

Enzymes feel the squeeze: biochemical adaptation to pressure in the deep sea

Jacob R. Winnikoff,
Telissa M. Wilson,
Erik V. Thuesen and
Steven H.D. Haddock

(Monterey Bay Aquarium
Research Institute, USA;
University of California, USA;
and The Evergreen State
College, USA)

To the human observer, the deep sea is as extreme an environment as Earth has to offer. Below about 200 metres, there is no light from the surface, the water can be frigid (-2 to 5°C), oxygen and food are scarce, and the pressure is staggering. Of course, to the countless species that inhabit the deep sea, these conditions are not so extreme, and in a statistical sense, they fall fairly close to average, since the deep comprises the planet's largest habitat by volume. Despite its expanse, we know little about how life persists in an environment so different from our own. Only in the last half-century has technology emerged that allows us to collect and study live deep-sea animals. Diversity, Evolution and EcoPhysiology of Ctenophores (DEEPC, deepc.org, a US NSF-supported research effort) is opening a window on biochemistry in the deep, and specifically on its relationship to high pressure. By determining structural constraints on enzyme function under pressure, we aim to inform models focusing on deep-sea animal colonization, and to find general patterns of protein adaptation with possible applications in protein engineering and biocatalysis.

A short history of pressure physiology

The first investigations on the effects of high pressure on enzymes were undertaken in the early 1940s. Scientists at New York University and Princeton measured the intensity of light emitted by bacterial bioluminescence over a range of hydrostatic pressures. Through a series of experiments, they were able to measure reaction rate changes of the enzyme luciferase and calculated volume changes of the enzyme. Over the following decades, high pressure studies continued on marine bacteria, but most high pressure investigations on the enzymes of animals have been carried out on fishes. The metabolic enzymes of deep-sea fishes generally show smaller effects of high pressure than those of their surface-dwelling relatives. High pressure affects both intrinsic characteristics, such as active site volume and substrate affinities, as well as extrinsic factors, such as the effects of pH and osmolytes. Studies of high pressure effects have only been carried out on the enzymes of a few species of bottom-dwelling invertebrates. By examining a unique group of gelatinous animals called ctenophores, or 'comb jellies' (see Box 1), DEEPC aims to understand evolution and diversification in the deep-sea habitat.

Deep questions

The crucial molecular-evolutionary question for the EcoPhysiology branch of DEEPC is How are proteins structurally adapted to high pressure? This leads to a wealth of sub-queries: Is the adaptive evolution mostly *parallel* or *convergent*? How many different solutions have evolved in

ctenophores to solve the same biophysical problem? Do they vary across protein families or across species? To take a biophysical lens to the initial question, namely, which intrinsic properties of proteins tend to evolve? Because we are biologically focused on ctenophores, we also want to resolve their adaptations to high pressure at an organismal level, requiring us to ask: Which components of ctenophore metabolic pathways are most affected by pressure?

One way to approach the evolutionary questions above is with phylogenetic theory and transcriptomics. To measure convergent evolution and identify metabolic 'weak links' heavily affected by pressure, we are using a phylogenetic nonlinear regression analysis, which detects depth-correlated variation in gene sequences and isolates it from the phylogenetic background. Once this analysis is complete, we will be able to score the most commonly substituted amino acids and structural regions proteome-wide. The regressions will also indicate which proteins contain the most depth-correlated substitutions. To answer our questions empirically, we are focusing on metabolic enzymes, such as pyruvate kinase (PK) and malate dehydrogenase (MDH) – components of the vital glycolytic and Krebs pathways. In the cases of PK and MDH, we can make use of prior findings that these enzymes have high-volume transition states and are thus kinetically inhibited by pressure. We also benefit from widely accepted assay chemistry. The latter is important, because precious little is straightforward about studying ctenophore biochemistry under pressure.

Specimens are collected to 20 metres depth by scuba diving, following 'blue water' protocols in which the divers swim in open water while tethered indirectly to a small

boat. Collections to 4000 metres are made using remotely operated submersible vehicles (ROVs) belonging to the Monterey Bay Aquarium Research Institute (Figure 1). The specimens are flash-frozen in liquid nitrogen at sea. When samples arrive at the lab, they enter the workflow pictured in Figure 2. This process involves cloning metabolic enzymes from complementary DNA (cDNA), expressing them in fully native form, with no amino acids artificially added to either terminus, and then assaying their activity across a 400-atmosphere pressure range. Subsequently, this analysis is repeated with point-mutated enzymes, to assess the effects of individual amino acids on pressure tolerance. In addition to assaying enzyme activity

under substrate saturation, we are also investigating the effect of pressure on the apparent K_m (affinity constant). The cloning and expression steps of our workflow mark our departure from traditional ecophysiology studies, most of which assay tissue homogenates.

From molecule to ecosystem

Preliminary results of phylogenetic nonlinear regression have been consistent with a hypothesis from previous work that larger amino acid side chains decrease the overall compressibility of deep-adapted proteins by filling their interior spaces. A typical example is the substitution of

Meet the ctenophore

Ctenophores are a small (~300 species) group of gelatinous marine organisms, which diverged from the rest of known animals near the very beginnings of multicellular life. Their bodies superficially resemble those of well-known jellyfish (cnidarians), but they are evolutionarily far removed. Ctenophores have a body plan fundamentally different from a cnidarian, they are propelled by eight paddle-like rows of fused cilia rather than a pulsing bell (hence the common term 'comb jelly'), and many use sticky cells on their tentacles to capture prey, in contrast to cnidarian stinging cells.

Ctenophores are uniquely suited to a study of protein adaptation to high pressure because they occupy niches throughout the ocean, from the surface to >7 kilometre depth (where ambient pressure is 700 atmospheres), and from the poles to the equator. Some species have remarkably broad tolerances as well, spanning thousands of metres depth, where others are constrained to the uppermost tens of metres. Phylogenetics suggest that ctenophore lineages experienced several recent and independent range shifts, producing closely related species that live under contrasting physical conditions. These species enable comparative analyses where we can consider pressure and temperature as independent factors affecting protein evolution, while controlling for the background of phylogenetic relatedness.



Figure 1: Ctenophore collection using the remotely operated vehicle (ROV) *Doc Ricketts* and blue-water diving techniques. Scuba divers can collect between the surface and 20 metres depth, while *Doc Ricketts* captures specimens and video down to 4000 metres. Ctenophore size is exaggerated in this schematic. Illustration by Kelly Lance, MBARI.

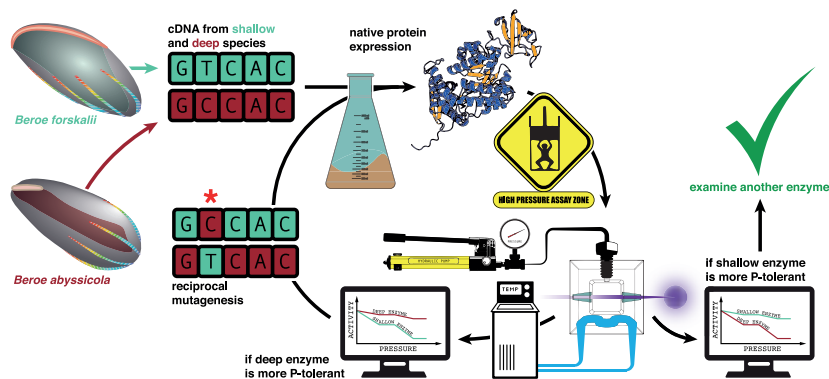


Figure 2: Workflow for experimentally probing pressure adaptation of enzymes. Wild-type enzymes are expressed from cloned cDNA of deep- and shallow-living species and assayed under pressure. To determine the additive effects of particular amino acids on pressure tolerance, pairs of enzymes undergo cycles of reciprocal site-directed mutagenesis until their respective pressure tolerances switch, i.e. until the deep-adapted enzyme is less pressure tolerant than its shallow counterpart.

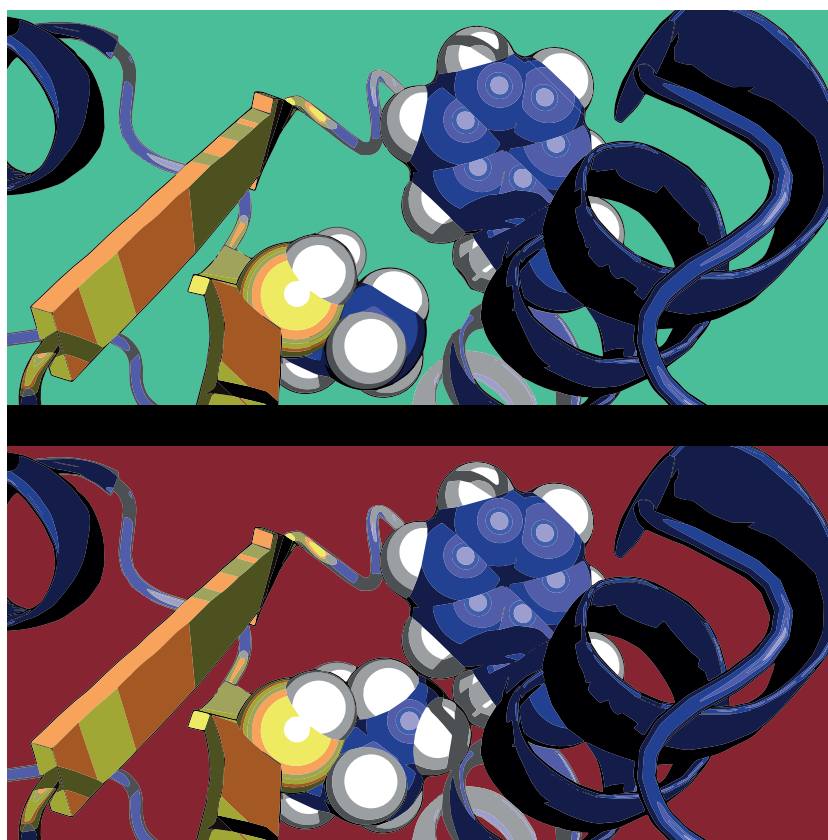


Figure 3: Example of the void-filling hypothesis for pressure adaptation. Predicted structures for PK of *Beroe forskalii* (top) and its deep-living sister species *Beroe abyssicola* (bottom) are shown at a site found to be significantly depth-correlated by phylogenetic nonlinear regression. At this site in the enzyme's core, isoleucine is substituted for valine in the deep species, adding a methyl group that occupies an otherwise empty space and inhibits compression. This is one of four similar sites detected in this enzyme: two with I → V substitutions and two with D → E.

Why clone?

Most deep-living ctenophores are fragile, rare and over 95% water by mass. Given the time and resources needed to collect these animals, the direct analysis of tissue homogenates would make replication difficult and collection costs exorbitant. Cloning also provides sequences of our genes of interest in every individual cloned, so we can examine variants of metabolic enzymes within an individual or between members of the same species collected at different depths. Molecular cloning offers another major advantage for our study, in the form of site-directed mutagenesis. Residue by residue, we can artificially 'adapt' proteins from shallow species toward exhibiting deep pressure-tolerant phenotypes, and *vice versa*. This moves a step beyond most studies in this field, which correlate protein sequence to function. We can actively manipulate sequence to establish causal relationships describing which protein sites most affect pressure tolerance.

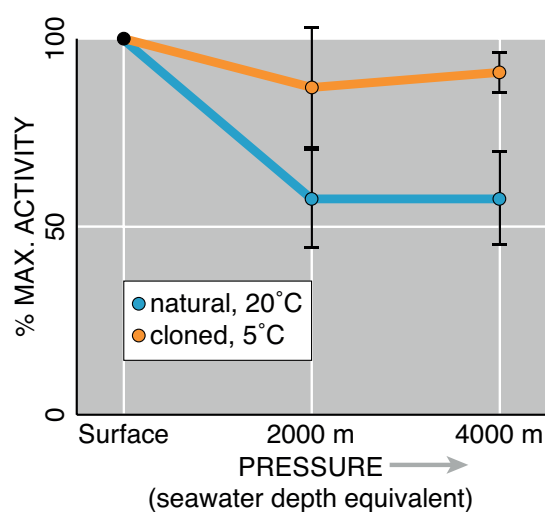


Figure 4: Activity of natural and native-cloned pyruvate kinase (PK) from *Beroe forskalii*, measured in duplicate assays at 20°C and 5°C, respectively. Results are displayed as percentage of activity measured at initial 1 atmosphere (sea level) pressure.

valine for isoleucine in the structure of PK from surface-dwelling ctenophore *Beroe forskalii* (Figure 3). Initial assays of the same enzyme reflected a decrease in activity when both native-cloned and specimen-derived samples were pressurized to 200 and 400 atmospheres, 2000 and 4000 metres depth, respectively. These trends were consistent with PK pressure studies conducted on deep- and shallow-living fishes.

Through such biochemically focused research questions, we aim to learn about ctenophore ecophysiology at all scales, from individual molecules

The tool box

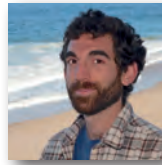
In the process of investigating pressure physiology in ctenophores, DEEPC will develop some tools of interest to the broader comparative biochemistry community. The first is a cold-shock-induced native protein expression system based on the *Escherichia coli* cold-shock promoter and a specialized high-specificity variant of the Tobacco Etch Virus (TEV) protease. This plasmid facilitates highly stable protein expression in bacteria or yeast at 15°C, easy purification using a His-tag, and then cleavage of that tag precisely at the protein's native N-terminus. The second tool is the phylogenetic nonlinear regression-based algorithm mentioned above, packaged as a transcriptome-wide association utility. Any researcher with transcriptomes and quantitative trait data for several related organisms will be able to use this tool to identify amino acid sites associated with the trait, in an analysis controlled for phylogenetic background signal.

to the vast marine ecosystems of which ctenophores are a part. At a molecular level, we want to find out what aspects of protein structure confer pressure tolerance, and how ubiquitous these elements are. We predict that they will be mostly convergent among the ctenophores, and are prepared for a surprise when we ultimately compare them to pressure adaptations in other deep-sea animals. Ctenophores diverged from other animals so long ago, and protein evolution is so path-dependent, that it is possible they evolved largely different means of coping with pressure. On an ecosystem scale, we hope to determine how large a role pressure has in dictating invertebrate species' ranges in the open ocean, by comparing enzyme functional measurements with our ctenophore sighting records. Some species' natural depth ranges may come up hard against their physiological pressure limits, while others may be more constrained by different factors such as temperature, predators and prey.

Achieving both our biochemical and ecological aims should give insight into the evolutionary past and trajectory of ctenophores, since we will have an idea how much genetic change is needed to enable colonization upward or downward in the water column. More generally, DEEPC will illuminate the evolutionary fitness trade-offs that deep-sea invertebrates face. To offset the challenging extremes of the deep, such as low temperature and a sparse prey field, there are corresponding benefits, like environmental stability and nearly boundless mobility, which permits gene flow. Knowing how pressure stacks up against these benefits, and how it compares to other constraints, is a crucial step in understanding evolution in the largest, yet least known habitat on our planet.

Outside the realm of marine biology, our work has applications in chemical and biological engineering. As the reader likely recalls from introductory organic

chemistry, many synthetic reactions, notably organic reductions, are carried out at high pressure and are classically catalysed by precious metals. Biocatalysis by an enzyme, or better yet, biosynthesis by living microbes, is an attractive alternative due to lower reactor cost and greater selectivity, but not all enzymes perform well at the required pressure and temperature. By cataloging evolved solutions to this problem, we can gradually build an enzyme optimization model for the protein engineer's toolbox. ■



Jacob Winnikoff is a PhD student in Ecology and Evolutionary Biology at the University of California, Santa Cruz. He has been working on ctenophore physiology and evolution with Steve Haddock at the Monterey Bay Aquarium Research Institute (MBARI) since September 2016. Jacob is interested in a range of marine biochemical subjects, and has previously researched biochemical adaptation to temperature fluctuation in mussels, as well as bioactive secondary metabolites of cyanobacteria. Email: jwinnikoff@ucsc.edu.



Telissa Wilson is currently a master's student at the Evergreen State College in Olympia, WA. She has been working with Professor Erik Thuesen and has been focused on studies in ctenophore physiology since June 2014. Email: wiltel16@evergreen.edu.



Erik Thuesen is a Member of the Faculty at The Evergreen State College where he teaches Zoology, Marine Biology, Ecophysiology and the science of Symbiosis. He has worked previously at research institutions in Japan, California, Catalonia and Argentina. His investigations have focused on biochemical and physiological adaptations of organisms to life in different marine habitats and the study of marine biodiversity. Email: thuesene@evergreen.edu.



Steven Haddock studies marine diversity, molecular biology and bioluminescence at the Monterey Bay Aquarium Research Institute and UC Santa Cruz. He specializes in fragile gelatinous jellyfish-like creatures that are abundant in the water column of the deep sea and open ocean. In addition to conducting research expeditions around the world, he uses genetic methods to reveal the relationships between organisms and to understand the proteins that they use to make light. He also runs the Bioluminescence Web Page (biolum.eemb.ucsb.edu), the citizen-science project jellywatch.org, and has a textbook for teaching computing to scientists at practicalcomputing.org. Email: haddock@mbari.org.

Further reading

- Brown, D.E., Johnson, F.H. and Marsland, D.A. (1942) The pressure, temperature relations of bacterial luminescence. *J. Cell Phys.* **20**, 151–168
- Geringer, M.E., Drazen, J.C. and Yancey, P.H. (2017) Metabolic enzyme activities of abyssal and hadal fishes: pressure effects and a re-evaluation of depth-related changes. *Deep. Res. Part I* **125**, 135–146
- Somero, G.N. (2003) Protein adaptations to temperature and pressure: complementary roles of adaptive changes in amino acid sequence and internal milieu. *Comp. Biochem. Physiol. Part B Biochem. Mol. Biol.* **136**, 577–591
- Dahlhoff, E.A. and Somero, G.N. (1991) Pressure and temperature adaptation of cytosolic malate dehydrogenases of shallow and deep-living marine invertebrates: evidence for high body temperatures in hydrothermal vent animals. *J. Exp. Biol.* **159**, 473–487
- Morita, T. (2008) Comparative sequence analysis of myosin heavy chain proteins from congeneric shallow- and deep-living rattail fish (genus *Coryphaenoides*). *J. Exp. Biol.* **211**, 1362–1367