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Stress tolerance alteration in the freshwater cnidarian green hydra (*Hydra viridissima*) via symbiotic algae mutagenesis

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Abstract
Symbiotic organisms such as corals are threatened by changing climate because they are sensitive to stress, and may be unable to adapt quickly due to the long host generation time. Instead of selecting for stress tolerant hosts, manipulating their symbiotic microbes has been proposed because microbes affect holobiont phenotypes and they have rapid life cycles. Because it is time-consuming to isolate stress tolerant symbionts from the wild even when appropriate types exist, mutation and selection of symbionts is a promising alternative approach. Using green hydra (*Hydra viridissima*) and symbiotic algae (*Chlorella variabilis* NC64A), we found symbiont mutagenesis in vitro altered their UV-B resistance as well as that of holobionts receiving mutated algae. In addition, hydra UV-B tolerance was positively correlated to that of the algae they were hosting, as the hydra associated with UV-B tolerant algal strains exhibited higher UV-B resistance. However, chronic low-level UV-B selection decreased algal resistance to acute high-level UV-B, which was unexpected, and did not affect UV-B resistance of holobionts. The variations in algal UV-B tolerance and hydra UV-B tolerance were largely due to mutagenesis rather than selection. Our results suggest symbiont mutagenesis and trait-based identification may be more effective than assisted evolution in holobiont phenotype alteration, and it highlights the need to characterize symbiont traits in vitro that are correlated to stress tolerance they can confer to hosts, which may have application in conservation, agriculture and forestry.

Keywords Endosymbiosis · Green hydra · Stress tolerance · Holobiont · Mutagenesis

1 Introduction

Discoveries of new functions provided by symbiotic microbes to their hosts have caused researchers to reconsider the significance of symbiosis in ecology and evolution, especially in the context of climate change (Zilber-Rosenberg and Rosenberg 2008; Bordenstein and Theis 2015). It has become clear that endosymbionts not only benefit host species by providing nutrients, protection, and energy (Selosse et al. 2004; Wernegreen 2012; Heyworth and Ferrari 2015; Wagner et al. 2015; Ishikawa et al. 2016; Hamada et al. 2018), but also alter host physiology and biochemistry, modifying their development and secondary chemical metabolism, and thus their stress tolerance (Montgomery and McFall-Ngai 1994; Mondo et al. 2017). Because both the symbiont and the host determine the holobiont phenotype, Zilber-Rosenberg and Rosenberg [2] developed the hologenome theory which proposes that the holobiont should be considered as the unit of selection, and genetic changes in either entity help to maintain endosymbiosis stability under stress.

Stress tolerance of holobionts, which is critical for endosymbiosis stability, is a concern in changing environments (e.g., climate change) because malfunction in either the host or the symbiont may lead to endosymbiosis breakdown (Toby Kiers et al. 2010). The positive, intimate relationship between the two parties may lead to greater vulnerability of symbiont-bearing species under climate stress compared to non-symbiotic species. Some recent studies, however, demonstrated that endosymbionts vary in their effects on holobiont tolerance which could include increasing or decreasing host stress tolerance (Nougué et al. 2015; Kumari et al. 2018). For instance, habitat stress tolerance in some plants is greatly influenced by associated symbionts (Rodriguez and Redman 2008; Rodriguez et al. 2008). Given that the symbiont may play a significant role in holobiont stress tolerance, the hologenome theory suggests symbiont-bearing species may be able to respond to envrionmental change quickly through

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changing the types or relative frequencies of their symbionts (Zilber-Rosenberg and Rosenberg 2008). This could allow rapid "adaptation" of slow-growing symbiont-bearing species via changes in symbionts, which otherwise may not be able to survive environmental changes solely through evolution of the host (Császár et al. 2010; Palumbi et al. 2014). This brought in a new perspective on modifying holobionts' stress tolerance, which we can manipulate the symbiont rather than the host in the lab to create stress-resilient holobionts such as corals (van Oppen et al. 2015; Damjanovic et al. 2017).

It has been proposed that symbiont genomes in holobionts can shift rapidly in three ways: rare strain amplification, novel strain acquisition, and interspecific horizontal gene transfer (Zilber-Rosenberg and Rosenberg 2008). There is evidence supporting each of these scenarios. Studies of corals have shown switching to high thermal tolerance algae (novel strain acquisition) could increase host thermal tolerance, and the proportion of tolerant strains increased (rare strain amplification) after corals experienced heat stress (Kinzie et al. 2001; Jones et al. 2008; Gilbert et al. 2010). Similarly, plants hosting different endophyte strains varied in their abiotic stress tolerance (Rodriguez et al. 2008) which supports a role for symbiont traits in holobiont stress tolerance. Pinto-Carbó et al. (2016) discovered substantial horizontal gene transfer between closely related symbiotic bateria in plants, which could facilitate their evolution. Although horizontal transmission in aphids is not as common as in corals or plants, aphids maintain symbiont polymorphisms that contribute to their success in various enviornments (Russell and Moran 2005; Dunbar et al. 2007). Together these studies support the hypothesis that holobionts are able to quickly improve their stress tolerance simply through changing symbionts, and raise the potential of employing novel microbes to mitigate negative effects of climate change on symbiont-bearing species (Buddemeier et al. 2004; Rodriguez et al. 2008). However, these novel associations could be unstable due to high specificity of associations or symbiont-bearing species may not be able to acquire suitable symbionts due to a low abundance or diversity of tolerant symbionts in the local environment (Coffroth et al. 2010; Peixoto et al. 2017).

One poorly investigated but promising alternative approach to increase holobiont stress tolerance relies on symbiont evolution, which can be actively intervened in the lab (van Oppen et al. 2015). Their shorter generation time, higher variability, and larger populations make the symbiont a better target for directed selection as they likely can evolve faster than the host (Thomas et al. 2010). For example, the doubling time of symbiodinians is typically between days to months, but up to years for their coral hosts, and so it has been suggested that symbiodinians provide corals opportunities to persist at future elevated temperatures as more variation could be accumulated in the symbiont (Berkelmans and van Oppen 2006; Allemand and Furla 2018; Kumari et al. 2018). Chakravarti et al. (Chakravarti et al. 2017) succeeded in selecting for more thermally tolerant symbionts, but failed to detect any improvements in coral thermal tolerance when the algae were innoculated into hosts. Because interests of hosts and symbionts may conflict under stress, symbiont strains that have higher growth rates under stressful conditions may not confer higher stress tolerance to hosts (Baker et al. 2018). For instance, choosing strains that have lower physiological responses to stress (such as production of reactive oxygen species) may be a better way to obtain symbionts that could improve holobiont tolerance, although the symbionts may slow the growth of holobionts under normal conditions (Jones and Berkelmans 2010), and not compete well with other strains (Karim et al. 2015). Since standing genetic variation in the symbiont might not be enough for their adaptaion to occuring stress in time (Damjanovic et al. 2017), we decided to combine selection with mutagenesis. Researchers have succeeded in selecting for non-symbiotic, free living algal strains with specific traits via random mutagenesis (Cordero et al. 2011; Bonente et al. 2011), and so mutagenesis may potentially be used to generate genetic variability in algal symbionts, from which strains could be selected to improve holobiont stress tolerance. Ideally, it would be possible to predict holobiont phenotypes from symbiont phenotypes in order to rapidly screen a large number of candidate symbiont strains.

To explore whether we could alter holobionts' stress tolerance by symbiont mutagenesis and selection, we used green hydra (*Hydra viridissima*) and endosymbiotic algae (*Chlorella variabilis* NC64A) as our model system (Hamada et al. 2018). We used UV-C (200-280 nm) to induce mutations in the algae. We used UV-B (300-315 nm) as the selection factor in our experiments because of its detrimental effects on aquatic invertebrates (Cywinska et al. 2000), which can be attenuated by endosymbiotic algae via shading or mycosporine-like amino acids (MAAs) (Garcia-Pichel 1994; Shick and Dunlap 2002; Summerer et al. 2009). In this experiment, we addressed the following questions: 1) Does mutagenesis and/or selection alter algae stress tolerance? 2) Does symbiont mutagenesis and/or selection change holobiont stress tolerance? 3) How are symbiont and holobiont stress tolerances related? By answering these questions, we will be able to verify the feasibility of rapid holobiont phenotype alteration through symbiont mutagenesis and selection, without the need of cultivating holobionts during the strain-identifying process (Mueller and Sachs 2015).

2 Methods

2.1 Study species and culture conditions

Endosymbiotic algae (*Chlorella variabilis* strain NC64A) used in the experiment were provided by Dr. David Dunigan

(University of Nebraska-Lincoln, USA). This algal strain was isolated from *Paramecium bursaria* and cultivated in Modified Bold's Basal Medium (MBBM) (Kodama and Fujishima 2015). We cultured algae in a constant 20 °C walk-in culture room with a 25-watt, 55.88 cm-long fluorescent light placed 40 cm above the cultures for illumination, providing a 12:12 h light-dark cycle.

Aposymbiotic (hydra without algae) green hydra (*Hydra viridissima* strain 1695C) were provided by Dr. Daniel Martinez (Pomona College, CA, USA). This hydra strain was collected in Chile in its symbiotic state, then bleached and maintained in the lab for generations. We kept hydra in petri dishes in a constant 18 °C room. We cultured them in hydra medium (Lenhoff and Brown 1970) changed weekly and fed them brine shrimp (Brine Shrimp Direct, Ogden, Utah, USA) two times a week. Hydra received the same illumination as the algae.

The experiment involved two main parts (summarized in Fig. 1).

2.2 Algae mutation and selection

We followed a mutation-selection process on algae to determine if we could change algal UV-B resistance, and therefore hydra UV-B resistance (Tillich et al. 2012). We collected algae at log growth phase (10^5–10^6 cells/ml) and combined them to get homogenized algae suspensions for mutagenesis (Singh and Dikshit 1976). We filled 10 cm-diameter petri dishes with 15 ml of this suspension and subjected them to 0.7 mw/cm^2 UV-C exposure, for periods of 10, 15, 20, 25, 30, 35, 40, 45, 50, or 60 min in a dark room and kept two additional groups unexposed. After the exposure, we immediately transferred algae of each dish into a 50 ml test tube and placed them in a dark chamber to prevent photoreactivation (Singh and Dikshit 1976; Tillich et al. 2012). After 24 h, we transferred the tubes to the culture room, and left them there for three weeks until algae recovered or died. We also placed 20 polyps of symbiotic green hydra that hosted the original NC64A algae under the same UV-C strength for 30 min to check if the holobiont could survive the stress the unassociated algae received. We used algal cultures that recovered from the UV-C treatment for further experiments. We derived multiple algal cultures during the mutagenesis process, however, only some cultures survived and were used in the following steps. Cultures A and B were non-mutated algae; cultures C through I were mutated algae from recovered tubes. We divided each culture into a non-selected

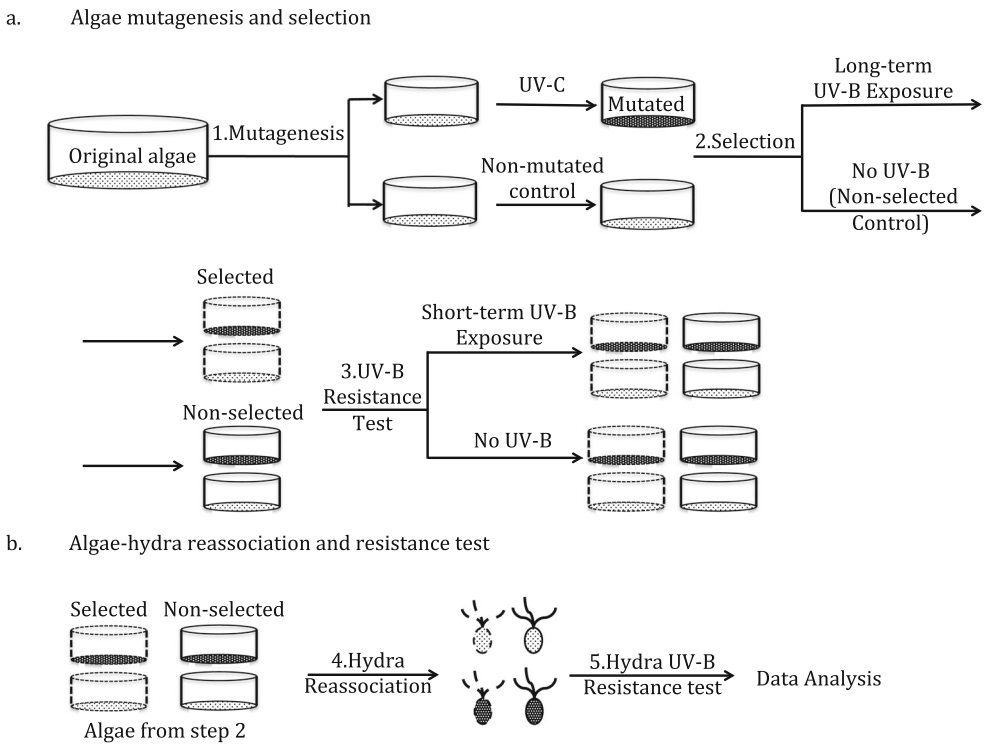

Fig. 1 Experiment flowchart of (**a**) algae mutagenesis and selection, (**b**) hydra-algae reassociation. The goal was to test whether we could alter symbionts' and holobionts' stress tolerance via symbiont mutagenesis and/or selection. Symbiotic algae NC64A was mutated with UV-C (step 1) and selected with UV-B (step 2). Both selected and non-selected populations were tested for UV-B resistance (step 3) and inoculated into hydra (step 4). Hydra were than tested for their UV-B resistance (step 5) which allowed the stress tolerances of symbionts and holobionts to be compared. Light fill indicates algae that have not been mutated, dark fill indicates mutated algae, solid lines indicate algae that have not undergone selection and dashed lines indicate algae that have undergone selection

(control) population and at least one UV-B treatment population (one for all but culture F which had three) and inoculated them into continuous culture independently. Each culture was an aerated 250 ml glass side arm flask containing 200 ml of MBBM. Medium was added at a rate of 100 ml/day (Carter Cassette Manostat Digital Pump, Anderson Process, Brookfield, WI USA) and discharged from the outlet. Cultures were kept at 18 °C with the same illumination as described above. The UV-B treatment group received an additional 0.15 W/m^2 UV-B irradiance for 12 h daily. After two months, we collected algae into test tubes and cultured them for approximately another eight generations before UV-B resistance tests to control for acclimation effects (Lohbeck et al. 2012).

2.3 Algae UV-B resistance tests

We centrifuged each test tube with algae, removed the supernatant, and re-suspended algae with deionized water, three times, to get rid of nutrients for algae growth. We divided each population (culture and selection treatment combination [selected and non-selected]) into three groups: 0 h time point group, 48 h time point control group, and 48 h time point short-term UV-B treated group. There were three replicates for the 0 h time point group and the 48 h time point control group respectively (2 ml aliquots in 10 ml glass test tubes), and at least 12 replicates for the 48 h time point short-term UV-B treated group. We placed the 48 h time point control group at 18 °C with the same illumination as described above for 48 h; the 48 h time point short-term UV-B treated group received the same illumination plus 0.2 W/m^2 UV-B. For each population, we centrifuged the tubes, removed the supernatant, added 1 ml methanol, sealed the tube with parafilm, sonicated it for 1 min (40 kHz), kept it in the dark at 5 °C overnight, and measured optical density at 663 nm (OD0hrs). The OD reading was used as an estimate of algae density because density is linearly proportional to OD (Jia et al. 2015). We used a higher intensity for the tests of algae UV-B tolerance because the goal of the selection experiment was to generate differences in growth and survival while allowing long-term cultures but the goal of the assays of tolerance phase was to have UV-B levels intense enough to quantify UV-B tolerance for a range of algae and holobionts.

2.4 Regreened hydra UV-B tolerance test

For each population (culture and selection treatment combination [selected and non-selected]), we microinjected algae into aposymbiotic hydra. We picked and cultured hydra that turned green in hydra medium. We placed 25 to 75 re-greened non-budding polyps of similar size from each algal culture and selection treatment at 18 °C with the same illumination as described above plus 0.1 W/m^2 UV-B, and tracked the population sizes over 48 h.

2.5 Statistical analyses

We performed a pair of ANOVAs (proc mixed, SAS 9.4) to determine the dependence of the change in OD for each population ($\ln[OD_{48hrs}/OD_{0hrs}]$) on selection, culture, selection×culture and population(selection×culture) in control or UV-B test conditions. We used adjusted means partial difference tests to discriminate among means for significant factors. To estimate tolerance to UV-B, for each population, we randomly assigned a UV-B test pair to each control test pair to create 5000 pseudo-datasets and calculated relative change in OD over 48 h ($\ln([OD_{UVB,t=48h} / OD_{UVB,t=0h}] / ([OD_{con,t=48h} / OD_{con,t=0h}])$). We calculated quartiles and 95% confidence intervals (CI) from these 5000 randomizations. We also calculated the average non-selected and selected algal UV-B resistances (excluding culture E that only had selected algae survive). We considered non-selected and selected populations to differ in their UV-B resistances when their 95% CIs did not overlap.

We used another ANOVA (proc glimmix, binomial distribution, logit link) to test whether hydra survival with UV-B exposure depended on selection and selection×time. We did not include cultures that did not have both non-selected and selected algae associated with hydra. We used survival analysis (proc phreg, Cox proportional hazard model) to test how hydra mortality under UV-B depended on algal culture and selection.

To test how algal survival under UV-B test conditions was related to hydra survival under UV-B test conditions when associated with those algae, we performed a correlation (proc corr) between the estimates of change in OD of algal populations under UV-B test conditions as the predictor and the Cox proportional hazard model estimates for hydra as the response. We repeated this correlation only including cultures that had both non-selected and selected populations associated with hydra.

3 Results

We ended up with surviving algal cultures that had been exposed to UV-C for 0 ($N = 2$, culture A, B), 15 ($N = 1$, culture C), 20 (N = 1, culture D), 30 ($N = 4$, culture E,F,G,H), or 35 min (N = 1, culture I). In addition, some algal populations were lost later during the experiment (non-selected culture E population) and some surviving populations were not associated with hydra (non-selected culture C, D, and I populations). All the hydra died within one day after UV-C exposure.

The change in OD for algae in control test conditions did not depend on selection ($F_{1,29} = 1.0$, $P = 0.3367$), culture ($F_{7,29} = 1.6$, $P = 0.1698$), selection×culture ($F_{7,29} = 1.5$, $P = 0.1992$), or population(selection×culture) ($F_{2,29} = 0.2$, $P = 0.8215$; Fig. 2a). In UV-B test conditions, selected algae had larger reductions in OD on average ($F_{1,164} = 13.4$, $P < 0.0001$) and reductions depended on culture ($F_{7,164} = 13.3$, $P < 0.0001$). But selection only significantly reduced OD for some populations (selection×culture, $F_{7,164} = 1.9$, $P = 0.0777$; population(selection×culture), $F_{2,164} = 3.1$, $P = 0.0463$; Fig. 2b). UV-B resistance of algae was lower with selection on average and for three of the selected cultures, which were culture A and two replicates of culture F (Fig. 2c).

Hydra survival in UV-B test conditions varied among cultures ($F_{6,121} = 8.6$, $P < 0.0001$) but did not depend on algal selection ($F_{1,121} = 2.3$, $P = 0.1358$) or their interaction ($F_{4,121} = 1.8$, $P = 0.1385$) (Fig. 3).

Algal UV-B resistance and hydra UV-B resistance were significantly positively correlated ($r = +0.52$, $P < 0.05$) whether all populations were included or only algae cultures with both selected and non-selected types (Fig. 4).

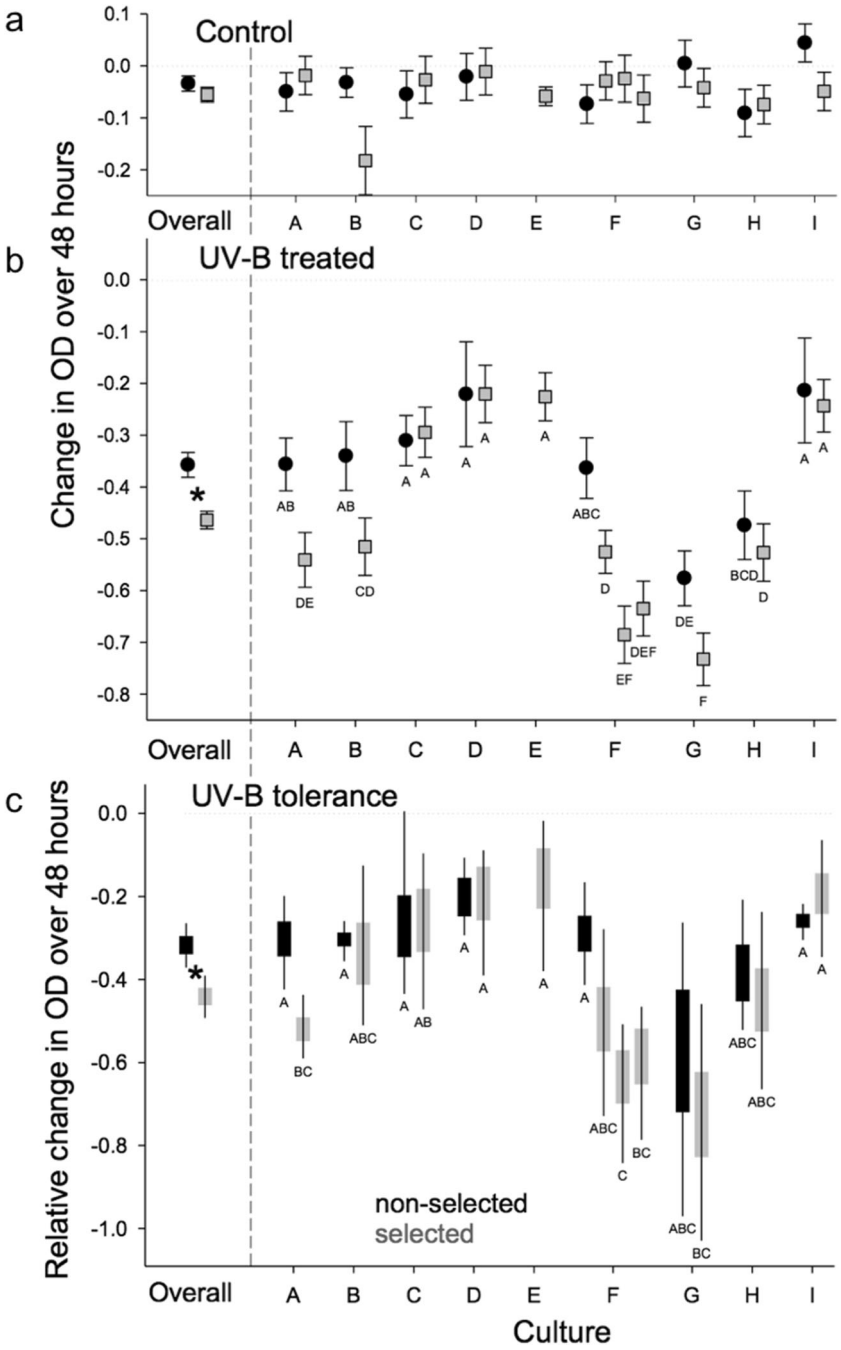

Fig. 2 The change in OD at 663 nm (ln [$OD_{t=48h}$ / $OD_{t=0h}$]) for different non-selected (dark symbols or lines) or selected (light symbols or lines) populations of algae from different cultures in (**a**) control and (**b**) UV-B test conditions. Means ± se. Means with the same letters did not differ in post-hoc tests. * indicates $p < 0.05$. (**c**) Algal tolerance to UV-B in test conditions (ln ([$OD_{UVB,t=48h}$ / $OD_{UVB,t=0h}$] / ([$OD_{con,t=48h}$ / $OD_{con,t=0h}$])) estimated by randomization. Thick bars indicate quartiles. Thin lines indicate the 95% CI. * indicates 95% CI that do not overlap for non-selected vs. selected algal populations from the same culture. A,B = 0 min UV-C (non-mutated), C = 15 min, D = 20 min, E,F,G,H = 30 min, I = 35 min. Overall indicates the average of cultures with both selected and non-selected populations (excludes E)

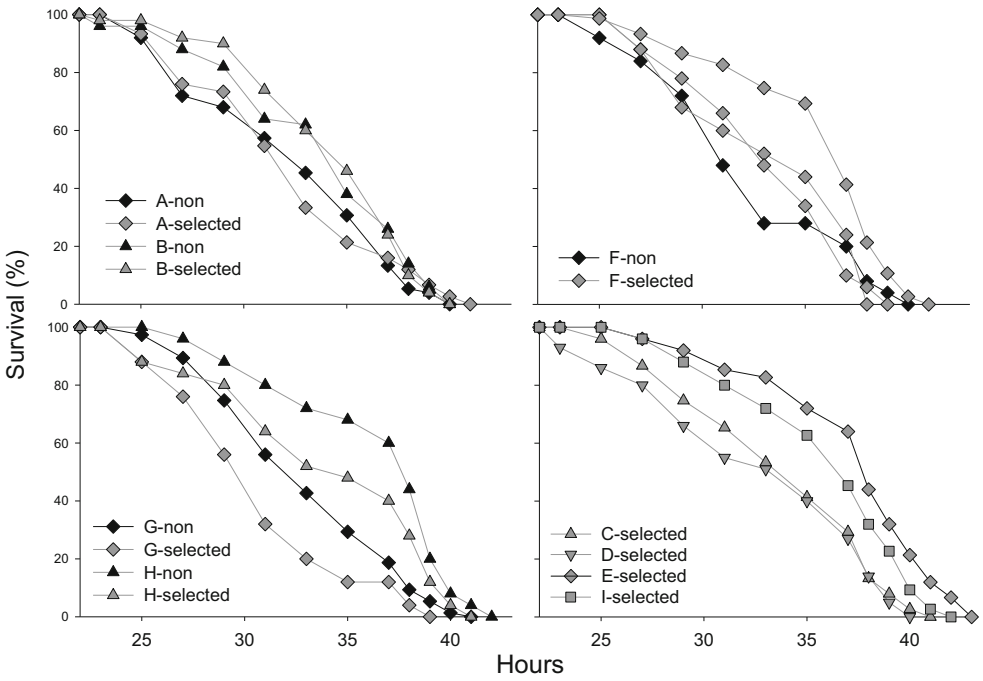

Fig. 3 The survival of hydra associated with different non-selected (dark symbols or lines) or selected (light symbols or lines) populations of algae. A,B = 0 min UV-C (non-mutated), C = 15 min, D = 20 min, E,F,G,H = 30 min, I = 35 min

4 Discussion

The hologenome theory proposes that rapid genetic changes in symbionts' genes may allow holobionts to adapt to new environments quickly, which implies artificial modification of symbionts' genes could be employed to develop holobionts with increased stress tolerance (Zilber-Rosenberg and Rosenberg 2008). Targeting the symbiont rather than the holobiont for selecting stress-tolerant holobiont is likely to be more efficient because of their shorter generation time, larger quantities that can be collected, ease of cultivation, and possibly more resilience to mutagenesis (Foster 2007; Császár et al. 2010; Thomas et al. 2010). Our results explicitly demonstrated that we can alter a holobiont's stress tolerance by mutating their associated symbionts, and we revealed a positive correlation between the symbiont's and the holobiont's stress tolerance. By following the mutation-selection process that had been conducted on free-living microbes (Cordero et al. 2011; Bonente et al. 2011; Tillich et al. 2012), we successfully created symbiotic algal populations that varied in their UV-B resistance, which could be conferred to hosts. In addition, we can predict the impacts of symbionts on holobiont stress tolerance before we inoculate them. Interestingly, we found it was mutagenesis but not selection on symbionts that affected holobiont stress tolerance. The results support the hologenome theory, and suggest symbiont mutagenesis combined with identification of symbionts with particular traits could be an efficient approach to acquire desired holobiont phenotypes. Because we could cultivate the symbionts in vitro, it was easy to generate such variations and identify algae that confer higher resistance to hosts.

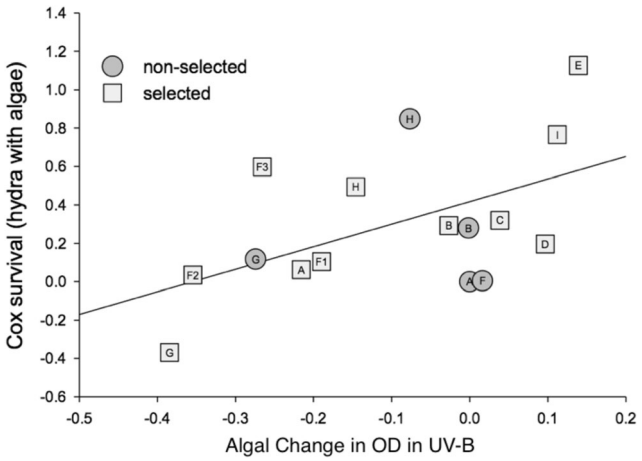

Fig. 4 The correlation (line) between algal change in OD in UV-B (Fig. 2b) and Cox survival coefficient for different non-selected (dark circles) or selected (light squares) populations of algae. A,B = 0 min UV-C (non-mutated), C = 15 min, D = 20 min, E,F,G,H = 30 min, I = 35 min. Non-selected culture A set at the origin

Our experiment indicates that random mutations can be induced in symbionts independent of hosts and influence holobiont phenotypes, suggesting we can use isolatable symbionts that subsequently can be associated with hosts to improve holobionts' stress tolerances (Voss et al. 2015). When compared with the non-mutated algal cultures, mutated algal cultures exhibited significantly different UV-B resistance (either higher or lower), as did green hydra associated with mutated and non-mutated algae. This provides new insights into holobiont trait alteration, because most past research tested various naturally occurring strains for their impacts on holobiont traits, and mutagenesis was only applied to identify

genes that are responsible for parasitism vs. mutualism (Freeman and Rodriguez 1993; Redman et al. 1999). For example, Rodriguez et al. (Rodriguez et al. 2008) found naturally occurring fungal strains differ in the stress tolerance conferred to host plants. Similarly, coral thermal tolerance is also influenced by the types of symbionts they host (Berkelmans and van Oppen 2006). In a previous work, different naturally occurring algal strains were associated with different naturally occurring hydra strains, and it was found that holobiont thermal tolerance varied across algae strains, with NC64A conferring intermediate tolerance (Ye et al. 2019). It is possible that the choice of this non-native algal strain influenced our results here because other researchers have shown that this can have an impact on some holobiont traits (Hamada et al. 2018). Here we demonstrated that in addition to employing naturally occurring symbionts, we can further generate variations based on a known beneficial symbiont strain and choosing those that confer greater stress tolerance to hosts. Perhaps the next logical extension is to identify, or also maybe modify, functional genes in symbionts that contribute to improved holobiont stress tolerance or production, such as heat shock genes or enhanced nitrogen-fixing genes.

In addition, our results also indicated symbionts are more suitable for a mutation-selection process than holobionts, because we discovered free-living symbiotic algae but not holobionts survived mutagenesis. We found all symbiotic hydra died after a 30-min UV-C exposure (although algae may survive in free-living format); in contrast, free-living algae recovered from the same exposure conditions. Kalafatić et al. (2016) observed a similar pattern that the symbiotic algae exhibited higher tolerance towards different xenobiotics compared to the hydra host, indicating the symbiont is a stronger partner than the host under stress. The greater chances of mutation due to higher mutagen dose, plus larger symbiont population sizes that could receive mutagenesis, suggest the potential of generating induced mutations is higher in the symbiont than in the host (Springman et al. 2010). These mutations may be especially likely to generate novel traits in symbionts because their genome size is usually smaller and more compact than their free-living relatives, retaining mostly functionally critical genes (McCutcheon and Moran 2012; Bennett et al. 2014). Symbionts significantly contribute to holobiont functional traits, so changes in these genes could easily alter symbionts' phenotypes, and thus holobionts' phenotypes (Leonardo and Mondor 2006; Friesen 2013; Su et al. 2013). Modifying cultivable symbionts is not only feasible but also seems to be more practical than modifying the host or the holobiont directly to acquire desired holobiont phenotypes.

While we found mutagenesis altered algal UV-B resistance, we failed to detect any UV-B resistance improvement due to selection. In fact, some algae populations showed lower UV-B resistance after they experienced long-term UV-B exposure, indicating stress induced damage rather than adaptive responses in these algae, which was unexpected. In contrast, some previous studies detected either positive or no effect of long-term selection on microorganisms' fitness under stress. For example, Lohbeck et al. (Lohbeck et al. 2012) demonstrated adaptive evolution of the coccolithophore alga *Emiliania huxleyi* to elevated CO_2 in 500 generations, and Huertas et al. (Huertas et al. 2011) found 12 algal strains of a variety of species were able to improve their thermal tolerance after 8 to over 100 generations under elevated temperature. Thermal adaptation was also observed in free-living algal coral symbionts, as their fitness was higher at elevated temperature after 80 generations of selection (Chakravarti et al. 2017). Yet a longer selection experiment on the green algae *Chlamydomonas* at elevated CO_2 for over 1000 generations failed to detect any adaptive responses, even though they found some new traits occurred, probably as the result of accumulated neutral mutations (Collins and Bell 2004). Sexual reproduction has not been observed in *Chlorella* so our study almost certainly involved selection on asexually reproducing algae, but other studies included algae that can undergo sexual reproduction (Billard and Inouye 2004; Blanc et al. 2010), which could help to explain the range of selection responses reported in the literature but not the negative effect of selection in our study. In addition, negative carry over effects might occur, in which long term exposure to stress in ancestors decreases offspring fitness (Ross et al. 2016). An alternative explanation could be that impacts of lower magnitude, chronic UV-B and higher magnitude, acute UV-B differed (because of different objectives for the selection vs. assay stages), so our selection process did not select for algae that had relevant tolerance in our assays. Because of the unexpected outcomes of the selection experiment, we propose for future symbiont selection experiments that the selection criteria and final testing criteria should be the same, especially if the mechanism underlying their effect on host, and in turn, holobiont traits are unknown.

In newly associated hydra, we detected effects of symbiont mutagenesis but not symbiont selection on holobionts' UV-B resistance, and mutated algae could either improve or decrease hydra UV-B tolerance. Hydra that received different populations of algae had dissimilar UV-B resistance, and the positive correlation between algal UV-B resistance and symbiotic hydra UV-B resistance suggests algae are able to confer their UV-B resistance to hosts. Our results indicate detrimental mutations could occur through symbiont mutagenesis and reduce host fitness. In addition, algae from the same culture conferred similar UV-B tolerance to hydra no matter they were selected or not. This resembles the results of some previous studies. For example, Giauque et al. (Giauque et al. 2019) found naturally occurring stress tolerant endophytic fungi improved plant fitness under stress; and Chakravarti et al. (Chakravarti et al. 2017) observed higher thermal tolerance for selected free-living algal strains but the selection effect disappeared

when algae were associated with coral hosts. Because both biochemical and physiological attributes affect algal UV-B resistance, certain algal strains that differ when free-living may not vary within the host as forming endosymbiosis changes their morphology or their spatial distribution (Pasaribu et al. 2015). This might also explain why Chakravarti et al. (Chakravarti et al. 2017) failed to detect coral thermal tolerance improvement after they inoculated corals with symbionts having highest growth rates at elevated temperature. Our results together with those from previous studies suggest symbiont selection might not be effective in improving holobiont stress tolerance, particularly if tolerance-conferring traits of symbionts are not clearly identified (van Oppen et al. 2015).

Indeed, our results not only show that experimentally induced variations in symbionts could generate various holobiont phenotypes, but also suggest stress tolerant symbionts confer greater tolerance to hosts. Depending on the species and traits of interest, predicting the holobiont phenotype based on the symbiont phenotype is possible. For instance, experimentally replacing naturally occurring bacterial symbionts of aphids with more heat tolerant bacteria increased aphid thermal tolerance (Moran and Yun 2015). In our case, hosting a stress tolerant symbiont population was likely to increase the holobiont fitness under stress. There are, however, symbiont traits other than stress response that can predict symbiont effects on holobiont performance. In endopyhte-plant symbiosis, fungi traits related to resource use and habitat characteristics can predict up to 53% of their effects on plants (Giauque et al. 2019). Previously, Mueller and Sachs (Mueller and Sachs 2015) proposed engineering animals and plants with microbes, but their method ("host-mediated selection") is indirect and requires cultivating the holobionts. Here we propose direct manipulation of symbionts, which is faster and more economical efficient, because more symbionts can be screened in a shorter time. Moreover, instead of conducting selection on symbionts, generating and identifying symbionts with certain functional traits via mutagenesis could be more effective in finding symbionts that enhance holobiont-stress tolerance.

5 Conclusions

To the best of our knowledge, our experiment is the first to employ symbiont mutagenisis to improve holobiont stress tolerance, which highlights the ability of holobiont evolution via rapid symbiont evolution. It also reveals a novel approach that can be employed to engineer holobionts. Compared with conventional host-targeted breeding and genetic engineering, symbiont-based approaches are time-efficient and transferrable (i.e., can be applied to multiple species) (Coleman-Derr and Tringe 2014), and has been applied in agriculture using non-mutagenesis methods (Hart and Trevors 2005). Because microbiomes can improve plant stress tolerance and they have been successfully applied to promote plants growth under unfavorable conditions (Redman et al. 2002; Mei and Flinn 2010), we propose that symbiont mutagenesis, evaluation and inoculation could potentially be applied to achieve desired traits of these and other organisms (Mueller and Sachs 2015) for agricultural or conservation purposes. Although there are important policy dimensions and ecological consequences (e.g., reduced biodiversity) to consider, this strategy could be applied to conservation or agriculture by creating and cultivating strains in the lab then releasing them to increase holobiont stress tolerance in the field (van Oppen et al. 2015). Moreover, altered traits due to newly associated symbionts may be inherited vertically for some holobionts (Bright and Bulgheresi 2010). In our case, we found that holobiont and symbiont abiotic tolerances were positively correlated but that generation and screening of symbiont variants was a more effective approach for improving holobiont tolerance than directed host-mediated symbiont selection (Mueller and Sachs 2015). Having a better understanding of correlations between microbes' functional traits and their effects within holobionts may allow assessment of numerous symbiont strains without inoculating them into the host, or even gene editing the symbionts to produce holobiont-enhancing symbiont strains. In addition, altering the fitness of the holobionts via symbionts may come at the cost of their fitness in other situations. Future research should investigate the mechanisms by which symbionts affect holobiont stress tolerance in order to identify symbionts with traits that are likely to improve their fitness under stress, and evaluate their effect in the host comprehensively.

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