Host-symbiont respiration related to symbiont density; anemones from Key Largo from (AnemoneOA project)

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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)

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Abstract
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Table of Contents

- Dataset Description
  - Methods & Sampling
  - Data Processing Description
- Data Files
- Related Publications
- Parameters
- Instruments
- Project Information
- Funding

Dataset Description

Results of laboratory experiments examining the respiratory capacity in a natural population of the sea anemone *Exaiptasia pallida*. This dataset details the relationships between host and symbiont respiration per symbiont density. 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

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 O2 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 O2 consumed hour−1. Dark respiration rates were then subtracted from net photosynthetic rates (moles O2 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:

$$CS\ Specific\ Activity\ U\ mg{-}1$$

$$= \triangle 412OA - \triangle 412blank \times Vreaction \times D / 13.6 \times L \times Vsample \times P$$

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 (SensiMixTM 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 Ct 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.

[table of contents | back to top]

Data Files

File
naturalKLpop.csv (Comma Separated Values (.csv), 6.46 KB) MD5:3e6a2e5b8acbf7c8174ee19db745afba Primary data file for dataset ID 649708

[table of contents | back to top]

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

[table of contents | back to top]

Parameters

Parameter	Description	Units
sample	sample ID number	unitless
holobiont_resp_total	total holobiont (anemone)respiration	micromoles of oxygen per hour
prot_normal_resp	Host protein-normalised holobiont respiration	micromoles of oxygen per hour per milligram protein
host_CS_activity_product_total	Total host citrate synthase activity; the product of host citrate synthase activity and total host protein	Units per milligram protein X milligrams
host_CS_activity	Host citrate synthase specific activity	units per milligram protein
total_host_protein	Total host protein	milligrams
symb_CS_activity	Symbiont citrate synthase specific activity	Units per milligram protein
total_anemone_CS_activity	Total holobiont citrate synthase activity	Units
host_DNA_total	Total host DNA content	micrograms
host_symb_dens_normal	Host protein-normalised symbiont density	million cells per mg protein
symb_biomass_fraction	Symbiont biomass fraction (calculated as total symbiont protein [mg]/total host+symbiont protein [mg])	dimensionless
symb_respir_fraction	Symbiont respiration fraction (calculated as total symbiont citrate synthase activity [U]/total host+symbiont citrate synthase activity [U])	dimensionless
genome_ratio_CO1	Mitochondrial:nuclear genome ratio calculated using CO1 (QiaAmp DNA Mini Kit)	dimensionless
genome_ratio_AT96	Mitochondrial:nuclear genome ratio calculated using ATP6 (QiaAmp DNA Mini Kit)	dimensionless
genome_ratio_CO1_Pro	Mitochondrial:nuclear genome ratio calculated using CO1 (Promega Wizard Kit)	dimensionless

[table of contents | back to top]

Instruments

Dataset-specific Instrument Name	fluorescence microscope
Generic Instrument Name	Fluorescence Microscope
Dataset-specific Description	Used to visualize cells' chlorophyll a fluorescence. [EVOS system, ThermoFisher (Life Technologies), Waltham, MA, USA; excitation: 628 ± 20 nm, emission: 692 ± 20 nm].
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	Improved Neubauer hemocytometer
Generic Instrument Name	Hemocytometer
Dataset-specific Description	Used along with fluorescence microscope to quantify microbial densities.
Generic Instrument Description	A hemocytometer is a small glass chamber, resembling a thick microscope slide, used for determining the number of cells per unit volume of a suspension. Originally used for performing blood cell counts, a hemocytometer can be used to count a variety of cell types in the laboratory. Also spelled as "haemocytometer". Description from: http://hlsweb.dmu.ac.uk/ahs/elearning/RITA/Haem1/Haem1.html.

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	microplate reader
Generic Instrument Name	plate reader
Dataset-specific Description	Measured baseline absorbance at 412nm. (Fluostar Omega, BMG, Cary, NC, USA)
Generic Instrument Description	Plate readers (also known as microplate readers) are laboratory instruments designed to detect biological, chemical or physical events of samples in microtiter plates. They are widely used in research, drug discovery, bioassay validation, quality control and manufacturing processes in the pharmaceutical and biotechnological industry and academic organizations. Sample reactions can be assayed in 6-1536 well format microtiter plates. The most common microplate format used in academic research laboratories or clinical diagnostic laboratories is 96-well (8 by 12 matrix) with a typical reaction volume between 100 and 200 uL per well. Higher density microplates (384- or 1536-well microplates) are typically used for screening applications, when throughput (number of samples per day processed) and assay cost per sample become critical parameters, with a typical assay volume between 5 and 50 µL per well. Common detection modes for microplate assays are absorbance, fluorescence intensity, luminescence, time-resolved fluorescence, and fluorescence polarization. From: http://en.wikipedia.org/wiki/Plate_reader, 2014-09-0-23.

Dataset-specific Instrument Name	NanoDrop spectrophotometer
Generic Instrument Name	Spectrophotometer
Dataset-specific Description	ThermoFisher, Waltham, MA, USA
Generic Instrument Description	An instrument used to measure the relative absorption of electromagnetic radiation of different wavelengths in the near infra-red, visible and ultraviolet wavebands by samples.

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)

[table of contents | back to top]

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.

[table of contents | back to top]

Funding

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

[table of contents | back to top]