Symbiont removed and anemone reinfected with one of two strains of Symbiodinium from (AnemoneOA project)

Website: https://www.bco-dmo.org/dataset/649728
Data Type: experimental
Version: 1
Version Date: 2016-06-17

Project
» Ocean Acidification: Understanding the Impact of CO2 and Temperature on the Physiological, Genetic, and Epigenetic Response of a Model Sea Anemone System with Different Symbionts (AnemoneOA)

Contributors	Affiliation	Role
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Abstract
Symbiont removed and anemone reinfected with one of two strains of Symbiodinium from (AnemoneOA project)

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Dataset Description

Results of laboratory experiments examining the respiratory capacity of the sea anemone *Exaiptasia pallid*a, infected with either homologous ITS2-type A4 Symbiodinium or a heterologous isolate of Symbiodinium minutum (ITS2-type B1). This dataset details the differences between host-symbiont combinations. Respiratory demand was assessed by both whole organismal oxygen consumption as well as in vitro mitochondrial activity via the enzyme citrate synthase.

Methods & Sampling

Full details of the experimental design are provided in Hawkins *et al.,* 2016

Data Processing Description

Anemone Husbandry, Symbiodinium Removal, and Re-infection:

Anemones were maintained in 15-L tanks supplied with constantly running natural seawater (1-micron-filtered and UV-sterilized, 26°C, 1 L min^{-1} flow rate). Irradiance (100 µmol photons m^{-2} s^{-1}) was provided on a 12 h:12h light:dark cycle (lights on at 06:00) provided by cool white LEDs (Cree XP-G2; LED Supply, Randolph, VT, USA). Aposymbiotic *E. pallida* were obtained by subjecting a subset of anemones to a menthol treatment (Wang et al., 2012; Matthews et al., 2015), with dark-incubation. Aposymbiosis was confirmed by the complete absence of *Symbiodinium* using a fluorescence microscope [EVOS system, ThermoFisher (Life Technologies),

Waltham, MA, USA; excitation: 628 ± 20 nm, emission: 692 ± 20 nm]. Aposymbiotic animals were maintained in a 30-L aquarium in the dark, and checked monthly for the presence of algal symbionts. Novel *E. pallida*-*Symbiodinium* associations were established by exposing aposymbiotic anemones to one of two different *Symbiodinium* species (~1000 cells mL^{-1} in 500 mL seawater) according to the methods of Hoadley et al. (2015). One group ($n > 30$ animals) was incubated with a homologous, monoclonal *Symbiodinium* culture established from *Symbiodinium* originally isolated from the same *E. pallida* population and maintained in semi-continuous growth in f/2-Si culture media (Guillard, 1973) for period of at least 1 year. Another set of anemones ($n > 30$) was exposed to a heterologous culture of *Symbiodinium minutum* (ID: Pk702) originally isolated from the octocoral *Plexaura kuna*. Algae were noted as ITS2-types A4 and B1 for the homologous symbiont and *S. minutum* isolates, respectively. Newly symbiotic animals were maintained in separate flow-through tanks for at least 3 months before any physiological analysis was undertaken. A third group of aposymbiotic anemones ($n = 24$) were placed under lights for 8 weeks without being exposed to any *Symbiodinium* cells. These anemones remained aposymbiotic (as confirmed by fluorescence microscopy). To confirm symbiont genetic identity in re-infected animals, *Symbiodinium* DNA was extracted from 5 to 10 anemones per group, and the ITS2 region was amplified as described by LaJeunesse (2002). In all instances, anemones contained the appropriate ITS2-type.

Measurements of Respiration and Photosynthesis as In-vivo Oxygen Flux:

Respiration and photosynthesis of individual anemones [oral disk diameter >4mm for natural *E. pallida* ($n = 53$), 4–6mm for re-infected animals ($n = 19$ per *Symbiodinium* species)] was measured in sealed glass scintillation vials fitted with an internal stir bar and an oxygen sensitive optode (Fibox 4, PreSens Gmbh, Regensburg, Germany). Vials were immersed in a constant 26°C water bath and maintained in darkness for 15min. Illumination was provided for 20 min at an irradiance of 200 µmol photons m^{-2} s^{-1} after which, the LEDs were turned off for a final 30-min period to allow measurement of steady-state dark respiration. Background O_2 flux was determined using vials containing 1-µm-filtered, UV-sterilized seawater, and was found to be negligible. After respirometry assays, each anemone was snap-frozen in liquid nitrogen, and stored at −80°C. Rates of holobiont dark respiration were calculated as moles O_2 consumed $hour^{-1}$. Dark respiration rates were then subtracted from net photosynthetic rates (moles O_2 produced $hour^{-1}$ during the light-phase) to generate values for gross photosynthesis. Respiration- and gross photosynthesis rates were normalized to soluble animal protein and symbiont cell number, respectively.

Anemone Processing and Symbiodinium Density Analysis:

Anemones were thawed in their 2-mL screw cap vials and 0.6–1mL ice-cold lysis buffer (25mM Tris, pH 7.8, 1mM EDTA, 10% glycerol [v/v]) was added to each vial. Anemones were then homogenized in a chilled bead-beater (FastPrep -24, MP Bio, Santa Ana, CA, USA) for 60s at a speed of 6m s^{-1} with a 5-mm-diameter stainless steel bead. The homogenate was inspected visually (100× magnification) to confirm anemone tissue disruption and *Symbiodinium* cell integrity. A 100-µL sample was removed, fixed with 5µL glutaraldehyde (8% [w/v] stock solution in water), and stored at 4°C in the dark for later quantification of *Symbiodinium* cell densities. The remaining homogenate was centrifuged at 3000 × g for 30 s. Two hundred microliters of the supernatant were removed for animal DNA extraction (see below) and the pellet was re-suspended. The sample was then centrifuged for 5min at 700 × g to separate the *Symbiodinium* cells from the remaining anemone material. The *Symbiodinium* pellet was immediately snap-frozen in liquid nitrogen and transferred to a −80°C freezer. The supernatant ("anemone fraction") was centrifuged at high speed (16,100 × g, 20min) to remove particulates, and aliquots of the clear supernatant were snap- frozen and stored at −80°C.

In order to remove residual animal protein, *Symbiodinium* pellets were thawed, re-suspended in 1 mL ice-cold wash buffer (as lysis buffer above, but with the addition of 0.01% [v/v] Triton X-100), and centrifuged for 5 min at 700 × g. The supernatant was discarded and the pellet re-suspended in fresh ice-cold wash buffer. This procedure was repeated four times, after which the supernatant was clear and the pellet dark brown, with little evidence of contaminating anemone material. *Symbiodinium* cells were finally re-suspended in 300µL ice-cold lysis buffer in a clean 1.5-mL tube containing a 200-µL-volume of 0.5- mm-diameter acid-washed glass beads. Cells were lysed in a chilled bead-beater (see above) for 3 min at a speed of 6.5 m s^{-1}. The lysate was inspected visually (100× magnification), before centrifugation to remove particulates (16,100 × g, 20 min). Aliquots of the supernatant were then snap-frozen in liquid nitrogen and stored at −80°C.

Total soluble anemone and *Symbiodinium* protein were determined using a linearized Bradford assay (Ernst and Zor, 2010). To test the effectiveness of the algal washing steps described above, a standard curve was constructed by spiking 12 *Symbiodinium* pellets from similar-sized anemones (in triplicate) with 24–1200 µg anemone protein "contamination" originating from crude homogenates that had been gently centrifuged (500

× g for 5 min) to remove algal cells. Algal pellets were then washed and a 50-µL sample was removed and fixed for cell counts. The remaining cells were lysed as described above. *Symbiodinium* protein content was then measured for each pellet, normalized to cell number (see below), and plotted against the respective amount of anemone material added.

Symbiodinium densities were quantified using an Improved Neubauer hemocytometer and a fluorescence microscope to visualize cells' chlorophyll *a* fluorescence (see above). Field of view was determined using the EVOS operating software (4× objective), and cells were counted using the "Analyze Particles" tool in ImageJ (NIH, Bethesda, MD, USA). At least 6 independent images were analyzed for each sample, and cell numbers were normalized to anemone protein content.

Biochemical Analysis of Mitochondrial Function:

Anemone and *Symbiodinium* aerobic capacity was quantified as the activity of the TCA cycle rate-limiting enzyme citrate synthase (CS), measured according the methods of Srere (1969) modified for use with small marine invertebrates. All samples were analyzed within 1 month of freezing. Representative results of assay optimization and validation procedures are provided in ESM Figure S1. Briefly, 20µL of thawed anemone or *Symbiodinium* supernatant (diluted to yield 2–6µg of protein) was added in triplicate to wells in a 96-well plate (Greiner Bio- One, Monroe, NC, USA) alongside triplicate blanks (20 µL lysis buffer) and positive controls [20 µL citrate synthase (1 U mL^{-1} in lysis buffer; Sigma-Aldrich, St. Louis, MO, USA)]. One hundred and seventy microliters of assay buffer (111mM Tris, pH 7.8, 0.11% [v/v] Triton X-100) containing 294µM 5,5′-dithiobis- (2-nitrobenzoic acid) (DTNB; Sigma-Aldrich, see above) and 588 µM acetyl-coenzyme A (Sigma-Aldrich, see above) were then added to all wells. DTNB stock solutions (5 mM) were made fresh in 0.1 mM Tris buffer, pH 8.0. Acetyl-coenzyme A solutions were prepared at a concentration of 12.35 mM in distilled water, stored in aliquots at −80°C, and used within 6 months.

To control for non-CS-specific reaction products following the addition of assay buffer, baseline absorbance (λ = 412 nm) was recorded for 3min using a microplate reader (Fluostar Omega, BMG, Cary, NC, USA) maintained at a temperature of 26° C. The CS-catalyzed reaction was initiated by adding 10 µL oxaloacetate to each well (OA; Sigma-Aldrich, see above; 12 mM stock solution prepared fresh in distilled water and stored on ice), giving a final concentration of 600 µM OA. Sample absorbance was monitored at 412 nm for a further 3 min, and CS enzyme activity was derived using the following equation:

$$CS\ Specific\ Activity\ U\ mg^{-1} = {}^{\triangle 412}_{OA} - {}^{\triangle 412}_{blank} \times {}^{V}reaction \times {}^{D}/13.6 \times L \times V_{sample} \times P$$

Where 412 is the linear rate of change in 412-nm absorbance prior to and after the addition of OA, V is the volume (mL), D is the sample dilution factor, 13.6 ($mM^{-1}\ cm^{-1}$) is the 412-nm extinction coefficient for the reaction product, L is the optical path-length (cm) and P is the sample protein concentration (mg mL^{-1}). Total *Symbiodinium* and animal CS activities were calculated as the product of the respective specific activity and total protein contents.

Quantification of Anemone Mitochondrial Copy Number:

Anemone genomic DNA was extracted using two independent methods. For some animals (n = 33), DNA was extracted using a Wizard Genomic DNA Extraction kit (Promega Corporation, Madison, WI, USA). The DNA pellet was washed with ethanol, air-dried under sterile conditions at 30° C, and finally dissolved in 50 µL nuclease- free water (BioExpress, Kaysville, UT, USA). DNA from a second group (n = 20) was extracted and purified using a QiaAmp DNA Mini kit (Qiagen, Germantown, MD, USA). DNA concentration was measured using a Quant-iT PicoGreen assay (ThermoFisher [Invitrogen], Waltham, MA, USA) and purity was determined spectrophotometrically as the 260/230 nm and 260/280 nm absorbance ratios (NanoDrop , ThermoFisher, Waltham, MA, USA). The 260/280nm ratio was consistently >1.8, but the 260/230 nm ratio was variable, particularly in samples purified using the Wizard Promega kit (range = 1.4–2.2), however, this had a negligible effect on the efficiency of subsequent QPCR reactions.

Primer Design and Standard Curve Construction:

Oligonucleotide primers are provided in the referenced publication (Hawkins et al. 2016). Primer validation was undertaken using end-point PCR, with each reaction containing 20 ng anemone DNA in a 20-µL mix of 1× Standard Mg-free PCR buffer, 0.25 U Taq DNA polymerase, 0.25 µM (*CO1* and *ATP6*) or 0.5 µM primers (*EF-*

1-α), 2.5 mM MgCl2, and 0.25 mM dNTPs. Cycling conditions were 94°C for 2 min, followed by thirty cycles of 94°C for 15 s, 60°C for 30 s, and 72° C for 30 s, with a final elongation at 72° C for 10 min. Agarose gel electrophoresis confirmed a single PCR product for each primer set and, after product purification, amplicons were sequenced in both directions using the respective PCR primers. Electropherograms were inspected visually to confirm the reliability of base-calling and sequences were compared to NCBI Genbank or *Aiptasia* genome databases. In all cases the sequences aligned most strongly with those used for primer design. Longer sequences of each of the three target genes was amplified and subsequently cloned in order to create material for QPCR standard curves.

QPCR Analysis:

Duplicate 2-µL aliquots of extracted DNA (5–10 ng μL^{-1}) were added to a 18-µL reaction mix (SensiMix™ SYBR Hi-ROX; Bioline, Taunton, MA, USA) such that the final mix contained 0.25 µM *CO1* or *ATP6* primers, or 0.5 µM *EF-1*-α primer. Gene- fragments were amplified using an AB-7500 real-time QPCR system [ThermoFisher (Applied Biosystems), Waltham, MA, USA], with the following cycling conditions: 94° C for 10 min, followed by 40 cycles of 94°C for 15 s, 60°C for 1 min, and 72°C for 15 s. A melt-curve analysis (60–94°C in 0.3°C increments, 30s per step) was carried out in order to detect non-specific amplification products. A single PCR product was detected in all cases, with a melting temperature within 1°C of the theoretical melting temperature of the sequenced amplicon as determined by a web-based tool (OligoCalc; Kibbe, 2007). Baseline values were determined automatically and threshold value was set manually at 0.04 (maintained across all samples and standards). Amplification efficiencies were 93–96% in all instances. The number of *CO1, ATP6,* and *EF-1*-α sequences per 20-µL reaction was determined by comparing mean C_t values for each sample to the respective log-dilution standard curve, and *CO1*/*EF-1*-α and *ATP6*/*EF-1*-α ratios were then calculated.

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Data Files

File
reinfections.csv (Comma Separated Values (.csv), 4.40 KB) MD5:427eb5e11305cac84a1359bb34116428
Primary data file for dataset ID 649728

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Related Publications

Hawkins, T.D., Hagemeyer, J.C.G., Hoadley, K.D., Marsh, A.G., Warner, M.E. (2016). Partitioning of Respiration in an Animal-Algal Symbiosis: Implications for Different Aerobic Capacity Between Symbiodinium spp. Frontiers in Physiology 7, 128. doi: 10.3389/fphys.2016.00128
General

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Parameters

Parameter	Description	Units
sample	Sample ID number	unitless
symbiont_id	Symbiont identity; i.e. Homologous ITS2-type A4 Symbiodinium or Symbiodinium minutum ITS2-type B1	text
anemone_resp_rate	Host protein-normalised holobiont respiration rate	micromoles of oxygen per hour per milligram protein
host_CS_act	Host (anemone) citrate synthase specific activity	Units per milligram protein
symbiont_CS_act	Symbiont (Symbiodinium) citrate synthase specific activity	Units per milligram protein
photo_output_symb	Net photosynthesis per symbiont cell	picomoles oxygen per hour per cell
symbio_density	Host protein-normalised symbiont density	million cells per mg protein
symbio_biomass_fraction	Symbiont biomass fraction (calculated as total symbiont protein [mg]/total host+symbiont protein [mg])	milligrams per milligrams(dimensionless)
symbio_resp_fraction	Symbiont respiration fraction (calculated as total symbiont citrate synthase activity [U]/total host+symbiont citrate synthase activity [U])	dimensionless
resp_biom_quotient	Symbiont respiration fraction/Symbiont biomass fraction	dimensionless

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Instruments

Dataset-specific Instrument Name	Fluorescence Microscope
Generic Instrument Name	Fluorescence Microscope
Dataset-specific Description	[EVOS system, ThermoFisher (Life Technologies), Waltham, MA, USA; excitation: 628 ± 20 nm, emission: 692 ± 20 nm]. Field of view was determined using the EVOS operating software (4× objective), and cells were counted using the “Analyze Particles” tool in ImageJ (NIH, Bethesda, MD, USA).
Generic Instrument Description	Instruments that generate enlarged images of samples using the phenomena of fluorescence and phosphorescence instead of, or in addition to, reflection and absorption of visible light. Includes conventional and inverted instruments.

Dataset-specific Instrument Name	oxygen sensitive optode
Generic Instrument Name	Optode
Dataset-specific Description	an oxygen sensitive optode (Fibox 4, PreSens Gmbh, Regensburg, Germany).
Generic Instrument Description	An optode or optrode is an optical sensor device that optically measures a specific substance usually with the aid of a chemical transducer.

Dataset-specific Instrument Name	ThermoFisher QPCR system
Generic Instrument Name	Thermal Cycler
Dataset-specific Description	AB-7500 real-time QPCR system [ThermoFisher (Applied Biosystems), Waltham, MA, USA], with the following cycling conditions: 94◦ C for 10 min, followed by 40 cycles of 94◦C for 15 s, 60◦C for 1 min, and 72◦C for 15 s.
Generic Instrument Description	A thermal cycler or "thermocycler" is a general term for a type of laboratory apparatus, commonly used for performing polymerase chain reaction (PCR), that is capable of repeatedly altering and maintaining specific temperatures for defined periods of time. The device has a thermal block with holes where tubes with the PCR reaction mixtures can be inserted. The cycler then raises and lowers the temperature of the block in discrete, pre-programmed steps. They can also be used to facilitate other temperature-sensitive reactions, including restriction enzyme digestion or rapid diagnostics. (adapted from http://serc.carleton.edu/microbelife/research_methods/genomics/pcr.html)

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Project Information

Ocean Acidification: Understanding the Impact of CO2 and Temperature on the Physiological, Genetic, and Epigenetic Response of a Model Sea Anemone System with Different Symbionts (AnemoneOA)

Coverage: Lewes, Delaware

The projected rise in carbon dioxide (CO2) in the atmosphere is considered a primary threat to marine systems throughout the world due to both ocean acidification and rising ocean temperatures. Coral reefs are very sensitive to these projected changes in the earth's climate, with continued losses in growth as well as disruption (also known as bleaching) in the symbiotic relationship between the algae (Symbiodinium) living within a diversity of host animals, including stony corals, soft corals and sea anemones. While much information has been gleaned as to how acidification may affect stony corals, considerably less is known about the interactive effects of acidification and temperature to other symbiotic anthozoans.

To this end, this proposal will investigate the long-term impacts of elevated CO2 and temperature on the model sea anemone, *Aiptasia pallida*, while harboring four different genotypes of Symbiodinium. The primary goals of this project are (1) to determine the sensitivity and capacity for acclimation in molecular and physiological processes while exposed to elevated CO2 and temperature, and (2) to assess the degree to which acclimated adult animals may confer (or transfer) an imprinted physiological characteristic to the next generation of asexual offspring. A series of long-term experiments will be conducted with each animal/algal combination (holobiont) in order to collect initial (3 month) stress markers and genomic data and then follow animal response and asexual reproduction through several generations for one year. The possibility for enhanced resilience or acclimation will be measured by tracking the recovery of each holobiont, followed by repeated exposure to elevated temperature while held in high CO2. This project will tease apart fine scale mechanisms of

stress, acclimation, or amelioration that may vary as a function of algal genotype and host animal response, and the degree to which environmental imprinting may pre-acclimate propagules. Project results will provide information regarding how future acidification and warming will affect cnidarian-algal symbioses, and the fundamental profile of their flexibility in stress response processes across organismal, metabolic, genomic and epigenetic scales.

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Funding

Funding Source	Award
NSF Emerging Frontiers Division (NSF EF)	EF-1316055

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