The Roles of Endosymbionts and Hosts in Adaptive Response to Stress

Siao Ye

Abstract

Endosymbiosis is such an intimate interaction that impacts on either partner may disrupt the other, which makes endosymbiotic species quite sensitive to environmental changes, such as elevated temperature, decreasing pH, etc. In some circumstances, the changes are so rapid that the whole ecosystem is under threaten as the endosymbiosis is disrupted, for example, the coral reefs experience bleaching when the sea temperature exceeds certain threshold. The hologenome theory proposed by E. Rosenberg and I. Zilber Rosernberg suggests that endosymbionts can adapt to such changes and confer their resistance to hosts, as they usually have shorter generation time and larger population size compared with hosts, and thus the holobiont, which is the host plus the endosymbiont, is the unit under selection.
My dissertation research uses green hydra *Hydra viridissima* as the model system, in which green algae provide carbonate to hydra while the hydra provide CO_2 and proteins in returns. The hydra can be bleached and survive without algae, and can re-associate with algae later, allowing manipulations on both the host and the endosymbiont. To date I have compared survival rate of symbiotic hydra and aposymbiotic hydra (i.e bleached hydra that algae have been deprived) under heat stress, and tried to select for UV-B tolerant algae. The result shows high variation exist across both symbiotic and aposymbiotic hydra populations, and there is correlation between the thermal tolerance of these two groups. In addition, the aposymbiotic hydra is more tolerant than their symbiotic ancestral strains. This provides a direct evidence that holobiont stress tolerance is correlated to host stress tolerance, which is usually overlooked in the past. For the artificial selection experiment, the selected algae didn't show improved tolerance under UV-B. However, different mutated algal strains exhibit various UV-B tolerance, indicating there is possibility that a stress tolerant mutation can be induced.
The next step would be testing UV-B resistance of hydra receiving these selected algae to see if hologbiont stress tolerant is related to algal stress tolerance. I will also compare acclimation/deacclimation rate of symbiotic/aposymbiotic hydra, as well as adaptation rate of algae within/without host in the future.

INTRODUCTION

Coral reefs, an ecosystem hosting over a quarter of the marine species of the world, are experiencing massive bleaching since the last century. Studies show corals lose their colors as they disassociate with the algae residing in them when stressed by warming sea temperature (Brown 1997;Plaisance et al. 2011). The high mortality of corals following bleaching is critical as it threatens a large amount of associated marine species on which local human communities depend. Since corals host endosymbiotic algae within their tissues, it makes them highly vulnerable to environmental changes, as endosymbiont malfunction can decrease the host's fitness, or vice versa. Rising temperatures and high light intensity impair the endosymbiotic algae, zooxanthellae, within the host, and cause bleaching (Coles and Jokiel 1978; Brown 1997). Coyte et al. (2015) suggested that cooperation could destabilize ecosystems through mutual downfall, in which one species decline generates negative

impacts on the other. This calls for more researches to understand how the symbiosis breaks down in unfavorable environments and the consequences of such breakdowns. One of the ways to circumvent such breakdowns may be if the endosymbiotic alga within the coral is induced to have high tolerance to those environmental stress factors that causes the disassociation of alga from coral. Hence, I propose to study responses of both the host and the endosymbiont to stress, and try to find out whether adaptation in the endosymbiont alone could mitigate damages on the host, as this may shed light on further conservation strategies through manipulating of either the host or the endosymbionts.

Endosymbionts have been found in distinctive species groups, and show great impacts on host stress tolerance in many cases. For example, endophyte-infected grasses are modified in terms of morphological and biochemical attributes, thus they are able to persist in harsh habitats that lack water and nutrients, or of high heat and salinity (Malinowski and Belesky 2000; R. Rodriguez and Redman 2008; R. J. Rodriguez et al. 2008). With enhanced drought tolerance, plants are able to utilize more broad niches and expand their geographic range (Clay and Schardl 2002; Afkhami, McIntyre, and Strauss 2014).

In some insect-bacteria symbioses, although the role of the bacteria is unclear, significant decrease of the endosymbiont due to high sensitivity to stress compared with the hosts results in endosymbiosis breaking down, which could also generate negative impacts on the hosts (Fan and Wernegreen 2013; Wernegreen 2012). An extreme example was found in pea aphid with its endosymbiotic bacteria, *Buchnera aphidicola*, in which one mutation in the bacteria decreases host thermal tolerance significantly (Dunbar et al. 2007). Since endosymbionts seem to play such a critical role in host stress tolerance, some major questions I would like to ask include:

1) Do both hosts and endosymbionts contribute to the stress tolerance of the endosymbiont-host association? If so, how is this combined tolerance related to their individual tolerances?

2) Can endosymbionts gain and confer higher stress resistance to hosts in a short period?

3) How does presence of endosymbionts affect the extent and rate of host adaptation/acclimation?

4) Do hosts constrain endosymbiont's adaptation rate?

I will investigate these questions using green hydra *Hydra viridissima* and the symbiotic green algae *Chlorella variabillis* NC64A strain.

Studies on coral reefs have shown that different types of zooxanthellae confer different levels of thermal tolerance to the host; specifically, corals associated with the more prevalent zooxanthellae, type of Clade C, improve their thermal tolerance after they switch their endosymbionts to clade D zooxanthellae (Berkelmans and van Oppen 2006; Cunning, Silverstein, and Baker 2015). It has been found the latter tends to be more common in harsh environment and comes at a cost for corals under normal conditions (Fabricius et al. 2004; Jones and Berkelmans 2010; Shahhosseiny et al. 2011). Buddemeier et al. (1993; 2004) hypothesized that bleaching might provide an opportunity for corals to adapt to rising temperature, since new association between coral-zooxanthellae can form in bleached but surviving corals. The assumptions that there is variation in algae tolerance and corals can establish symbiosis with non-native endosymbionts have been tested and verified (Kinzie et al. 2001; Toller et al. 2001; Yuyama and Higuchi 2014). Meanwhile Rosenberg and Rosenberg presented the coral probiotic theory as they observed that bacterial community present on the surface or within corals can determine whether corals will bleach when infected with pathogens, and this was extended as the hologenome theory (Reshef et al. 2006; Zilber-Rosenberg and Rosenberg 2008). In the hologenome theory, the host and the symbiont are viewed as a unit (holobiont) upon which the process of selection starts, and include not only endosymbionts but also other microorganisms associated with the host that contribute to the phenotype of the holobiont (Zilber-Rosenberg and Rosenberg 2008). The hologenome theory points out that since symbionts can be horizontally acquired and vertically transmitted, hosts can gain new traits by associating with novel symbionts. As the hologenome includes both hosts and symbionts genes, changes in both partners could contribute to variation in hologenome, and specifically, rapid genetic changes in the symbionts part would allow fast adaptation to happen in host-symbiont unit.

Although debates are still going on about the hologenome concept (Ainsworth et al. 2007; Brucker and Bordenstein 2014; Chandler and Turelli 2014; Moran and Sloan 2015), there are growing evidences that symbionts play a critical role in host life history beyond corals. Depending on the symbiont's character, the holobiont's phenotype may vary, and thus host fitness is affected. Studies on wasps and Drosophila show distinctive symbiotic bacteria drive the isolation between populations,

by either changing mating preferences or reducing hybrid fitness (Sharon et al. 2010;Brucker and Bordenstein 2013). These experiments suggest speciation could be caused by microbiome-host incompatibility, a new type of isolation in which not only the host's allele but also the symbiont's matter (Norris 1996). Even though symbiont may not necessarily drive speciation, they can still generate intraspecific variation. For instance, gut bacteria have been discovered to correlate with obesity, as slim mice gained weight after they received gut bacteria from fat mice (DiBaise, Frank, and Mathur 2012). In addition, lack of appropriate symbionts to cryptic organ formation in mice and squids further proves an additive effect of the host and symbionts which generate a phenotype of holobiont under selection (Montgomery and McFall-Ngai 1994; Eugene Rosenberg and Zilber-Rosenberg 2011; Wostmann 1981).

Establishing endosymbiosis could improve hosts fitness under some conditions, as one can be susceptible to certain stress that otherwise is not fatal. Strychar and Sammarco (2009) observed that while zooxanthellae were damaged by elevated temperature, coral cells experienced little cell death. This indicates that endosymbiosis breakdown could be a result of malfunction in only one partner, which are algae in this case.

It is widely accepted that high stress can damage photosynthetic systems in algae, and the excess electron is transported to targets that finally turn into reactive oxygen species (Smith, Suggett, and Baker 2005; Lesser 2011). Heat treatment on sea anemone and corals shows that the more sensitive the algae are, the more likely the hosts will bleach, and algae will produce a high amount of reactive oxygen species (ROS) under stress (Dykens et al. 1992； Fitt and Warner 1995; Perez, Cook, and Brooks 2001; Tchernov et al. 2004). A similar pattern of high sensitivity to stress in endosymbionts has been explored in arthropod-bacteria endosymbiosis (Wernegreen 2012). Wernegreen and Fan (2013) discovered that a four-week heat shock can effectively eliminate more than 99% of endosymbiotic bacteria in ants, while the hosts didn't show any obvious symptoms. A study on pea aphids also showed significant decrease in bacteriocytes and primary endosymbiont Buchnera after heat-stressed, and it was found that fecundity was positively correlated to number of bacteriocytes (Montllor, Maxmen, and Purcell 2002). While infection of a second endosymbiont increased numbers of bacteriocytes retained after heat stress, the fecundity was also partially recovered. On the other hand, endosymbionts can also constrain low-temperature tolerance of host. A predatory bug

Macrolophus pygmaeus acquire better freezing tolerance after they are treated with antibiotics, which eliminates all the endosymbionts within it. Another example that well illustrates endosymbionts constrain host niche is the experiment on *Artemia* (Nougué et al. 2015). *Artemia* is a kind of crustacean that can be found in high salinity environments but is not able to survive at low salinity. In the wild, *Artemia* mainly predates on algae, and symbiotic bacteria are involved in digesting algae. The experiment showed that isolated endosymbionts grow better on high salinity medium compared with low salinity one. Meanwhile, axenic brine shrimp has higher survival when fed on yeast than algae. This indicates though brine shrimp could tolerant low salinity, the inability of their symbiotic bacteria to persist in such condition limits their nutrients intake in the wild, and thus their niches are restricted to only high salinity water body. In these cases, endosymbionts appear to be the weaker partner facing the stress compared with hosts, so it is reasonable to expect that resistance improvement in endosymbionts can better mitigate damages on the holobiont than that in hosts.

In addition, endosymbionts are considered to have the potential to achieve resistance in a short term while the hosts may not be able to. This is mainly based on the generation time hypothesis, which suggests species with short generation time evolve faster because more genes with duplicate errors that can be accumulated giving same time length. The higher copy of errors correlated to shorter generation time has been observed in many phylogenetic distinctive species, from bacteria to invertebrates, plants and vertebrates (Mooers and Harvey 1994; Li et al. 1996; Soria-Hernanz et al. 2008; Thomas et al. 2010; Weller and Wu 2015). For example, Soria-Hernanz et al. (2008) found accelerated substitution rates in annual plants compared with perennial ones. Rodents, with shorter generation time than primates, also exhibit higher molecular evolutionary rates (Li et al. 1996). Thus the relative short generation time of endosymbionts compared with hosts indicates the former has higher mutation accumulation rate per unit time, and faster response to selection. In addition, population size of endosymbionts could be greater in the order of magnitude than hosts because one single host can be associated with a large amount of endosymbionts, thus there could be more beneficial mutations arising in endosymbionts (Desai and Fisher 2007). Taking these into consideration, endosymbionts are viewed as a hope for hosts persistence under stress (Zilber-Rosenberg and Rosenberg 2008; Gilbert et al. 2010).

My study on green hydra aims to discern the role of endosymbionts and hosts when the holobionts

are under stress, and to find out how endosymbionts could affect host response to unfavorable conditions. By manipulating endosymbiotic *Chlorella* and hydra, I hope to find answers to questions regarding holobiont adaptation in a stressed environment.

FOCAL SPECIES

Green hydra (*Hydra viridissima*) is a freshwater invertebrate that forms stable endosymbiosis with green algae (*Chlorella)*. It is a simple creature consisting of a cylindrical body and 5 to10 tentacles, which are no more than 4 cm in length (but usually ~1 cm). Green hydra are carnivores that prey on small crustaceans, and reproduce both sexually and asexually, with a doubling time about 3-4 days (Muller-Parker and Pardy 1987). The sexual reproduction in green hydra occurs periodically, while asexual reproduction dominates most of the time (McAuley1984; Kaliszewicz 2010). Green hydras are distributed widely and can be found in water bodies with low nutrients. The symbiotic *Chlorella* resides in the gastrodermal epithelial cells of green hydra, and provides carbohydrates to the host. In return, they obtain nitrogen from the host (Mews 1980; McAuley 2016). Since I am not able to isolate and culture the algae from the hydra as described by Kovacević (2010), I use *Chlorella variabillis* , strain NC64A isolated from paramecium (Hoshina, Iwataki, and Imamura 2010) to construct endosymbiotic hydra (Kessler, Huss, and Rahat 1988). This strain has a doubling time around 20 hours (Koltin 1988), which is pretty short compared with its host.

The ease of collection and mass culture of hydra make it an ideal species to study photosynthetic endosymbiosis in the lab compared with other species such as corals or sea anemone (Kovacevic 2012). More importantly, green hydra can be bleached and survive without endosymbionts, and re-establish endosymbiosis with *Chlorella* by injecting algae into the cavity (Pardy 1983). This means we can manipulate both the host and the algae separately or together, dissociate and associate particular host and algae, and thus partition responses of each partners under stress.

SOURCES OF HYDRA AND ALGAE

Symbiotic hydra: Green hydras from six sources are maintained in the lab. Two strains are kindly provided by Dr. Daniel Martinez at Pomona College, CA. One is 1695C collected in Chile; the other is GBR01b collected in Britain. The other four strains were purchased from biological supply companies in U.S.A. These include: 1) Connecticut Valley Biological Supply Company, collected from local ponds

in Massachusetts,U.S.A. 2) Carolina Biological Supply, collected from ponds in Nevada, U.S.A. 3) VWR International, 4) Niles Biological, collected from local ponds in Sacramento, California.

Aposymbiotic hydra: All derived from above strains by treating symbiotic hydra with 10^{-6} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in M solution, which is a photosynthetic inhibitor. The hydras are placed at 5 cm from a 30 watt light (Pardy 1983). *Artemia* (brine shrimp) are fed to them every three days. The bleached hydras are kept in cultivation for several generations and any re-greened hydra are discarded. The major difference between the derived hydras and the original symbiotic hydras is they don't have algae inside and are heterotrophic (Muscatine and Lenhoff 1965a, Muscatine and Lenhoff 1965b) .

Algae strain: Dr. David Dunigan at University of Nebraska provided this algae strain.

Maintenance: Both symbiotic hydra and aposymbiotic hydra are cultured in M solution at 18°C and fed with brine shrimp every three days, a 25-watt fluorescence light is used for illumination with a 12:12 hr light:dark cycle (Lenhoff and Brown 1970). Hydras are placed in petri dishes and the medium is changed after feeding.

Chlorella variabillis is cultured in MBBM solution at 20°C, and on MBBM agar plat as stocks. 25w fluorescence lights are used for illumination with a 10:14 hr light:dark cycle (Kodama and Fujishima 2015).

PROGRESS TO DATE

Phase1: Contribution of hydra and algae to the holobiont's thermal tolerance

Background: Studies on corals show that different types of algae in the same type of host can cause holobiont phenotypic variation (discussed above), while the role of host morphology and behaviors are overlooked, which could also be essential in their thermal tolerance. It hasn't been tested how host sole attributes are related to holobionts' performance under stress, as corals almost cannot survive without algae. There could be intrinsic differences in hosts which make some populations more susceptible to stress than others, suggested by observations that some types of corals are more susceptible to bleaching (Loya et al. 2001;Baird et al. 2009). In addition, direct comparison of responses to stress between animals with endosymbiotic algae and corresponding aposymbiotic

animals is lacking. Only a couple experiments on paramecium show that algae enhance host tolerance of UV, oxidative stress, and heat (Iwatsuki, Nishidoi, and Suehiro 1998; Hörtnagl and Sommaruga 2007; Summerer et al. 2009). Though some studies on sea anemone and their aposymbiotic peers showed differences in biochemical characters and transcriptomes under stress, they didn't compare their fitness directly (Dykens et al. 1992; Sabourault et al. 2012). One observation of algal impact on host was made by Karntanut and Pascoe, who found that algae contribute to hydra's tolerance of heavy metal at low concentration, suggested algae can improve host's tolerance to some extent (Karntanut and Pascoe 2005). Attempts to compare symbiotic and non-symbiotic coral larvae showed either improved performance or no effect in the symbiotic individuals, but this could be distorted due to low density of algae presented in the larvae (Baird et al. 2006; Ohki et al. 2013).

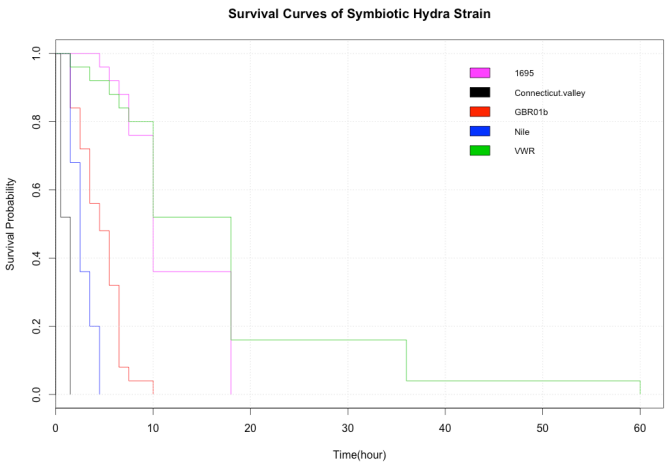
Here the main object is to find out how the holobiont's tolerance is correlated to stress tolerance of the host and alga respectively in green hydra, as well as whether presence of alga improve or reduce hydra's fitness under stress**. I hypothesized that as there could be differences among hydra-algae holobiont in their thermal tolerance, tolerance of each holobiont corresponds to tolerance in both the host and the endosymbiont, which means the most resistant hydra associated with the most resistant algae will generate the most resistant hydra-algae combination. Also, aposymbiotic hydra may show less thermal tolerance than their symbiotic peers.**

Manipulation:

1) 25 hydras of each symbiotic and correspondent aposymbiotic hydra were heat shocked at 35°C for an hour, and survival was recorded at intervals of 0hr, 1hr, 3hr, 5hr, 8hr, 24hr, and daily thereafter, for five days in total. For heat shock, 50 ml beakers were pre-warmed with 20ml of M solution in water bath, and each hydra strain was transferred into one beaker all at one time. After one hour, all beakers were taken out and cooled in room temperature.

2) Planned to generate various hydra backgrounds with one algae strain, and various algae backgrounds with one hydra strain. The former can be achieved by squashing all 6 strains of green hydra, and microinjected into aposymbiotic hydra strain 1695C. The latter can be achieved by squashing one hydra strain 1695C, and microinjected into all aposymbiotic hydra strains. The heat shock will be done as described above.

(a)



(b)

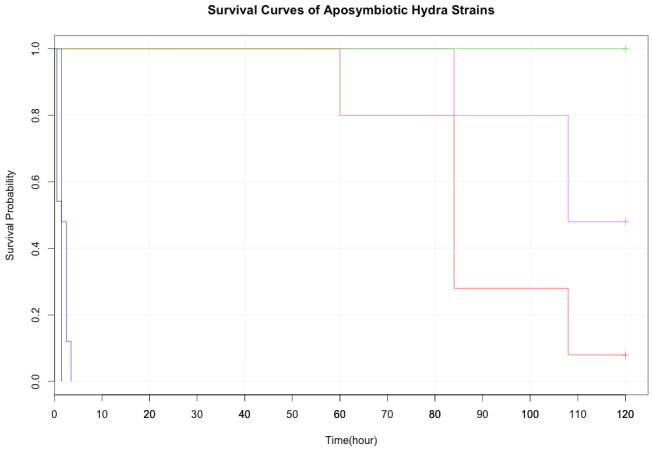


Figure 1. (a) Survival curves of symbiotic hydra after heat shocked at 35°C for an hour. After the heat shock, data were collected every hour in the first 8 hours, and then at 12 hours and 24 hours after heat shock, thereafter every 24 hours, for a 5-day monitoring period. (b) Survival curves of aposymbiotic hydra after heat shocked at 35°C for an hour. Observations were made at same intervals as those for symbiotic hydra.

Result to date:

The hydra-algae holobionts show high variation in thermal tolerance. For some strains, all individuals died within hours after the heat shock treatment, e.g., the Connecticut valley strain died out in 3 hours. In contrast, 60 percent of 1695C were still alive at the end of the observation, and may persist longer (Fig. 1a). The same pattern appears in aposymbiotic hydra, as variation occurs across different strains (Fig.1b). In addition, the ones that show higher heat tolerance in symbiotic state also have higher heat resistance when algae are deprived. A linear regression between symbiotic hydra thermal index and aposymbiotic hydra thermal index is performed. The aposymbiotic hydras also show higher fitness compared with their endosymbiotic ancestral strains. At this stage I am still working on establishing same hydra background with different algal strains and same algal background with different hydra strains. Hydras that received alga have turned green, but not yet to the degree as the wild type. It may take longer time for the alga to proliferate in the hydra, so if there is variation caused by algae and how this would relate to hydra thermal tolerance in symbiotic state is unclear for current stage.

Preliminary Conclusion: Hydra-algae holobionts from different sources exhibit variation in their thermal tolerance, which could be a result of local adaptation. Meanwhile, the correlation between symbiotic hydras' thermal tolerance and aposymbiotic hydras' thermal tolerance clearly demonstrates that host's stress tolerance is critical in determining the holobiont's stress tolerance, which is often overlooked. As hosts and symbionts may show different resistance to a type of stress, the lower limit of the holobiont to that stress could be more dependent on the more sensitive partner. The data here also indicate that endosymbiotic alga impairs, rather than improves, hydra thermal tolerance, as the aposymbiotic hydras have higher fitness under heat stress. While the data of how different algae strains may influence hydra thermal tolerance is unavailable at this point, it suggests alga contribute to the holobiont stress tolerance, though negatively in this case. An alternative explanation to the difference between aposymbiotic and symbiotic hydra thermal tolerance could be DCMU treatment to get rid of alga select for more robust individuals, as some hydras died during the treatment. I may add control groups in which aposymbiotic hydra strains receive their original alga and result in exactly the same algae-hydra combination, and test their thermal tolerance to see whether it is the bleaching procedure that increases their thermal tolerance, or it is the intrinsic difference between aposymbiotic and symbiotic hydras.

Phase2: Fast adaptation to stress in algae can increase hydra's stress tolerance

Background: As results in phase one show different thermal tolerance of symbiotic hydra, it suggests hydras thermal tolerance could be controlled by both alga and hydra itself.

To test whether endosymbionts can assist hosts to survive under stress within a short period, I partitioned the question into two parts: 1) Can endosymbionts acquire resistance to stress rapidly and independently? 2) If endosymbionts can acquire such resistance, can hosts benefit from it?

Manipulation:

1) Algae mutation: I took 30 algae samples from alga mass culture (NC64A strain), and exposed them to a 45-watt UV-C (100-280 nm) germicidal light in petri dishes for 30 minutes at a distance of 40 cm. Then all mutated algae were transferred into test tubes and immediately placed in darkness for 2 days. After the darkness treatment, algae were subjected to 12:12 hour light:dark cycle. The mutated algae tuned pale within one week. 2) Continuous culture and selection: After culturing for

another two to three weeks, 10 re-greened algae samples from the 30 mutated algae samples were picked and inoculated into continuous culture respectively. Two original samples (non UV-C treated) were also inoculated. Each culture was kept in a 250 flask, with 12:12 hour light:dark cycle. Airflow was filtered for bubbling the algae, and the medium turn overate was 5 days per flask. These continuous cultures were subjected to 25-watt UV-B light at a distance of 10 cm. The UV-B light period started at 5 hours/day, and increased gradually until 18 hours/day within two weeks. A control group of these picked alga that were kept in the same condition without UV-B light. 3) Alga UV-B tolerance test: Both control group and treatment group were taken. The algae were washed three times with DI water by centrifuging. Then they were re-suspended in DI water and adjusted to OD 0.5 at absorbance of 663 nm. Three milliliters of the suspension were transferred to small test tubes and sealed, three replicates for each strain. Then all tubes were placed 3 cm away from a 25-watt UV-B light in random orders. Tubes were shaken several times during the irradiance, and OD was measured after 48 hours. 4) Re-establishing alga-hydra endosymbiosis: All control and UV-B selected alga were injected into aposymbiotic hydra strain 1695C, so I could achieve holobionts that only differed in algae selection history. 5) Hydra UV-B tolerance test: 25 hydra of each alga-hydra combination will be subjected to a 25-watt UV-B light at a distance of 10cm. The survival will be recorded in following 4 days at 0, 1, 3, 5, 8, 12, 24, 48, 72,and 96 hours after treatment start. 5) Data analysis: First UV-B selected alga and control group will be compared to see whether the selection could increase their tolerance, and see whether they are different in response from the non-mutated original one. Hydra resistance will be analyzed by survival analysis, and ranked in similar way to heat shock index. A correlation between alga resistance and hydra resistance will be analyzed to test whether alga tolerance could affect the host.

Result to date: 1) 8 out of 12 attempts of continuous culture were successful, in which number 1 and number 4 are from same original strain, number 5, number 6, number 7 are from same original strain. The mutated algae have been under selection for about two months. The preliminary result shows UV-B does impair algae, as those exposed to UV-B show lower OD compared with the control group . Moreover, variation in algae performance is greater when placed under UV-B. Unexpectedly, the selection doesn't increase algae UV-B tolerance; actually the selected algae tend to perform worse under intense UV-B (Fig 2).

Preliminary conclusion: The two-month selection didn't improve algae performance under UV-B. This could be due to lower vigor in selected algae, as they were exposed to UV-B continuously. UV-B is known to affect the pigment complexes of algae in a deleterious manner so the algae that survive the exposure may have a pigment system which cannot support maximum photosynthesis so high growth rates are not found. However, OD change under control condition in selected algae is not significant compared with non-selected algae. One way to avoid the problem could be culturing the selected algae under normal condition for a while, and then comparing their UV-B resistance with non-selected. It could also be that algae are not sensitive to current UV-B strength in selection, or more time is needed for this procedure.

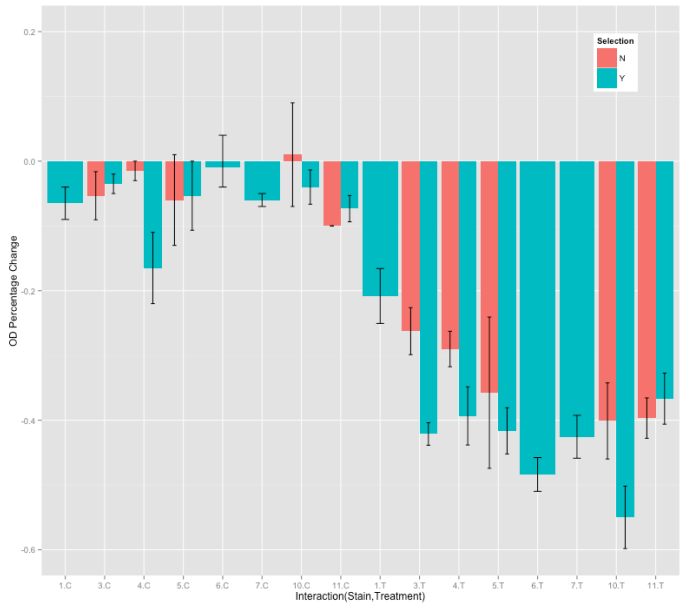


Fig 2. UV-B Resistance in Selected vs. Non-Selected Algae. 8 strains of UV-B selected (Y) or non-selected (N) algae are exposed to 48 hours of UV-B treatment (T), or under similar condition without UV-B as control groups (C). Chlorophyll is extracted with methanol both before and after treatment, and optical density (OD) is measured at 663nm. Strains 1 and 4 share the same control group; strains 5,6,7 share the same control group.

The high variation among the mutated algae, nevertheless, suggests UV-C did induce various mutations in the endosymbiotic algae in free-living state. Though no extreme high/low UV-B tolerant strain is detected in the experiment, we can expect to encounter one if we mutate enough algae. The variation in mutated algae suggests endosymbiont's genetic diversity could be induced independently of the host, and this diversity could cause variation in holobiont when they form endosymbiosis with the host, even it's unlikely for the algae to gain better resistance within a few months under stress. To further investigate how this variation may affect holobiont stress tolerance, I am injecting the algae into hydra. Certain strains of algae have established stable endosymbiosis with hydra, while the rest are still under constant microinjection.

PROPOSED IMPROVEMENT

In previous experiments, I checked intrinsic differences within hosts and within algae, and how these variations could impact the performance of the holobiont under stress. It is a systematic study on one species that checks upon both partners in stressed environment. In addition, I was testing one of the critical questions in hologenome theory, which is whether adaptation in endosymbionts could help hosts to persist in the harsh environment, which means selection acts on a host plus endosymbiont level. A further complement to above study is to check host's role in holobiont's adaptation/acclimation, which is largely ignored. Although it is assumed hosts are not able to adapt at the same rate as their endosymbiotic partners do, it is possible they can acclimate to environmental changes. To my knowledge, no explicit experiment has been conducted to test **how presence of endosymbionts affects host acclimation, and more importantly, the resistance to deacclimation.** Deacclimation resistance is considered an important trait for species persistence under current capricious climate, as species may lose gained acclimation during short intervals between two stressed periods (Arora and Rowland 2011). For example, plants with slow deacclimation rate can survive in the winter when encountered an unexpected warm period, while those with low deacclimation resistance could die during the following low temperature, as the latter lose their cold tolerance in the warm period.

H1. There is a difference in capacity of acclimation between hosts and endosymbionts, and presence of algae impacts deacclimation rate.

Manipulation:

Determination of acclimation capacity: 1) Hydra and algae acclimation: 20 aposymbiotic hydras and 20 symbiotic hydras are put in temperature controlled chamber at 18°C with 12:12 light:dark cycle. The temperature will be raised 2°C every two days until reach 30°C for acclimation. Then hydras will be kept at that temperature for three weeks. During this process, hydra could die due to high temperature, so the survived hydra will be fed with brine shrimp every three days, which stimulate asexual reproduction through budding. A control group for each type is maintained in the similar condition except that the temperature is 18°C. Similar procedure is conducted on algae. 2) After the three-week acclimation, biochemical characters can be measured in both hydra and algae to check

their acclimation capacity. This could be done by either measuring reactive oxygen species (ROS) or superoxide dismutase.

ROS measurement: After the acclimation, 3 hydras from each population will be sampled and placed in 32°C for 1 hour. ROS can be measured according to protocol given by Edward Owusu-Anasah et al. (2008). Basically, 2', 7'-dichlorofluorescein is a dye that can be oxidized to a green fluorescence product in cell, and can be monitored by fluorescence microscope. The more ROS produced within tissue, the stronger the fluorescence will be, and the value of fluorescence strength is defined as FS. The acclimation ability (RFS) will be expressed as FS in non-acclimated hydra minus FS in acclimated hydra (Vega, Palta, and Bamberg 2000). Similar procedure can be conducted on algae (Wietheger 2012). High ROS presented in the tissue will suggest low tolerance in the target.

SOD measurement: Instead of measuring ROS in the host/algae, we can measure SOD activity. Higher SOD activity means better acclimation ability in the target. ROS can be measured as Chade et al. (2014) did by using SOD colorimetric assay kit. The reaction of the dye with superoxide anion can be inhibited by SOD, which can then tell the activity of SOD in the sample by a colorimetric method. The sample procedure is similar to ROS measurement. The data will be analyzed by ANOVA.

Acclimation limit determination: After the acclimation at 30°C for hydra (both symbiotic and aposymbiotic) and algae, the temperature will be raised at 1°C every 2 days. Twenty hydras will be tracked individually and the temperatures at which they die or not being able to take in food and bud will be recorded. For algae, 20 replicates with starting OD 0.5 will be cultured. OD will be measured every two days, and the final temperature at which OD ceases to increase/decreases will be recorded. The upper thermal tolerance of symbiotic hydra, aposymbiotic hydra and algae will be compared by ANOVA.

Deacclimation rate: Hydra and algae acclimated at 30°C will be transferred to 18°C. After 24 hours and 48 hours, deacclimated hydra and algae will be heat-shocked at 35°C for 30 minutes. Acclimated hydra and algae will be used as a control group. ROS/SOD will be measured, and deacclimation rate will be calculated as FS of deacclimation/FS of acclimation.

H2. Adaptation of endosymbionts is constrained by the host

The endosymbiont population size is usually regulated by the host to some extent, or the host could be killed due to over proliferation of the endosymbiont (Bossert and Dunn 1986). Since one host usually keeps a constant number of endosymbionts inside, endosymbiont populations in a free-living phase could be larger than those kept within hosts. Given the same chance of generating beneficial mutations, the larger the population is, the more likely that population will gain a beneficial mutation. This assumes more variation and beneficial mutations are expected in a free-living phase endosymbiont population compared with that in the host. Moreover, studies on nucleotide substitution in endosymbionts suggests the rate may be decreased by hosts as they can control DNA replication and cell division of endosymbionts, and help to repair mutations in the endosymbionts by transporting hosts' protein (Bossert and Dunn 1986;Silva and Santos-Garcia 2015). Exposed to the environment directly without protection from the host, the free-living state endosymbiont experience stronger selection, which means more advantages in beneficial mutations, and thus higher probability in their fixation (Patwa and Wahl 2008). The strength of selection on endosymbionts within hosts could also be constrained by tolerance of the hosts, if the host tolerance limit is lower than that of the endosymbiont. On the one hand, once the selective pressure is above host's tolerance, it will kill the host as well as the endosymbiont in it, so the endosymbiont will never has a chance to experience a higher selective pressure, otherwise it could withstand without the host. On the other hand, the small population of endosymbionts within the host also provides deleterious mutations higher possibility to fix as a result of drift. Combined together, beneficial mutations are more likely to arises and be fixed in a larger population, here is the free-living phase endosymbionts. On the contrary, deleterious mutations are more likely to be fixed in a smaller population, here is endosymbionts residing in the host. Based on the reasoning, endosymbionts with strict vertical transmission may adapt more slowly than those with horizontal transmission.

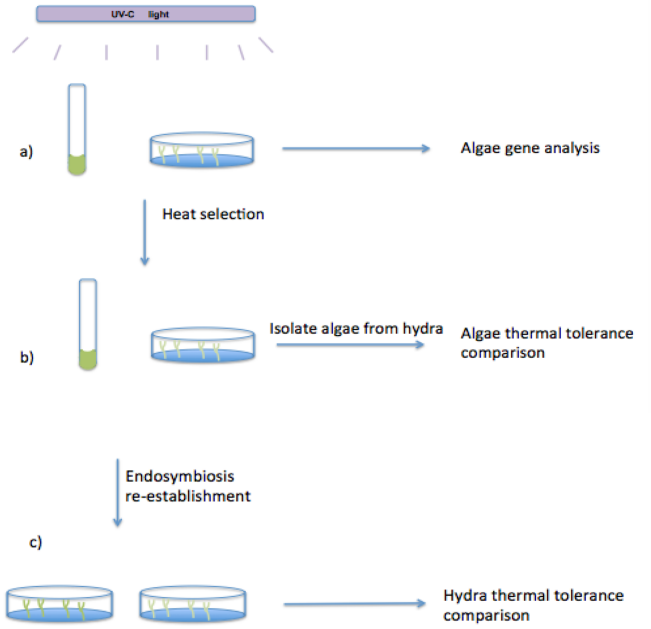


Fig 3. Comparing free-living state and endosymbiotic state algae adaptation. a) Mutate algae with/without hydra. Compare mutation rate. b) Select algae with/without hydra under elevated temperature determined by their upper thermal limits. After selection, isolate the algae and culture in liquid solution. Compare quantum yield of algae selected with/without hydra. c) Inject both algae into hydra respectively, and compare hydra thermal tolerance.

Manipulation: 1) Mutate algae in both free-living phase and endosymbiotic phase with UV-C. For free-living state algae, 3 replicates of 5 ml algae in log growth phase will be exposed to a 40-watt UV-C light at a distance of 40cm for 30 minutes. For endosymbiotic phase algae, 3 replicates of 40 hydras will be treated with similar UV-C exposure as to free-living state algae; however, the exposure length will be adjusted according to hydra endurance. 2) Gene sequences between the mutated algae in two states can be compared to see whether there are more mutations in one than in the other. 3) The mutated free-living algae and hydra will be cultured at their upper thermal limit, which gained from previous study, for two month. Algae will be cultured in test tubes, and half volume of algae culture will be replaced by fresh medium every week so algae will have enough nutrients. Hydra will be fed with brine shrimp every two days. The hydra population in each replicate will be maintained at around 80, and the excess will be discarded. A control group of non-mutated algae will be maintained at 18°C. 3) After two months, isolate the algae from both treated hydra according to protocol used by R. Hill (2014), and culture in liquid medium for 2 weeks. Heat shock algae isolated form hydra, heat-treated algae, control algae, at 35°C for 2 hour, then use microscopy-PAM to measure quantum yield Fv/Fm (Ralph, Larkum, and Kühl 2005). 4) 4) If there are differences in algae thermal tolerance, further analysis on thermal tolerance related genes, such as genes coding heat shock proteins will be conducted. 5) Infect aposymbiotic hydra with algae selected in free-living state and algae selected in endosymbiotic state respectively by microinjection. After they form stable endosymbiosis, compare their thermal resistance by heat shock and survival analysis in a similar way in previous experiment (Fig 3).

INTELLECTUAL MERITS

As more endosymbionts are discovered to be crucial to host life history and contribute to phenotypic variation, it is necessary to investigate endosymbiosis establishment and breakdown. Based on the hologenome framework, my work aims to disentangle roles of endosymbionts and hosts in holobiont adaptation by manipulating both the endosymbionts and the hosts. It will give answers to whether endosymbionts can assist host adaptation to stress and how they may impact the adaptation of the holobionts. With these results, we can also explore potential conservation strategies on endosymbiotic species such as coral reefs.