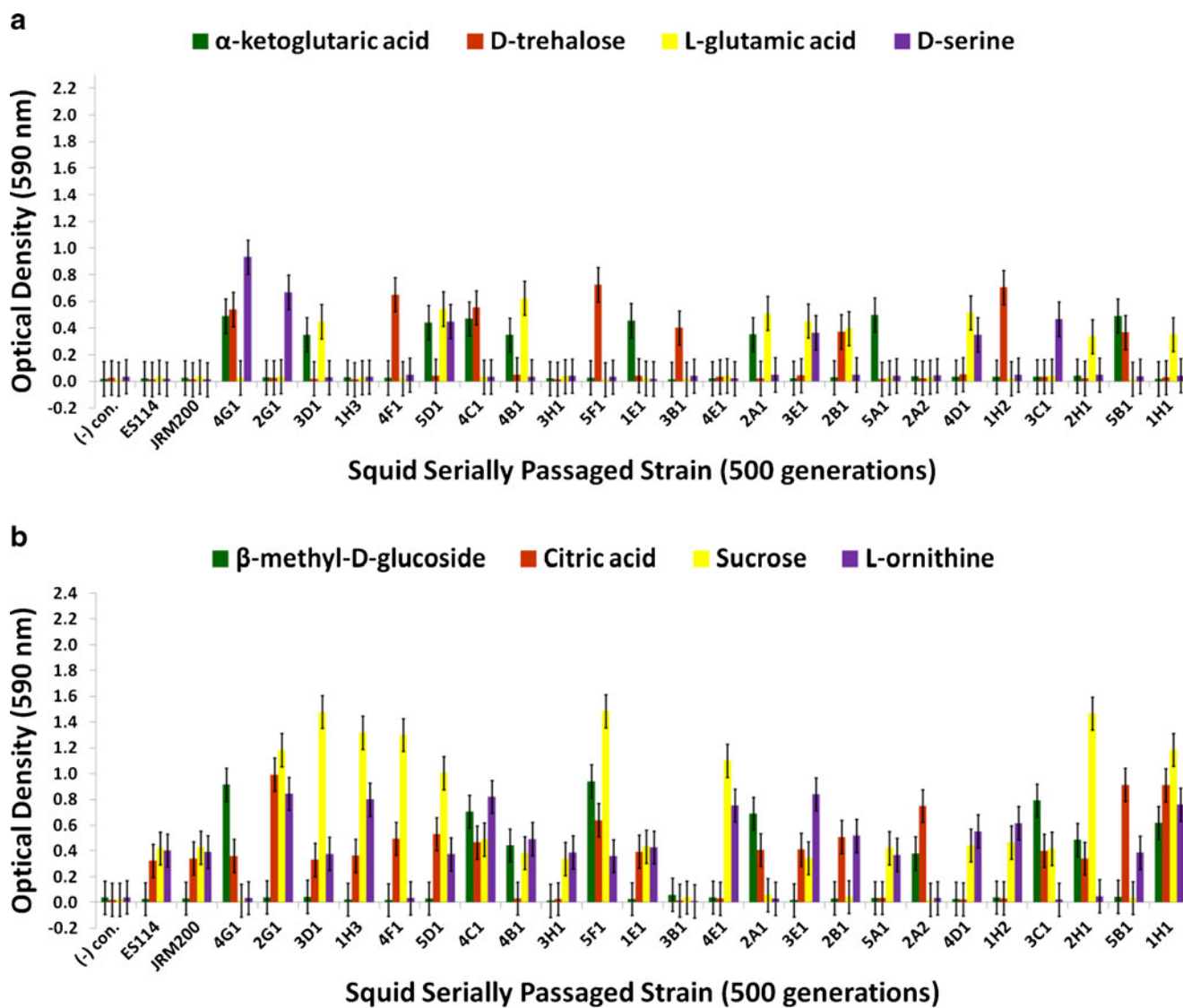


Fig. 6 Optical density (590 nm) measurements in **a** α -ketoglutaric acid, D-trehalose, L-glutamic acid, and D-serine, and **b** β -methyl-D-glucoside, citric acid, sucrose, and L-ornithine of ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations ($n=3$). Error bars represent

Fisher LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]

Vibrio populations under specific environmental parameters (e.g., free-living versus host-associated lifestyles). Curiously, no wild isolates from *E. scolopes* and *E. tasmanica* grew on *N*-acetyl-D-galactosamine, L-fucose, and L-ornithine. L-Fucose residues may also serve as attachment sites for bacteria to other cells, such as fucose-sensitive hemagglutinin A (*fshA*) in *V. cholerae* [49]. In summary, ET convergence was observed with α -ketoglutaric acid, D-trehalose, L-glutamic acid, D-serine, β -methyl-D-glucoside, citric acid, and sucrose (Figs. 4a, b and 6a, b). As with motility and biofilm, evolutionary distinction is emerging among the lines in carbon source metabolism.

Bioluminescence

Bioluminescence in *V. fischeri* is necessary for sepiolid squid colonization, as *lux* null mutants display defects in colonization ability [6, 50, 51]. Negative control *E. coli* K12 MG1655 was never luminous. Mean and maximum mean bioluminescence (RLUs [$\text{Log}_{10}(\text{CFUs/mL})$] $^{-1}\text{mL}^{-1}$) (Fig. 7a, b) and notable characteristics of the “growth-light” curves for the wild isolates and experimentally evolved lines were determined (Tables 1 and 2). All ET strains are brighter than all ES ones (Fig. 7a). ET wild strains are 10.4-fold and 15.6-fold brighter (higher power) than ES strains in mean and maximum mean bioluminescence, respectively. Furthermore, ET *V. fischeri* produce light before ES strains (ET: 195 min versus ES: 225 min) at lower cell densities (ET: 4.80×10^7 CFUs/mL versus ES: 1.00×10^8 CFUs/mL), ET symbionts achieving total photon emission maxima higher (ET: 4,294.2 RLUs versus ES: 264.5 RLUs) and later (ET: 327 min versus ES: 294 min) at higher cell densities (ET: 1.06×10^9 CFUs/mL versus ES: 4.67×10^8 CFUs/mL) than ES isolates in the process (Table 1). A trend in ET growth curves is the appearance of a lag phase, while this growth phase is completely lacking in ES symbiont reproduction. ET symbionts appear to be evolving a quorum-sensing machinery to maximize light production throughout bacterial growth, including producing light earlier at lower populations, prolonging length of time cells are luminous, surging light intensity or wattage, and increasing cell density at which maximum light emission occurs to amplify total amount of light output at an instant.

On average, mean and maximum mean bioluminescence increased 2.5-fold in the derived lines relative to the ancestor. All derived lines increased in mean and maximum mean bioluminescence as a result of adapting to novel host *E. tasmanica* (Fig. 7b); however, modifications of other traits suggest light intensity (i.e., power or wattage, photons emitted per cell per second) was not the only important factor influencing the evolution of bioluminescence. Population growth and quorum sensing apparatus may also have been affected. Some lineages challenged with a novel ET host developed a lag phase, a feature reminiscent of ET symbiont

convergence (Tables 1 and 2). Perhaps the ET lag phase is a cost or tradeoff associated with elevated photon production. Moreover, the time point that the evolved lines first ignited occurred earlier and at lower cell densities relative to ancestral *V. fischeri* ES114 and unevolved *V. fischeri* JRM200, while maximal light output occurred later at higher cell densities. Evidently, polymorphisms exist for traits affecting population growth, bioluminescence, and quorum sensing (Fig. 7b; Table 2), as the various lineages are different from each other as well as from the ancestral state, perhaps due to differences in autoinducer sensitivity. As with motility, biofilm formation, and carbon sources demonstrating hyperpolymorphic utilization, bioluminescence displays convergent evolution to ET wild isolates yet simultaneously exhibits ecological diversification among the various lineages.

Discussion

Convergent Evolution

Convergent evolution implies that axenic squid light organs subject colonizing *V. fischeri* to similar selection pressures, and multifarious genetic changes exist to produce the same phenotypic solutions necessary for the symbionts to successfully respond to the evolutionary challenges imposed by a host [52]. Motility plays an integral role in the colonization of sepiolid squid by *V. fischeri* [53] and allows host-associated bacteria to reach the destination and surface desired for further colonization or attachment. Motility results suggest that adaptation to novel host *E. tasmanica* by *V. fischeri* JRM200 mandated first a decrease in motility and then a subsequent boost in biofilm formation. The latter may not have been possible until the first occurred due genetic constraints or evolutionary contingency (i.e., biofilm potential does not increase until mutations decreasing motility happens first) [54], or decreased motility may have been the more immediately important and necessary evolutionary change to adjust to a new host, with a surge in biofilm capacity coming later as a reinforcing adaptation. Interestingly enough is the realization that *V. fischeri* did not show statistically meaningful host adaptation to *E. tasmanica* until 400 generations, yet motility decreased by a significantly different amount by 100 generations [7]. Like motility, biofilm formation is also indispensable in squid colonization for *V. fischeri* [55, 56]. Motility and biofilms from wild symbiont populations in *Euprymna* squid hosts suggest convergent evolution between experimentally evolved lines in *E. tasmanica* and natural ET *V. fischeri* (Figs. 1a–d and 2a–d). ET *V. fischeri* form more prolific biofilms and swarm less than ES *V. fischeri*, while ES *V. fischeri* produce more restricted biofilms and move faster (Figs. 1a and 2a). Perhaps, symbionts in *E. tasmanica* are selected for better host tissue attachment and elevated stress tolerance (more sticky and hardy), while those in *E. scolopes*

are evolutionarily honed for chemotaxis (more nimble locomotion and elevated nutrient sensitivity). Biofilm formation is positively correlated in many host-associated vibrios with colonization potential, immunity avoidance, and eukaryotic cell attachment in hosts [57–59]. Biofilms are known to increase bacterial survival against environmental stress [58–61].

The artificial variation generated from the experimentally evolved lines is congruent with ET natural variation, as *V. fischeri* “domesticated” in *E. tasmanica* appear more phenotypically similar to field ET *V. fischeri* in motility and biofilm production and less like their original ES *V. fischeri*. Figure 2c suggests a threshold event or critical period was

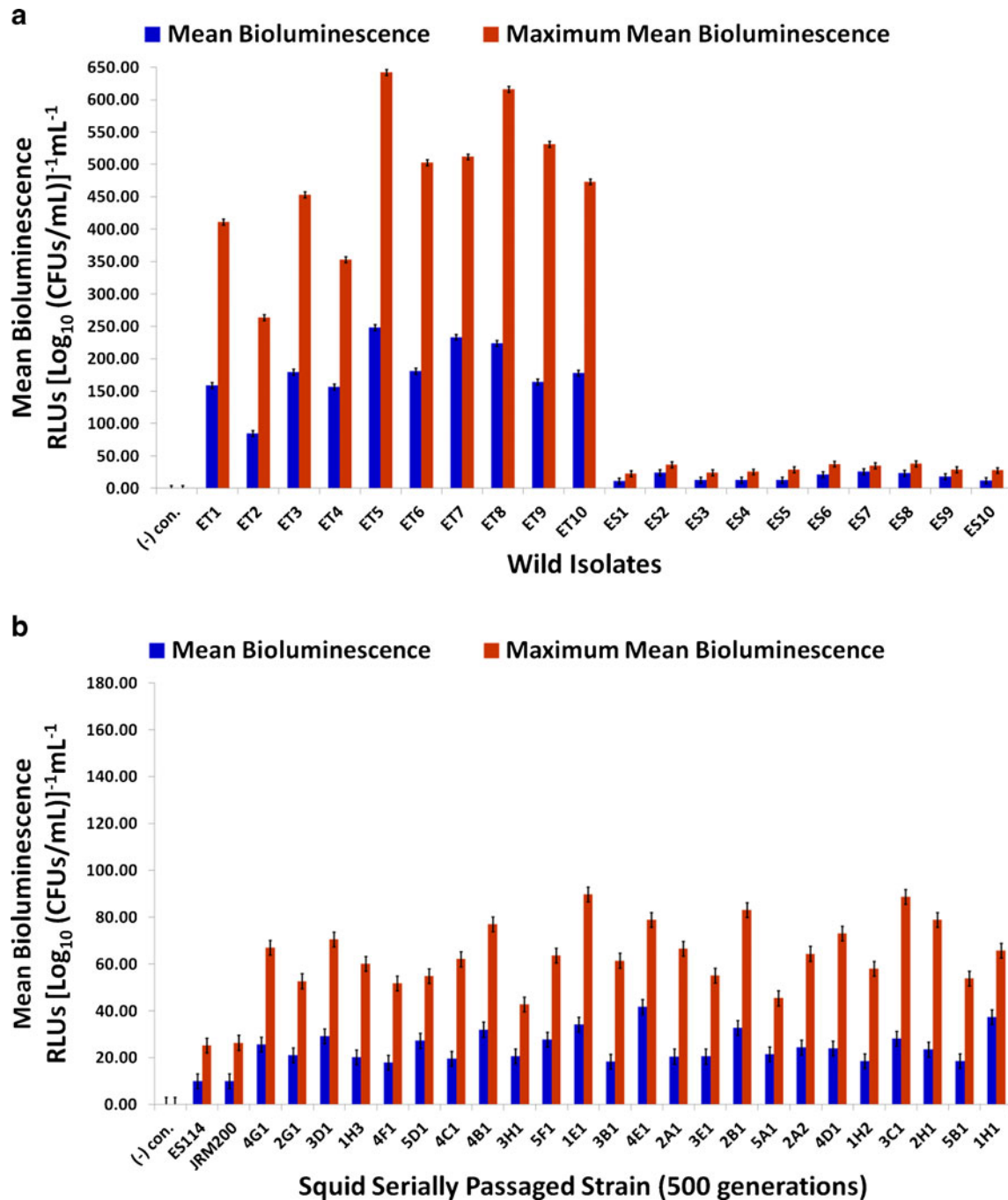


Fig. 7 Mean and maximum mean bioluminescence for a wild *V. fischeri* strains from *E. tasmanica* (“ET”) and *E. scolopes* (“ES”) specimens collected from the field and **b** ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations ($n=6$). Error bars represent Fisher

LSDs with Bonferroni correction for multiple pairwise comparisons (type I experimentwise error rate $\alpha=0.05$). If error bars overlap between any particular pair of comparisons, they are not significantly different from each other [28]. *E. coli* K12 MG1655 was used as the negative control

Table 1 Notable characteristics of the “growth-light” curves from wild *V. fischeri* strains from *E. tasmanica* (“ET”) and *E. scolopes* (“ES”) specimens collected from the field ($n=6$)

Strain	Time light emission began (min)	Log10 [CFUs/mL] where light was first emitted (\pm SE)	Log10 [CFUs/mL] where light was first emitted (\pm SE)	Maximal light output (RLUs) (\pm SE)	Log10 [CFUs/mL] where maximal light output occurred (\pm SE)	Lag phase (length)
ET1	210	7.797 (\pm 0.033)	300	3,597.9 (\pm 29.3)	8.754 (\pm 0.046)	No
ET2	240	8.111 (\pm 0.116)	360	2,464.5 (\pm 20.9)	9.347 (\pm 0.127)	No
ET3	180	7.510 (\pm 0.057)	270	3,835.1 (\pm 23.5)	8.464 (\pm 0.140)	No
ET4	150	7.231 (\pm 0.056)	300	3,072.5 (\pm 33.4)	8.696 (\pm 0.053)	No
ET5	180	7.492 (\pm 0.134)	300	5,609.8 (\pm 38.1)	8.737 (\pm 0.077)	Yes (1.0 h)
ET6	210	7.786 (\pm 0.082)	330	4,570.8 (\pm 25.7)	9.088 (\pm 0.060)	No
ET7	210	7.835 (\pm 0.051)	360	4,809.9 (\pm 19.9)	9.398 (\pm 0.146)	No
ET8	210	7.815 (\pm 0.086)	330	5,606.6 (\pm 39.5)	9.096 (\pm 0.048)	Yes (1.0 h)
ET9	180	7.418 (\pm 0.087)	360	4,963.8 (\pm 29.7)	9.338 (\pm 0.122)	Yes (1.0 h)
ET10	210	7.818 (\pm 0.054)	360	4,411.4 (\pm 25.4)	9.317 (\pm 0.092)	No
Mean	195 (\pm 5.8)	7.681 (\pm 0.056)	327 (\pm 5.4)	4,294.2 (\pm 162.6)	9.024 (\pm 0.072)	–
ES1	210	7.793 (\pm 0.109)	270	191.5 (\pm 0.4)	8.375 (\pm 0.048)	No
ES2	240	8.152 (\pm 0.094)	300	316.1 (\pm 5.6)	8.716 (\pm 0.077)	No
ES3	240	8.168 (\pm 0.078)	360	226.8 (\pm 3.6)	9.275 (\pm 0.058)	No
ES4	270	8.407 (\pm 0.093)	330	229.9 (\pm 4.1)	9.045 (\pm 0.093)	No
ES5	240	8.207 (\pm 0.084)	300	250.4 (\pm 2.4)	8.752 (\pm 0.035)	No
ES6	180	7.542 (\pm 0.121)	240	306.9 (\pm 2.5)	8.170 (\pm 0.068)	No
ES7	240	8.111 (\pm 0.052)	300	305.9 (\pm 1.1)	8.696 (\pm 0.033)	No
ES8	240	8.216 (\pm 0.059)	330	345.6 (\pm 0.9)	9.033 (\pm 0.071)	No
ES9	210	7.780 (\pm 0.055)	270	242.8 (\pm 1.5)	8.369 (\pm 0.047)	No
ES10	180	7.628 (\pm 0.117)	240	228.6 (\pm 2.6)	8.254 (\pm 0.073)	No
Mean	225 (\pm 4.8)	8.000 (\pm 0.032)	294 (\pm 6.5)	264.5 (\pm 8.5)	8.669 (\pm 0.099)	–

Negative control *E. coli* K12 MG1655 is omitted from the tables, since it produced no light

SE standard error

reached by 400 generations, since a sharp and sudden expanse in biofilm growth precipitously arises across many of the lineages in different media in a manner suggestive of second messenger signaling, signal transduction cascades, and quorum-sensing autoinducers [62]. The decrease in motility is more progressive and continued throughout the 500 generation time period. Not only was convergent evolution observed in biofilm development and motility between the derived lines and wild isolates but an inverse relationship was also noted. The inverse relationship between biofilm development and motility is known to have a biochemical basis within the genus *Vibrio*, specifically the second messenger cyclic diguanylate (c-di-GMP) [56, 59] and is a topic for future research. Generally, high intracellular concentrations of second messenger c-di-GMP correlate with increased biofilm formation, while lower quantities are associated with elevated motility (e.g., *V. cholerae*) [63]. Inverse relationships between motility and sessility in microbial lifestyles along a “c-di-GMP” continuum within hosts is an intriguing topic to partially explain competitive dominance and resulting tradeoffs observed in *Vibrio* symbionts colonizing *Euprymna* [9, 10].

Possibly, c-di-GMP intracellular pool levels as a “host” point of selection may be dissipated by sympatry of several *Sepiola* host species [1], not permitting for “c-di-GMP” ecological specialization for swarmer or biofilm ecotypes due to more homogenization of host environments in a geographical area. A scattering of few mutations affecting biofilm gene expression and c-di-GMP regulation drives convergent evolution and adaptive radiation in *Pseudomonas fluorescens* in structured microcosms, while simultaneously provoking astounding diversity [64, 65].

As with motility and biofilm formation, some convergent evolution appears to be occurring with carbon metabolism for substrates characteristic of evolutionary fluid allocation and redeployment in the *V. fischeri* physiological repertoire, when weighted against wild symbiont light organ populations from field-caught *E. tasmanica* and *E. scolopes* specimens (Figs. 4a, b, and 6a, b) [37–40, 44, 45]. Analyzing wild *V. fischeri* isolates from *Euprymna* squid hosts provides compelling evidence that citric acid and sucrose metabolic capabilities are abandoned in favor of L-glutamic acid, β -methyl-D-glucoside, D-trehalose, α -ketoglutaric acid, and D-serine for an

Table 2 Notable characteristics of the “growth-light” curves from ancestor *V. fischeri* ES114, unevolved JRM200, and the 24 lines evolved through the novel Australian squid host *E. tasmanica* for 500 generations ($n=6$)

Strain	Time light emission began (min)	Log ₁₀ [CFUs/mL] where light was first emitted (\pm SE)	Log ₁₀ [CFUs/mL] where light was first emitted (\pm SE)	Maximal light output (RLUs) (\pm SE)	Log ₁₀ [CFUs/mL] where maximal light output occurred (\pm SE)	Lag phase (length)
ES114	210	8.152 (\pm 0.041)	270	224.8 (\pm 4.5)	8.886 (\pm 0.014)	No
JRM200	210	8.165 (\pm 0.014)	270	234.1 (\pm 3.3)	8.876 (\pm 0.004)	No
Mean	210	8.159 (\pm 0.004)	270	229.5 (\pm 2.4)	8.881 (\pm 0.003)	–
4G1	150	6.624 (\pm 0.006)	270	514.0 (\pm 20.8)	7.769 (\pm 0.045)	Yes (1.5 h)
2G1	180	7.551 (\pm 0.005)	360	481.5 (\pm 8.4)	9.280 (\pm 0.008)	No
3D1	150	6.869 (\pm 0.004)	390	644.5 (\pm 8.6)	9.246 (\pm 0.113)	Yes (1.0 h)
1H3	180	7.595 (\pm 0.016)	390	566.3 (\pm 8.3)	9.539 (\pm 0.110)	No
4 F1	180	7.496 (\pm 0.041)	330	461.9 (\pm 0.1)	9.068 (\pm 0.053)	No
5D1	210	7.817 (\pm 0.051)	450	568.0 (\pm 8.4)	9.770 (\pm 0.053)	No
4C1	150	6.924 (\pm 0.009)	330	536.3 (\pm 0.7)	8.746 (\pm 0.025)	Yes (1.0 h)
4B1	180	7.514 (\pm 0.046)	300	663.6 (\pm 3.1)	8.705 (\pm 0.044)	No
3H1	210	7.801 (\pm 0.030)	300	366.0 (\pm 9.0)	8.705 (\pm 0.004)	No
5F1	150	7.192 (\pm 0.004)	360	583.7 (\pm 37.2)	9.295 (\pm 0.084)	No
1E1	150	6.897 (\pm 0.033)	360	806.3 (\pm 13.1)	9.058 (\pm 0.079)	Yes (1.0 h)
3B1	180	7.702 (\pm 0.007)	300	540.8 (\pm 0.1)	8.916 (\pm 0.004)	No
4E1	150	7.272 (\pm 0.012)	390	749.7 (\pm 19.1)	9.592 (\pm 0.064)	No
2A1	180	7.491 (\pm 0.093)	390	631.9 (\pm 1.8)	9.597 (\pm 0.044)	No
3E1	180	7.459 (\pm 0.049)	330	490.8 (\pm 2.7)	9.038 (\pm 0.020)	No
2B1	150	6.896 (\pm 0.034)	360	741.6 (\pm 19.2)	9.008 (\pm 0.056)	Yes (1.0 h)
5A1	180	7.544 (\pm 0.077)	270	372.2 (\pm 11.1)	8.344 (\pm 0.070)	No
2A2	180	7.487 (\pm 0.068)	330	575.0 (\pm 12.0)	9.045 (\pm 0.045)	No
4D1	180	7.501 (\pm 0.051)	330	651.5 (\pm 16.5)	9.022 (\pm 0.062)	No
1H2	180	7.615 (\pm 0.007)	330	518.3 (\pm 0.7)	9.061 (\pm 0.015)	No
3C1	210	7.807 (\pm 0.041)	300	767.5 (\pm 11.0)	8.732 (\pm 0.044)	No
2H1	180	7.535 (\pm 0.014)	300	684.0 (\pm 4.7)	8.756 (\pm 0.001)	No
5B1	180	7.578 (\pm 0.012)	300	461.0 (\pm 0.6)	8.702 (\pm 0.001)	No
1H1	180	7.420 (\pm 0.005)	390	622.6 (\pm 10.4)	9.579 (\pm 0.007)	Yes (0.5 h)
Mean	175 (\pm 4.0)	7.430 (\pm 0.038)	340 (\pm 9.3)	590.4 (\pm 24.5)	9.033 (\pm 0.093)	–

Negative control *E. coli* K12 MG1655 is omitted from the tables, since it produced no light

SE standard error

ET lifestyle. This is a trend observed in the 24 lines originally retrieved from *E. scolopes* and experimentally adapted to novel host *E. tasmanica*. The functions of these carbon sources in bacteria, the genus *Vibrio*, and in sepiolid squid–*V. fischeri* mutualisms have not been fully elucidated. D-Amino acids are largely restricted to peptidoglycan and teichoic acid in the bacterial cell wall [66], but D-serine could serve as an electron donor with appropriate isomerase function (e.g., mutase, racemase, or epimerase activity), as has been identified in *V. cholerae* [67]. β -Methyl-D-glucoside may serve as an alternative nutrient for bacteria when rapidly metabolizable and more energy efficient catabolites are unavailable [68], as is true for all less preferred carbon sources—but still utilized by *V. fischeri* on the Biolog GN2 microplate. β -Methyl-D-

glucoside utilization has been implicated in chemotaxis, motility, phosphorylation by the phosphotransferase system, and *rpoB* mutations affecting RNA polymerase, regulatory proteins, regulatory RNA three-dimensional structures, and translation initiation/termination complexes [69]. β -Methyl-D-glucoside may also be employed in membrane-derived oligosaccharide synthesis and metabolism of cellulose [69, 70], a polymer known to be a component of *V. fischeri* biofilms [56]. D-Trehalose may also function in protection against osmotic stresses [71].

L-Glutamic acid and citric acid increase *V. cholerae* host colonization potential in humans through the phosphotransacetylase–acetate kinase pathway (i.e., acetate switch) [72, 73], which drives ATP generation, recycles acetyl-

CoA when respiration and central metabolism are backlogged, and post-translational regulation of proteins, illustrating the effect carbon sources can have especially on animal host colonization when the substrates themselves are main metabolites easily shuttled or shunted into alternate biochemical pathways (The acetate switch is present in *V. fischeri* [74], but acetic acid did exhibit ET convergence.) [72]. L-Glutamic acid has also been reported to aid bacterial growth during iron limitation [75], which is noteworthy considering the squid light organ is low in available iron [76]. Furthermore, α -ketoglutaric acid is known to function as a substrate for the production of bacterial siderophores to assist iron acquisition in *Vibrio* [77]. α -Ketoglutaric acid is also believed to possess value against oxidative stress [78], and the squid light organ is known to initiate a respiratory burst via innate host immunity that produces toxic oxygen species [79]. Perhaps, the convergent evolution depicted in biofilm formation and these carbon sources are related, either to exploit new resources or increase resistance against unaccustomed stressors present in a novel host. Enhancing colonization and persistence in an unfamiliar host are other possibilities. How alternate carbon sources may be used by *V. fischeri* to manage against various host stresses (e.g., tenacious immune defenses) is a stimulating prospect for future study. For instance, amino acids may be more useful than monosaccharides for some cells against osmotic stress, while the former might be more beneficial to regulate high pH stress if nitrogen is not limiting (otherwise amino sugars such *N*-acetyl-D-glucosamine could be used if available) [80]. Although not relevant to the squid light organ, carbon source is known to affect bacterial susceptibility to heavy metal toxicity, including *V. fischeri* [81]. Convergent evolution coupling metabolism and stress together has been described in mutualisms between invertebrate hosts and microbial symbionts [82, 83].

For bioluminescence, the derived lines displayed convergence to ET wild isolates (Fig. 7a, b; Tables 1 and 2), implying that light emission may be associated with biofilm formation, motility, and the convergent carbon sources. *luxU* links bioluminescence to biofilm formation in *V. fischeri* [84], and *rpoN* controls motility, biofilm, luminescence, and squid colonization ability [85]. Bioluminescence may be tied to biofilm formation, motility, and central metabolism through integrated circuitries involving quorum sensing and c-di-GMP second messenger signaling [86], which is consistent with the simultaneous increase in luminosity, biofilm formation, and lower motility. Relative to the ancestor, light emission occurred earlier and at a lower cell density for the derived lines, while maximal light output ensued later and at a higher cell density (Table 2). Early quorum sensing at low cell densities and late quorum sensing at high cell densities implicate the AinS and Lux signal transduction relays in *V. fischeri*, respectively [87]. AinS quorum sensing has also been associated with the acetate switch [74]. Thus, evidence exists adaptation to *E. tasmanica* and conceivably to novel hosts in general

involves selection at multiple layers of cell signaling (e.g., GGDEF, EAL, and PilZ proteins), which could account for appearance of a lag phase [14, 88]. Mutations affecting AinS quorum sensing is known to affect growth curves [89]. Previous efforts failed to find bioluminescence differences among the evolved lines within *E. tasmanica*, and several replicate animals were not examined within a single evolved line (e.g., using $n=3$ squid hosts with 3B1 instead of one animal each with 3B1, 5B1, and 4F1) [7]. At the time of study, there were insufficient *E. tasmanica* hatchlings available for these experiments. However, derived lines that are significantly brighter at 500 generations relative to ancestral *V. fischeri* ES114 and unevolved *V. fischeri* JRM200 have recently been observed in *E. tasmanica* (unpublished data). Prior investigation showed the derived lines were more dim in the ancestral host *E. scolopes*, giving off less photons per \log_{10} (CFUs/mL) mL^{-1} [7]. *E. scolopes* hatchlings and adults are smaller (with concomitantly smaller light organs as well) than *E. tasmanica* throughout their entire life cycles. Therefore, *E. scolopes* individual hosts carry one to two orders of magnitude less symbionts than *E. tasmanica*, regardless of the ontogenetic stage. Hence, tradeoffs may now exist in the ancestral host *E. scolopes* where *V. fischeri* cell densities and quorum sensing are mistimed for motility, biofilm formation, and bioluminescence due to adaptation of the derived lines to Australian *E. tasmanica*, since symbionts can no longer reach the proper population levels in the Hawaiian squid at the appropriate times of host development, as the symbiont and host life histories are no longer accurately synchronized.

Diversification

Diversifying evolution or radiation predominates over convergent evolution in morphospace when profuse phenotypes prevail to be evolutionarily fit or successful to a particular selection pressure. A multitude of mutations to attain each of these prosperous possibilities in a complex, heterogeneous environment with vacant niches provide ecological opportunity, an attribute characteristic of island evolution, including Galapagos finches, Hawaiian silversword alliance, Hawaiian honeycreepers, *Anolis* lizards, and cichlids in their lake habitat islands [90–92]. Aside from the carbon sources displaying convergent evolution, the remaining carbon sources metabolizable by *V. fischeri* wild isolates depicted a general polymorphism, a tendency also reflected in the derived lines (representative examples in Figs. 3a–c and 5a–c). These polymorphic carbon sources are being used distinctly and variedly, both individually and as an assortment. These results are congruent with other microbial experimental evolution studies [52, 93], including with *E. coli* experiments with less than 800 generations of evolution in homogenous and unstructured environments [94]. This increased “domesticated” variation may be reflective of diversifying selection in the squid light

organ with incipient resource partitioning and changes in nutrient specificities toward carbon resources, perhaps even as part of cross-feeding or syntrophy between other *V. fischeri* ecotypes within the light organ of the same squid host individual [91, 94]. The implication of these results in nature are profound. *Euprymna* squid can live up to a year, which amounts to 1,500–2,000 *V. fischeri* generations [7]. Squid light organs are microcosms full of convoluted chasms with tremendous physical and biochemical complexity and heterogeneity [95], and a single *V. fischeri* clone has immense opportunity to evolve cross-feeding with either other *V. fischeri* subtypes or host cells within a host lifetime. The squid light organ microenvironment varies in spatial and temporal carbon source composition available to *V. fischeri* cells (e.g., *N*-acetyl-D-glucosamine at night and glycerol in the morning) [42]. Moreover, within the light organ of a single individual squid host, different *V. fischeri* subtypes will specialize in where they will settle and reside, with little mixing of individual *V. fischeri* variants within the light organ symbiont population despite the disturbance imposed by daily venting [42, 96]. In turn, this dissimilarity in the spatial localization and structured regional occupancy of the light organ by *V. fischeri* cells leads to differential gene expression in the symbionts, which can be a primer for the evolution of ecological differentiation.

An imperative realization is biofilm formation, motility, convergent carbon sources, and bioluminescence are also exemplifying ecological diversification. Despite the manifestation in convergence to wild ET isolates in these traits, the evolved lines are still becoming divergent from one another. Other laboratories have also proved convergent evolution facilitates ecological diversification in separate lineages, including on other carbon sources [52, 93]. Quorum sensing and biofilms beget diversity, including the modification of population growth (e.g., lag phase changes) [97, 98]. Research with *V. cholerae* verifies alternate carbon sources can be incorporated or substituted into the composition of a biofilm to access new ecological niches [99, 100]. Intriguingly, bioluminescence (i.e., quorum sensing) and *trans*-genetic regulation of the *lux* operon could function as a generator for *V. fischeri* adaptive radiation inside this symbiont's animal hosts, as cAMP receptor protein (a transcriptional regulator) may have a continuum of graded possible effects on *lux* operon gene expression due to the presence of different carbon sources in various light organ microenvironments within sepiolid squids and monocentrid fishes [62]. A captivating possibility is the utilization of the sepiolid squid-*Vibrio* symbiosis to investigate evolvability and versatility in *V. fischeri*. *V. fischeri* possesses three biofilm gene clusters, *syp*, *vpsII* like, and the cellulose operon, yielding over 30 genes that can be independently modified to stimulate biofilm niche differentiation to exploit squid light organ microenvironments in novel ways [101]. This evolutionary process is quite analogous to the customization of the myriad bony elements in the head region of

cichlids [102]. Additionally, modularity (as seen in convergent carbon sources, Figs. 4a, b, and 6a, b) increases the evolutionary diversification potential of a lineage, including for adaptive radiation [103, 104].

Squids as Host Habitat Islands

The use of biogeography theory to characterize microbial diversity has been previously applied, including mutualisms, pathogen–host interactions, and microcosms [105–110]. Axenic sepiolid squid hatchlings, first emanating from their eggs, essentially serve as sterile and mobile volcanic islands (i.e., host habitat islands) that subject their bioluminescent symbionts to severe genetic bottlenecks during colonization [7, 106, 108, 111, 112]. Only 6 to 12 *V. fischeri* cells of a 10^0 – 10^3 CFUs/mL seawater inoculum initiate symbiosis in an animal that ultimately reach an adult light organ carrying capacity of 10^8 – 10^{11} cells per host [113], providing pioneer *V. fischeri* colonizers with ecological release from the semi-starvation frequently encountered during the oceanic free-living phase. Such episodes of genetic bottlenecks with ensuing abundant proliferation and population recovery are called “founder flushes” [114], which provide opportunities for population movements across valleys via genetic drift to alternative adaptive peaks [90] and facilitate genetic revolutions, unique adaptations, founder effect evolution not normally possible, and novel independent evolutionary trajectories into vacant niches [e.g., island syndromes such as gigantism in the genera *Geochelone* and *Aepyornis*, nanism in *Mammuthus exilis*, and evolution of woody lifestyles by herbaceous plants] [90, 101, 115–118]. Founder flushes have been documented for bacteria, including *V. cholerae* and *Helicobacter pylori*, and are of significance in epidemiology [119]. Within the squid host, founder flushes lead to expanding *V. fischeri* niche breadth and ecological diversification (e.g., alternate metabolic utilization) from initial tight symbiont bottlenecks containing low genetic diversity, as has been observed in heterogeneous and complex microcosm environments with *P. fluorescens* [91, 120]. Moreover, the sepiolid squid light organ is an immensely specialized and complex structure [95], where *V. fischeri* populations can undergo ecological differentiation over the lifetime of the cephalopod host [7, 90, 101]. These conclusions are consistent with population genetics conducted with ES, ET, and *Sepiolo* wild isolates of *V. fischeri* from sepiolid squid hosts through vast scales of space encompassing the world's oceans and time spanning 20,000 generations of symbiont evolution [7, 33, 121, 122]. The presence of phenotypic convergent evolution and ecological diversification of *V. fischeri* serially passaged through *E. tasmanica* in motility, biofilm formation, metabolism, and bioluminescence when compared to wild isolates from this animal indicate host adaptive landscapes that have both smooth and rugged, multi-peaked topographies [123, 124],

providing this bacterium with tremendous ecological opportunities to exploit a vast N-dimensional niche hypervolume space within animal hosts [125]. Innumerable evolutionary trajectories and adaptive peaks are available in the fitness landscape of a novel host. Simply put, there are many ways *V. fischeri* can successfully colonize a squid, permitting extensive specialization, resource partitioning, and genetically distinct ecotype subpopulations to crystallize within an animal host—in essence adaptive radiation [120, 126, 127].

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