Assessing Functional Diversity Down the Water Column: The Effect of Hydrostatic Pressure on the Metabolic Enzymes of Ctenophores from Different Habitat Depths

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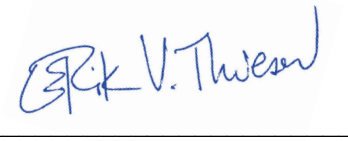

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ABSTRACT

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Deep-sea animals have evolved numerous biochemical strategies to thrive under high pressure. Hydrostatic pressure influences physiological performance as well as the evolution of deep-sea organisms. Our understanding of evolutionary changes in enzymes in the deep sea is incomplete and derived mostly from the metabolic enzymes of fishes. Though the function of enzymatic machinery often decreases with increasing pressure, this trend may be different in deep-adapted organisms. To better understand biochemical adaptations to high hydrostatic pressure in deep-sea animals, the phylum Ctenophora was chosen since phylogenetically distant species have independently evolved to inhabit the deep sea. Ctenophores from various habitat depths were examined to explore the enzymatic constraint of pressure. The metabolic enzymes Creatine kinase (CK), Malate dehydrogenase (MDH), and pyruvate kinase (PK) were assessed for pressure tolerances. The glycolytic enzyme pyruvate kinase (PK) has exhibited adaptive pressure resistance in deep-sea fishes and was targeted for comparison. Native enzymes from different ctenophore species were assayed at 1, 200, 400, and 600 bar. After being assayed at increasing pressures, enzymes were assayed again at atmospheric pressure (1 bar). Maximum rates of enzymatic reactions (Vmax) were recorded at each pressure increment and recovery to investigate the effects of hydrostatic pressure on metabolic functioning. When saturated with substrate, both CK and PK generally displayed enzymatic inhibition with incremental pressure. Decreased enzymatic activities were seen until the point of decompression (recovery), where enzymatic activities seemed to rapidly spike. This effect was more pronounced on PK than CK. Malate dehydrogenase showed stable or slightly increased activity with increased pressure and returned to initial activity after decompression. Extremely deep species living below 2000 m disrupted this relationship in a manner consistent with historic data collected from vertebrates.

Initial results support two intriguing hypotheses: (1) relationships between environmental conditions and enzymatic volume change parameters are consistent across the longest branches of the animal tree of life, and (2) pressure inactivation of an enzyme under saturating conditions is set by selective forces other than hydrostatic pressure of the habitat. Phylogenetically, these results indicate that adaptations to moderate depth (100 m) is not necessarily convergent at the scale of a single enzyme. The effects of pressure reported herein are novel for invertebrates, and they offer a good comparison to biochemical studies conducted on deep-sea fish. Further assessing functional diversity of ctenophore metabolism will indicate parallel or convergent protein adaptation in the deep sea. The importance of ecophysiology when seeking the criteria for choosing functional traits to understand processes within a community will be highlighted.

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CHAPTER 1: BACKGROUND

Introduction

The marine environment comprises the largest habitat by volume on the planet and is home to a large portion of Earth's gelatinous biomass (Lucas et al., 2014). The phylum Ctenophora is a small group of predatory gelatinous zooplankton that make up a significant portion of this biomass (Haddock, 2004). Ctenophores occupy an important ecological role throughout the oceans, from the surface to approximately 7000 meters, and from the poles to the equator (Harbison et al., 1978). The phylum is small, comprised of ~200 described species with more to be classified (Appeltans et al., 2012). Ctenophores are commonly referred to as 'comb jellies', due to the eight rows of fused cilia (combs) that propel their bodies through the water column (Dunn et al., 2015; Harbison et al., 1978; Mills, 1998-present). The phylum is mostly pelagic with the exception of one benthic order, Platyctenidae, whose members attach themselves to substrate such as rock, coral or sponges (Mills, 1998-present). Both benthic and pelagic ctenophores feature tentilla embraced by sticky colloblast cells rather than stinging nematocyst cells, characteristic of jellyfish (Cnidaria) (Leonardi et al., in press). Like other gelatinous zooplankton, ctenophore abundances fluctuate rapidly. Seasonal variability can cause large increases in ctenophore populations creating aggregates called 'blooms' (Mills, 1995, 2001). Ctenophore blooms can be particularly problematic because ctenophores are zooplanktivorous and ichthyoplanktivourous, feeding on a number of planktonic species and larval assemblages (Shiganova, 1998). Blooming events can cause extensive damage to ecosystem functioning, including economically important fisheries (Mills, 2001; Shiganova, 1998). For example, *Mnemiopsis leidyi* ,a ctenophore native to east coasts of North and South America, was accidentally introduced into the Black Sea in the 1980s and thus became a harmful invader into important spawning grounds of major Baltic fish stocks (Schaber et al., 2011; Shiganova, 1998).

Acceleration of the rate to which global change is occurring will be reflected in our oceans through increased acidity, temperature, salinity and hypoxia. Changes in ocean chemistry will have acute effects on marine invertebrates, such as ctenophores, and their trophic interactions (Mills, 1995, 2001; Thuesen et al., 2005b). Characterizing biodiversity in our oceans before

threats, such as anthropogenic climate change, bring negative irreversible damage is key in understanding how to combat the current climate crisis.

Until recently, most ctenophore research focused largely on species inhabiting shallow waters. Much of the information assembled on deep pelagic ctenophore species was procured within the last half century, when emerging technology allowed for collection and study of live, fragile deep specimens (S. Haddock et al., 2017; Mills, 1995; Robison, 2004). However, current ctenophore publications still focus largely on the surface-dwellers. This is likely due to the relatively robust and available nature of surface species and the recent attention they've received as blooms threaten food webs (Harbison et al., 1978; Schaber et al., 2011). The majority of the deep living ctenophores are undescribed, and little is known about their physiological functioning (Appeltans et al., 2012; S. H. D. Haddock et al., 2017).

This study will utilize physiological measurements to extrapolate characteristics of functional biodiversity found throughout the phylum Ctenophora. This research will address the large gap in knowledge of ctenophore ecophysiology, protein evolution, and functional biodiversity and will have applications across other marine groups.

Ctenophore taxonomy

Ctenophora is currently comprised of eight recognizable orders: Beroida, Cydippida, Lobata, Platyctenida, Cestida, Cambojiida, Cryptolobiferida, and Thalassocalycida. The ctenophore phylogeny has been constructed using morphometric and physiological signals of homology, however transcriptomic and genetic information is being incorporated to the phylogeny (Appeltans et al., 2012; Haddock, 2004; Mills, 1998-present). Ctenophora diverged from the rest of the known animals at the beginning of multicellular life, making the clade one of the oldest groups in Metazoa, which comprises all animals (Borchiellini et al., 2001; Wallberg et al., 2004; Whelan et al., 2017). Despite their gelatinous nature, ctenophores are unusually complex, containing distinct muscle and nerve cells (Dunn et al., 2015; Jékely et al., 2015). Ctenophore lineages have undergone recent independent range shifts, resulting in closely related species living under contrasting physical conditions, such as pressure and temperature (Dunn et al., 2015). Within their phylum, ctenophores have evolved numerous times on multiple branches to live in the deep. Both shallow and deep ctenophore species are represented within each family

(Haddock, 2007; Mills, 1998-present). Some species have remarkable tolerances in depth and temperature, while others are constrained to specific conditions (Harbison et al., 1978).

Since the establishment of the phylum, researchers have questioned the classification of ctenophore orders and have debated the position of Ctenophora within Metazoa. Ctenophore morphology and life stages superficially resemble well-known jellyfish, yet they are evolutionarily far removed (Borowiec et al., 2015; Dunn et al., 2015). Early phylogenies classified ctenophores and cnidarians as one group (Colenterata). This grouping was based on morphological attributes, the main being that both Ctenophora and Cnidaria only have 2 cell layers with jelly (mesoglea) in between the ectoderm and endoderm. The two groups differ greatly and have been recognized as such as technology advanced, yet the phylogenetic position of Ctenophora is still debated (Dunn et al., 2015; Haddock, 2004). Until recently, Porifera (sponges) was hypothesized as the earliest lineage within Metazoa, however, current gene analyses suggest Ctenophora be placed as the initial lineage in Metazoa (Borchiellini et al., 2001; Dunn et al., 2015; Simion et al., 2017). This new hypothesis challenges our understanding of early metazoan evolution because it implies that complex traits, present in ctenophores but absent in sponges, either evolved twice in Metazoa, or were independently, secondarily lost in the lineages leading to sponges and placozoans (Borowiec et al., 2015; Dunn et al., 2015). With support from the current genomic evidence, there has been increasing acceptance of the 'ctenophore-sister' hypothesis. Soundness of this hypothesis is subjected to systematic errors, biases and 'blind spots' in our conceptualization of evolutionary history (Wallberg et al., 2004; Whelan et al., 2017).

Ctenophore morphology and ecology

Approximately 200 ctenophore species are defined, and it is estimated that this only accounts for half of the extant species (Appeltans et al., 2012). The limited species number associated with Ctenophora lead to the clade's recognition as "quasi-cnidarians" or stunted bilaterians (Dunn et al., 2015; Haddock, 2004). However, they differ from cnidarians and bilaterians symmetrically; ctenophores are characterized by a unique rotational symmetry not found in other Metazoan clades. Ctenophores are also unique in that they are the only known organisms in Metazoa to have colloblasts. Colloblast cells are adhesive cells found along ctenophore tentilla and are used for prey capture (Leonardi et al., in press). Ctenophores are the largest organism that use cilia

(for locomotion. The cilia are arranged in comb (ctene) rows extend from the aboral end of the organism up the sides towards the oral end (Haddock, 2007; Mackie et al., 1988; Matsumoto et al., 1993). Species in the phylum range from 1.5 mm to 3 meters and vary morphologically.

Ctenophora is categorized by two classes, Nuda and Tentaculata (Mills, 1998-present). Beroida is the only order of Ctenophora that is classified in Nuda. Beroid ctenophores are melon or cone shaped and are distinguished from other ctenophores by the complete lack of tentacles. All other orders of Ctenophora have some form of tentacles and fall into the class Tentactulata. Members of Cydippida, Platyctenida, Cambojiida, and Cryptolobiferida, have long tentacles that can be retracted into a spherical or oval body. Lobata and Thalassocalycida have stunted tentacles, but their most distinct characteristic is their flattened cup-like lobes that extend from their bodies. Many of the lobed ctenophores have ciliated appendages called auricles, used for feeding. Cestida is a unique order within Tentaculata whose morphology hardly resembles other members of the phylum. Cestid ctenophores are flat and belt-shaped with shortened tentacles (Haddock, 2007; Mackie et al., 1988; Matsumoto et al., 1993). This study will focus mainly on three of the orders of Ctenophora; Beroida, Cydippida, and Lobata. Other orders within the phylum superficially resemble one of these three orders and use similar feeding strategies.

Differences in morphology have led to several variations in feeding, but three main feeding strategies exist for the phylum: engulfing, snagging prey items via tentilla, and utilizing lobes to capture prey (Haddock, 2007; Matsumoto et al., 1993; Tamm et al., 1995). Ctenophores use these different strategies to feed on a variety of zooplankton, such as copepods or even other ctenophores (Haddock, 2007; Matsumoto et al., 1993). Ctenophores that feed by engulfing belong solely to the order Beroidia. Beroid ctenophores lack tentacles their entire life cycle, thus they engulf prey whole or bite portions from prey. When prey items come into contact with the mouth, the lips, lined with tooth-like macrocilia, guide the prey into the gut (Haddock, 2007; Matsumoto et al., 1993; Tamm et al., 1995). Recent evidence has shown that Beroidia facilitate hunting through chemical and mechanical cues. These engulfers are particularly rapacious predators on other gelatinous plankton (Haddock, 2007). Ctenophores that facilitate feeding using their tentacles are mostly classified in Cydippida (Mills, 1998-present). These species deploy their tentacles and sit-and-wait, a strategy described by Tamm and Moss (1985), in the water column until prey are intercepted by the tentacles. Colloblasts adhere to the prey item,

similarly to a fly trapped in a spider's web, the tentacles retract, pulling the prey close to the organism's body, then the ctenophore rotates to bring its mouth to the prey (Haddock, 2007; Leonardi et al., in press; Moss, 1991; Tamm et al., 1985). Some species of ctenophores have a cydippid larval stage where this feeding strategy is employed; as those species' life stages change, so does their approach to feeding. The members of Lobata are an example of this transition in feeding strategy. As lobate ctenophores mature, they begin to use their auricles and oral lobes for feeding. Prey that come near the oral end of the animal are disturbed by the motion of the auricles and are trapped by the lobes, then the lobes bring the prey toward the mouth. Lobate ctenophores either passively or actively swim toward their prey, with the lobes facilitating not only feeding, but, in some species, propulsion through the water column (Haddock, 2007; Harbison et al., 1978; Matsumoto et al., 1993). Generally, ctenophores are considered a top tier zooplanktonic predator, thus is it important to characterize their functional diversity to better understand food webs, especially in the deep sea (Mills, 1995; Schaber et al., 2011; Shiganova, 1998).

Characterizing functional biodiversity from the surface to the deep

Marine biodiversity plays a critical role in ecosystem functioning at the surface of our oceans and at great depths. Provisioning of the services marine biodiversity delivers has and continues to change in the Anthropocene (Luypaert et al., 2020). Understanding the functional diversity of species will allow for better prediction of how environmental perturbations will affect marine environments. Biodiversity is a measure of variation at the genetic, species, ecosystem level and number of units in a system. Functional diversity is a component of biodiversity is defined by Tilman (2001) as 'the value and range of species and organismal traits that influence that ecosystem functioning.'(Tilman, 2001). Organismal or functional traits are 'morpho-physio-phenological traits that impact the fitness of individual species via their effects on growth, reproduction and survival, the three components of individual performance' (Violle et al., 2007). Measurements of functional biodiversity for the phylum Ctenophora have mostly been gathered from species at the surface. Even so, there is a large gap in knowledge regarding the phylum. Analysis of functional diversity in the deep pelagic ecosystem is necessary to accurately represent the phylum and to predict ecological variation in the most unknown habitat.

Metabolism as a proxy for functional diversity

Metabolic measurements of deep-sea organisms provide a window into deep pelagic functional diversity. Recovering metabolic indices of species is especially helpful when attempting to understand marine taxa that are difficult to observe. Metabolic rates can be used to understand ecological niches, phenotypic adaptations, and biodiversity (Childress et al., 2015; Gerringer et al., 2017a; Seibel et al., 2000; Thuesen et al., 1994). A supply of oxygen is required to facilitate organismal metabolism, thus metabolic measurements can be calculated from oxygen consumption rate (or carbon dioxide produced) per unit time (Thuesen et al., 1994; Thuesen et al., 2005a). The 'baseline' metabolic rate of an animal is measured while the organism is at rest, unstressed, and not actively digesting food (fasting). Metabolism is often studied using complimentary information such as the animal's life history stages, taxonomy, body mass and in the case of marine species habitat depth (Barnett et al., 2007; Childress, 1995; Dahlhoff, 2004; Pomerleau et al., 2015).

Metabolism in the deep

The deep sea is defined as ocean beyond the shelf break and depths greater than 200 meters (Mengerink et al., 2014). The deep pelagic realm is the largest biome on the planet in terms of area, biomass, and number of individuals (Robison, 2004). Despite the expanse of the deep, little is known about how life persists at great depths. Below 200 meters, oceanic waters become comparable to the arctic (-2 to 5˚C), light from the surface diminishes, food is scarce, conditions can be hypoxic, and hydrostatic pressure elevates to extremes with depth. These environmental conditions generally challenge metabolic functioning (Fengping et al., 2014; Gerringer et al., 2017a; Robison, 2004). However, deep pelagic animals exhibit many clear physiological and biochemical adaptations to sustain life under what we would consider extreme environmental conditions. It appears that metabolic rates of deep-sea animals have evolved in response to overriding environmental conditions. Traditionally, metabolism in the deep sea has been viewed as universally low and environmentally constrained (Childress et al., 1992; Childress et al., 1995; Seibel et al., 2007; Seibel et al., 1997a). With environmental factors playing such a large role in functionality, hypotheses focus on a perceived limitation of metabolism. These hypotheses also recognize that metabolism represents a cost. Elevated metabolic rate is not a benefit to an organism, and selection will not act to elevate metabolism in the absence of energy. With this in

mind, we can say that deep-sea organisms with lower metabolic rates are taking opportunities for energy savings (Seibel et al., 2007).

Visual interactions hypothesis

The metabolic rates for some groups of deep-sea species, like crustaceans, cephalopods and deep-ocean fishes, decline sharply with depth, yet in others, metabolic enzyme activities proceed as fast as ecologically similar shallow species at equivalent temperatures (Augustine et al., 2014; Childress, 1995; Gerringer et al., 2017a; Seibel et al., 2007; Seibel et al., 1997a; Thuesen et al., 1993b; Torres et al., 1994). Consolidated data available for metabolic rates in abyssal communities covers a diverse group of phyla, habitats, depths, and regions (Gerringer et al., 2017a). A consensus made using these datasets states that patterns of metabolism across a depth gradient reflect demand for energy for predator-prey interactions and such interactions are dependent on vision and light. Marked reduction in metabolic rates with depth have been retained in clades with image-forming eyes (Childress et al., 1985; Seibel et al., 2007; Seibel et al., 1997a; Torres et al., 1994; Torres et al., 1988). The visual interactions hypothesis suggests that in the absence of light, the evolutionary pressure for burst swimming, which is consistent with increased depths and the distances over which predator and prey interact, is reduced. This results in lower metabolic rates in some deep-sea taxa as compared to visually dependent surface taxa (Childress et al., 1985; Childress, 1995; Childress et al., 1979). Enigmatically, this pattern of metabolic decline corresponding with habit depth is not present in non-visual pelagic taxa, such as copepods, chaetognaths, and medusae (Childress et al., 1992; Thuesen et al., 1998).

Comparative ecophysiology studies on deep-sea taxa

Oxygen consumption rates and rates of enzymatic activities have successfully been used to characterize ecophysiological traits in and functional diversity in deep-sea organisms. Comparison of oxygen consumption rates and metabolic enzyme activities in some organisms has shown support for the use of certain enzymes as indicators of metabolic potential under overriding environmental conditions (Childress et al., 2015; Gerringer et al., 2017a; Thuesen et al., 1993b). Studies using enzymatic activities have shown that the maximum reaction rate (Vmax) can be temperature or pressure dependent (Fields et al., 2015; Hochachka, 2015; Somero, 2003). For instance, a study of metabolic enzymes in abyssal fishes showed evidence for protein adaptation under high hydrostatic pressure (Gerringer et al., 2017a; Gerringer et al.,

2017b). Currently, there are few studies on temperature driven metabolism in ctenophores, however, there are no data published on the effects of pressure on the metabolic enzymes of ctenophores.

Metabolic enzymes to measure whole animal metabolism

When determining metabolic profiles for deep-sea taxa, metabolic measurements can be retained using enzymatic data. An enzyme is a type of protein (biological macromolecules) that catalyzes a reaction. Enzymes can be useful because they retain function long enough after being frozen to run experiments. Due to the habitat restrictions and the fragile nature of deep-sea specimens, deep-sea researchers have utilized enzyme assays of tissues and whole organisms as an alternative method for characterizing metabolism (Childress et al., 2015; Gerringer et al., 2017a; Thuesen et al., 1993b). This method is commonly used when measuring metabolism in gelatinous zooplankton, such as ctenophores. Preliminary research by King and Packard (1975) showed significant correlation between electron transport chain activity and respiration in several members of zooplankton (King et al., 1975). The Electron Transfer System (ETS) is the pathway responsible for transfer of electrons to oxygen, the final electron acceptor. The ETS activity is responsible for oxygen consumption by both the cell and organism, and can be used as an index, or biochemical proxy for zooplankton respiration in the sea. ETS may be characterized as a multi-enzyme, multi-substrate system, and its activity is determined in substrate saturating conditions, i.e. at the maximal rate (Vmax) (Båmstedt, 1980). Subsequent research on marine fishes investigated the use of individual aerobic and anaerobic metabolic enzyme activities, and also found correlation with respiration rates (Childress et al., 1990; Torres et al., 1988). The same key metabolic enzymes were assayed and found to be good indicators of metabolism in pelagic chaetognaths, nemerteans, and annelids (Thuesen et al., 1993a; Thuesen et al., 1993b).

Our knowledge of deep-sea metabolic functioning is limited to a few enzymes and has mostly been extrapolated from fishes. Lactate dehydrogenase (LDH) citrate synthase (CS), pyruvate kinase (PK) and malate dehydrogenase (MDH) have been found to be appropriate indices of metabolic potential in the deep-sea (Childress et al., 1979; Gerringer et al., 2017a). LDH and CS catalyze the main reactions used in anaerobic and aerobic intermediary metabolism. LDH catalyzes the reaction responsible for converting pyruvate into lactate and is the terminal enzyme used for anaerobic glycolysis. CS is used in the first rate-limiting step in the Krebs cycle,

where it catalyzes the reaction between acetyl-CoA and oxaloacetate to form citrate (Båmstedt, 1980). The activity of each LDH and CS, is indicative of the metabolic poise of its respective pathway. The activity of the specific pathway relates to the overall physiological condition of the whole organism (Hochachka et al., 2002). Like LDH, PK is used in glycolysis. PK converts phosphoenolpyruvate to pyruvate, yielding one molecule of adenosine triphosphate (ATP), which is the final step in glycolysis. MDH is used in the citric acid cycle where it reversibly catalyzes the oxidation of malate to oxaloacetate; this reaction is performed in many other metabolic pathways (Gerringer et al., 2017a). The glycolytic enzymes LDH and PK are used as proxies to indicate burst locomotory capability and anaerobic capacity, whereas CS and MDH, are applied as indicators of routine metabolic rate and aerobic activity (Childress et al., 1979; Childress et al., 1992; Thuesen et al., 1993b).

Protein adaptation in response to environmental factors

Proteins are among the most important and most-studied biomolecules in biochemical research. Proteins are macromolecules that function in a range of biological processes. They are the cellular workhorses that provide cells with most of its structural elements. They are also responsible for the machinery required to generate energy and carryout various types of work (i.e. locomotion, transport, and biosynthesis). Proteins are comprised of amino acids grouped together to form a linear polymer often called a protein backbone. The sequence of amino acids is the primary structure of the protein, but in order to gain function, the protein must fold into a three-dimensional structure called a conformation. Bonds between differentiated functional groups attached to the protein backbone promotes folding into a native conformation. This native conformation could be a catalyst, regulator, structural element, or contributor to another function (Somero et al., 2017). Protein folding largely deals with charged functional groups, but outside, or environmental, conditions could promote folding.

Most proteins are marginally stable, always returning to or staying near a particular state, and must be so to maintain function, resulting in positive protein selection. Selection favors the structure that confers optimal functional properties on a system. Marginal stability is beneficial because there is an increased capacity to sense and respond appropriately to environmental changes. The natural tendency toward marginal stability in proteins allow for the protein to

interact with external forces. This leads to protein adaptation under unique conditions, which, in turn, drives evolutionary change (Somero et al., 2017).

Proteins delicately balance the stabilizing and destabilizing interactions between themselves and the environment. Flexibility in the conformation of a protein allows for adaptation to extreme environments (Hochachka, 2015; Hochachka et al., 2002; Somero et al., 2017). Such environments shift the 'mesophilic' characteristics of a protein to the respective extremes of temperature, hydrostatic pressure, pH and salinity. This shift enhances the intrinsic stability of the protein, which requires minute local structural changes to the protein. Specified proteins, or enzymes, have evolved over time to combat pressures associated with the surrounding environment. These pressures could be associated with a number of environmental forces, but temperature and pressure will be the main focus here because they are the two fundamental physical variables affecting all chemical reactions (Fields et al., 2015; Hochachka et al., 2002; Somero et al., 2017).

When environmental conditions change (e.g., temperature or pressure), the protein will only function to a certain degree before it ‘crashes out’ or denatures (Mozhaev et al., 1996; Somero, 1992; Somero, 2003; Somero et al., 2017). Like most chemical reactions, the rate an enzyme catalyzes a reaction increases as temperature increases or decreases. Enzymatic reactions are typically adversely affected by high temperatures; the reaction rate increases with temperature to a maximum level, then abruptly declines with further increase of temperature. The effect of temperature on proteins is well understood, however, the same cannot be said about the effect of pressure on proteins (Hochachka, 2015; Low et al., 1976; Somero, 2003; Somero et al., 2017).

Selection’s tendency to accommodate for environmental change is evident across all biochemical structures and allows life to prevail in a wide range of conditions. This is necessary to sustain efficiency, accuracy, and responsiveness. Often, the external environment brings about biochemical manifestations of stress which are applicable to all biochemical systems, unifying otherwise diverse organisms (Somero, 1992; Somero, 2003; Somero et al., 2017). Biological systems are perturbed by environmental stressors. Thus, selection is influenced by mechanisms responsible for achieving structural balance (Hochachka et al., 2002; Somero et al., 2017).

The effect of temperature on proteins

Thermodynamic relationships are universal. The states of all biological structures and their rates of reaction are affected by changes in temperature. In the context of proteins, temperature effects catalytic rates, acclimation of enzyme activities, protein thermal stability, and protein expression (Fields et al., 2015; Hochachka, 2015; Somero, 2003; Somero et al., 2017). Both high and low temperatures can lead to denaturation of a protein, which involves a change in the protein structure (generally unfolding) with the loss of activity. Interactions with water govern the thermodynamics of protein folding and stability because proteins must be bathed in solution to facilitate proper folding, and the aqueous solution surrounding the protein is affected by temperature. Structures of a protein are water soluble but contain a hydrophobic core. The sidechains of this core are buried to the interior of the protein, away from the surrounding water, which stabilizes the folded state of the protein. The burying of these amino acid chains is an endothermic reaction, requiring input from heat energy to break up the organized shell of water surrounding the protein. The net free energies of stabilization are low, about equal to energies associated with formation of a few noncovalent ("weak") bonds, thus protein structures are highly sensitive to temperature (Somero et al., 2017).

Sensitivity to temperature helps determine the success of organisms in all habitats. As a result, temperature plays an important role in determining biogeographic range limits of many organisms. Underlying this sensitivity of biological systems to temperature is the impact that changes in the thermal energy of the environment have on biochemical and thus physiological processes (Fields et al., 2015; Hochachka, 2015; Somero, 2003; Somero et al., 2017). The relationship between protein folding capacity and temperature has proven itself over a wide range of conditions and, like most chemical reactions, effect of temperature can be explained by the following equation:

$$k = Ae^{-E_a/(RT)},$$

K, the rate of a reaction, increases exponentially with temperature, *T*. *R* is the universal gas constant, *A* is a reaction-specific constant, and E_a represents the activation energy of the reaction. Depending on the value of E_a, rates of biochemical reaction will increase 2-3-fold with a 10°C increase in environmental temperature, exhibiting the 'Q10' relationship of thermal physiology. This is known as an Arrhenius relationship (Somero et al., 2017). When an enzyme is assayed in

vitro across a range of temperatures within "normal" physiological temperatures of the organism, it likely reacts in the expected exponential increase in reaction rate. Eventually, a "break-point" is reached and the activity of the reaction begins to decline due to loss of the protein's native structure. However, when metabolic rates of species adapted to different temperatures are compared, the Arrhenius relationship does not hold. For example, a cold-adapted polar fish living at 0°C does not have a metabolic rate 20-times lower than that of a desert lizard living at 40°C (Hochachka, 2015).

The effect of pressure on proteins

Unlike temperature, pressure works in two directions (i.e., presence or absence). The effect of pressure, however, is not as well understood as the effect temperature has on proteins, despite the fact that ninety-eight percent of Earth's habitable volume lies below 200 meters of water (Childress et al., 1995; Hochachka, 2015). The effect of pressure on proteins appear to be more distinctive than the effect of temperature; high pressure can inhibit some reactions and enhance others. Similar, to temperature, pressure induced changes to protein structure arise from regions primarily stabilized by hydrophobic and electrostatic interactions, or interactions between objects having electric charges. Hydrogen bonds are almost pressure insensitive. Structural changes from the hydrophobic effect cause the protein to fold in such a way that charged and polar sidechains are mostly located on the protein surface, where networks of hydrogen bonding interactions occur. Thus, pressure-adapted proteins may have structures relative to their native or heat-treated counterparts as a consequence of different functionality. Pressure, like temperature, can denature proteins. As the proteins change shape, water can penetrate the protein's interior. Some proteins are better able to resist this incursion of water, but the molecular mechanisms of how pressure is resisted aren't yet well understood. What we do know however, is that the effects of pressure and temperature on kinetics are both antagonistic in molecular terms (Hochachka et al., 2002; Somero et al., 2017).

Pressure increases linearly with depth and selects for enzymes that are resistant to volume changes during catalysis, an adaptation that reduces catalytic efficiency. Adaptations in enzymatic capacities allow for an organism to overcome pressure-induced inefficiencies in enzymes to maintain a minimum level of performance (Hochachka, 2015; Mozhaev et al., 1996; Somero, 1992). The effects of pressure can be brought about on a single enzyme-catalyzed

reaction, depending upon the temperature. Because of this dependency, temperature can be regarded as an entirely different physical parameter than pressure, from a functional and evolutionary standpoint (Low et al., 1975).

Enzyme kinetics

When considering the relationship between temperature or pressure on enzyme kinetics, it is often conceptualized in terms of enzyme substrate affinity, or the level to which the enzyme will bind to a substate. For an enzymatic reaction to occur, substrate must bind to the enzyme's active site (Engelking, 2015). The degree of participation of an enzyme is determined by enzyme affinities for key regulatory ligands, large molecules that bind to another. In the examples of temperature or pressure, we deal with a network of reactions whose degree of participation in metabolism is automatic and autocatalytically controlled (Hochachka et al., 2002).

Enzyme kinetics focuses on the factors, such as temperature or pressure, that influence the rates of enzyme catalyzed reactions (Engelking, 2015; Segel, 2013). The rate of a biochemical reaction is referred to as the velocity (V). Enzymes speed up the rate of reaction (V) by lowering the activation energy of a reaction. For this to happen the reacting substrate binds to the enzyme, forming an enzyme substrate complex before forming a product. To simply conceptualize this, we can view the reaction as a change from *A* to *B* (product). The rate of this change can be described by the following equation:

$$Rate = K\,[A]$$

(Engelking, 2015; Hochachka et al., 2002; Segel, 2013).

In the equation, the constant, *K* is dependent on the environment, which can be described in terms of temperature or pressure, and *A* is the starting substrate concentration. The rate at which new product is formed can be modified by changing the substrate or enzyme concentration or by changing the environmental condition (*K*) (Engelking, 2015; Segel, 2013). The enzyme concentration is assumed constant and the enzyme will only work to a certain speed, where it will reach a maximum velocity (Vmax). This means that the enzyme is saturated or "filled up" with substrate, preventing the reaction from occurring any more rapidly. Thus, Vmax signifies the turnover number of an enzyme, or the number of substrate molecules converted into product

by an enzyme molecule at the time the enzyme is fully saturated with substrate (Engelking, 2015; Hochachka et al., 2002; Segel, 2013; Somero et al., 2017).

What is known about maximum reaction rates of enzymes is mostly limited to in vitro analyses, however two hypotheses are clear; (1) both the substrate concentration and the number of substrate molecule each enzyme site converts to product per unit time tend to increase with temperature, which is constrained by hot- or cold-denaturation of the enzyme, and (2) effects of pressure vary significantly between species and enzymes (Hochachka, 2015). In both situations, functional characteristics and structural features must adapt. By measuring how well an enzyme is binding to substrate or how fast the enzyme can perform under such environmental conditions, adaptation to particular habitat settings becomes clear (Engelking, 2015; Hochachka et al., 2002; Segel, 2013; Somero et al., 2017). Selection for physiologically significant changes in enzyme function can be driven by small differences in habitat temperature or the habitat associated pressure. Genes belonging to specific functional groups are known to be particularly susceptible to temperature and high-pressure (Somero et al., 2017). Thus, evidence of positive selection should be shown in protein evolution in marine habitats, where both pressure and temperature are working on a protein (Childress et al., 1990; Gerringer et al., 2017a; Hochachka, 2015; Torres et al., 1988).

Activation volume

Temperature and pressure affect enzymatic activation volume by altering the kinetic energy of reactants. Effects from these environmental factors can result in conformational changes to the enzyme's active site (Engelking, 2015; Hochachka et al., 2002; Segel, 2013; Somero et al., 2017). Changes in enzyme conformation during catalysis often results in volume change of the system. Activation volume is the measure of conformational change, that is the difference between the partial molar volumes of the activated complex and the reactants, during an enzymatic reaction (Michels et al., 1992; Schuabb et al., 2014). Volume changes may derive from two sources: (1) "hydration density" effects due to changes in the exposure to solvent of protein groups which modify water density, and (2) 'structural' contributions arising from changes in the volume of the protein itself (Low et al., 1975).

Pressure activates, retards, or shows no effect on various reactions. This relationship can be given by the following equation:

$$\Delta V^{*} = 2.3\mathrm{RT}\,\frac{logK\ p_1 - logK\ _2}{p_1 - p_2}$$

Here, ΔV^{*} in the change of volume activation, R is the gas constant, K is the constant velocity at pressure $p1$ and $p2$. To simplify:

$$\Delta V^{*} = \frac{Volume\ of\ activated\ comple}{volum\ \ of\ reactants}$$

(Basilevsky et al., 1985; Hochachka, 2015).

If the volume of the activated complex is greater than the constituents outside of the complex, pressure retards the reaction and when the volume of the activated complex is less, the reaction rate is accelerated. When the volumes are equal, no effect is taken by the reaction (Basilevsky et al., 1985; Michels et al., 1992; Schuabb et al., 2014; Somero et al., 2017)

The role of ecophysiology in assessing functional diversity

When multiple environmental conditions shape taxa within a community, they can be reflected differently in trait composition of species. Species are different, but not equally different. By collecting information about species' functional traits, dissimilarities between species can be resolved, and by collecting average trait values from multiple species, community response can be predicted. Functional traits are often traits that influence organismal performance and/or species fitness. Functional traits describing physiological processes (e.g., respiration, metabolism) can be used as a surrogate of a function (e.g., enzymatic activity) or as the function itself (e.g., metabolism) (Violle et al., 2007). One strategy for determining a functional trait is identifying the physiological processes and mechanisms that allow species to cope with an environmental driver, and how organismal responses affect patterns in distribution, abundance, community structure and ecosystem processes (Diaz et al., 2007; Rosado et al., 2013). Environmental factors can be considered filters in that they constrain specific attributes of functional traits. The response of whole-animal performance to an environmental variable influences 'ecological performance'. A component of this is identifying the environmental drivers and the associated timeframe that driver affects a community. For example, organisms facing the same environmental conditions throughout their lifespans (e.g., high hydrostatic pressure and cold temperatures in the deep sea) over large spatial and temporal ranges are expected to have changes in trait values due to plasticity (Rosado et al., 2013). Also species

resistance to unfavorable environmental conditions can be determined by their tolerance strategies (Schleuter et al., 2010; Tilman, 2001).

Ecophysiological knowledge is fundamental when establishing the criteria for choosing functional traits. There has yet to be a consensus on what the most important functional traits are and the best way to measure them. However, establishing criteria for choosing functional traits and validating them is important in understanding functional diversity amongst understudied groups such as Ctenophora.

CHAPTER TWO: MANUSCRIPT

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Assessing Functional Diversity Down the Water Column: The Effect of Hydrostatic Pressure on the Metabolic Enzymes of Ctenophores from Different Habitat Depths

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1. Introduction

The deep pelagic environment comprises the largest habitat by volume on the planet. Within this habitat live hundreds of thousands of undescribed species whom have evolved numerous biochemical and ecological adaptations to cope with the associated environmental conditions (Robison, 2004). The habitat is characterized by cold (5˚C), dark waters, where food is scarce and organismal bioluminescence is the sole source of light and communication. Here, hydrostatic pressure increases linearly with depth, and rises to extremes. The hydrostatic pressure is so confounding that it affects the solubility of gasses. (Danovaro et al., 2017; Fengping et al., 2014; Mengerink et al., 2014; Robison, 2004). Biochemical and genetic studies have eluded that both physiological and structural adaptations are essential for life under high hydrostatic pressure (Campanaro et al., 2008). When considering hydrostatic pressure as a force driving biochemical adaption, several molecular- evolutionary questions arise. Here, we will focus on addressing the mechanisms for allowing proteins to be structurally adapted to function at high pressures. To investigate the influence of hydrostatic pressure on biochemical structures and physiological performance, the phylum Ctenophora was investigated.

Ctenophores, gelatinous macrozooplankton, are predators persistent at various levels of the marine water column, ranging from sea level to 7000 meters (Appeltans et al., 2012; Dunn et al., 2015; Harbison et al., 1978). Some species have remarkable tolerances in depth and temperature, while others are constrained to specific conditions (Fig. 1). The phylum is commonly referred to as “comb jellies”, due to their eight comb rows of fused cilia used for locomotion (Dunn et al., 2015; Harbison et al., 1978; Horridge, 1964; Mills, 1998-present). Ctenophora is a small (~200 described species) clade that diverged from Metazoa near the beginning of multicellular life (Dunn et al., 2015; Mills, 1998-present). Despite their gelatinous nature, ctenophores are complex, containing distinct muscle and nerve cells (Dunn et al., 2015; Wallberg et al., 2004; Whelan et al., 2017).

Ctenophora is uniquely suited to investigate protein adaptation in high pressure environments due to their evolutionary history. Ctenophore phylogenies suggest that lineages within Ctenophora have undergone recent, independent range shifts, producing closely related species living in contrasting physical positions (Winnikoff et al., 2019; Winnikoff et al., 2017). This

allows for comparative analyses where pressure and temperature are independent variables affecting biochemical adaptation in high pressure environments(Winnikoff et al., 2017). Previous studies have largely investigated pressure tolerances in the metabolic enzymes of fishes and previous pressure studies on invertebrates extremely rare (Childress et al., 1979; Childress et al., 1995; Gerringer et al., 2017a). By choosing a to investigate a series of enzymes whose physiological functions and regulatory properties are well documented, we can recognize patterns across clades. Previous studies that have investigated the influence of hydrostatic pressure on the physiological functioning of deep-sea taxa have measured activities of the metabolic enzymes creatine kinase (CK), malate dehydrogenase (MDH), pyruvate kinase (PK), lactate dehydrogenate (LDH), and citrate synthase (CS). In this study, CK, MDH, and PK were investigated for comparison. Comparative analyses can provide insight on how individual factors affect protein evolution while controlling the background of phylogenetic relatedness (Winnikoff et al., 2019; Yancey et al., 2015).

At a molecular level, aspects of protein structure that confer pressure tolerance and ubiquity of elements must be addressed. Convergence amongst ctenophore species is predicted, however, it is unclear how the clade will compare to pressure adaptations in other deep-sea organisms. Ctenophora diverged so early and protein evolution is so path dependent, that it's possible that different species within the clade have evolved variously different mechanisms for coping with hydrostatic pressure (Winnikoff et al., 2019; Yancey et al., 2015). To compensate for the extremes of the deep, such as low oxygen and temperature, or little prey availability, there are benefits. For example, environmental stability and boundless mobility in the deep promote gene flow (Seibel et al., 2007). Hydrostatic pressure interacts with these benefits, and its comparison to other constraints is key in understanding evolution in the least known habitat on our planet. By cataloging evolved solutions enabling function under extreme pressure, gradual construction of enzyme optimization models is possible for protein engineering.

Enzymatic rates and whole animal metabolism can be used as a proxy to investigate the influence of hydrostatic pressure on physiology. Enzyme activity measurements have correlated with metabolic rate, thus enzymatic activities can be indicative of metabolism. Measuring the enzymatic activities of delicate deep-sea animals serves as a control for the stress of capture and

pressure effects and allows examination of specimens that cannot be recovered alive (Childress et al., 1979, 2015; Gerringer et al., 2017a; Thuesen et al., 1993b).

Early literature reports a decline in metabolic rates of marine organisms as hydrostatic pressure increases linearly. This decline is to a greater degree than expected from the decrease in temperature (Childress et al., 2008; Childress et al., 1979; Childress et al., 1992; Gerringer et al., 2017a; Seibel, 2007; Seibel et al., 2007; Torres et al., 1988). The current pattern of enzyme activities and metabolism in the deep-sea shows that some groups, such as cephalopods, crustaceans, and pelagic fishes, demonstrate large declines in metabolic rate as hydrostatic pressure increases (Childress et al., 1979; Childress et al., 1995; Low et al., 1976; Seibel et al., 2000; Seibel et al., 1997a, 1997b; Torres et al., 1994; Torres et al., 1988). Benthic groups exhibit minimal to low declines, and gelatinous pelagic groups appear to show no decline in metabolic rate as depth increases (Augustine et al., 2014; Hochachka, 2015; Thuesen et al., 1993a; Thuesen et al., 1993b; Thuesen et al., 1994). This indicates that reduced metabolic rates are not necessary or a usual characteristic of adaptation of deep-sea animals. This coupled with decline in other metazoan clades can be explained by determining if environmental parameters that covary with depth are correlated with metabolism (Seibel et al., 2007). With pressure effects being so varied across groups, we can say that hydrostatic pressure itself has no effect on metabolism, and thus pressure may be a factor driving protein evolution (Winnikoff et al., 2019). Pressure in some ways can be limiting to metabolism, by reducing the efficiency of enzymes as a mode of biochemical adaptation to elevated pressure (Hochachka, 2015; Mozhaev et al., 1996; Somero, 1992; Somero et al., 2017). By determining structural constraints on enzyme function under increasing hydrostatic pressure, models focusing on deep-sea colonization can be made informed and efficient. This will allow for the discovery of general patterns of protein adaptation and possible applications in protein engineering and biocatalysts. Studying the effect of pressure on the metabolic enzymes of ctenophores permit relative studies between phyla in the deep sea.

2. Methods

2.1 Eco-diversity profiles in the phylum Ctenophora

The term eco-diversity will be used to encompass taxonomic, morphological and habitat diversity while sampling the phylum Ctenophora. Information regarding the sample site and collection details were recorded for every individual. Habitat data from MBARI's Video Annotation and Reference System (VARS) database, was gathered to evaluate minimum depth occurrences of species. The minimum depth of occurrence (MDO) is determined as the depth below which 95% of the population of each species lives (Childress, 1995). Species MDO calculations were made using VARS database (Schlining et al., 2006) referencing ROV observations and by personal communication for undescribed species. Sampling was guided by the framework of ctenophore phylogeny and the extended transcriptome sampling that has generated the baseline for the phylum. Using phylogenetic information provides critical context for examining diversification, colonization, and detecting evolutionary convergence and selection.

Depth is taken in terms of the minimum depth of occurrence (MDO) for all species.

2.2 General sample collection and processing

Field collection was facilitated through collaboration with the Monterey Bay Aquarium Research institute (MBARI). Ctenophores were collected for experimentation during several of MBARI's research cruises off the coast of California (2018-2020) and one cruise in Hawai'i (November 2018). Research cruises were organized by MBARI's Dr. Steven Haddock. Ctenophore species were targeted to cover the taxonomic, ecological, and functional diversity of the phylum Ctenophora. Ctenophores that were collected for physiological experiments were collected using three methods; each method targeted specimens from different habitat depths. Surface ctenophores were collected to 20 meters by blue-water SCUBA divers (Haddock et al., 2005). Intermediate samples were obtained using a Tucker trawl, an opening and closing mid-water zooplankton net (2.5 m2 standard or 1.0 m2). Samples collected by trawl were often damaged in their transit to the surface, thus only robust species were processed for experimentation. Deep species were mobilized using MBARI's remotely operated vehicles (ROV), the ROV *Doc Rickets*, *Ventana,* and mini ROV. Species obtained by ROV were often too delicate to undergo shipboard experiments and were processed immediately to retain optimal physiological functionality. Sample methods at sea followed methods that have been developed over many years, including recent developments in transcriptomics.

When in good condition, specimens collected for physiological experiments were documented photographically to retain morphological information and were entered in a catalogue. After ship-board physiological and behavioral experiments, somatic tissues were sub-sampled for genetic analysis. Specimens suited for on-ship experiments underwent respirometry to quantify the rate of oxygen consumption. Oxygen consumption rates were calculated using established techniques for measuring respiration. Samples used for respirometry were flash frozen in liquid nitrogen promptly after experimentation and stored in a -80˚C freezer on-board. Specimens too delicate for respiration were immediately frozen in liquid nitrogen after collection until enzyme activates could be measured by spectrophotometer. The frozen samples were shipped to The Evergreen State College's ecophysiology lab and transferred to a -80 °C freezer for storage. All specimens were analyzed within 6 months of capture.

2.3 Genetic analysis

Following taxonomic identification and experimentation, specimens underwent subsequent laboratory extraction. Simultaneous purification of RNA and DNA during extraction was facilitated using an extraction buffer. Sampled DNA was archived by MBARI to be used in direct gene sequencing and navigate targeted genes via a series of genomic software including *Geneius*.

2.4 Physiological experiments

Physiology data include parameters of functional diversity such as metabolic and biochemical indices characterized relative to the varied environmental conditions in which ctenophores are found. Direct enzyme measurements will provide quantification of metabolic potential.

Enzymatic profiles of anaerobic and aerobic potential will be used as indicators of eco-physical performance under increasing hydrostatic pressure. Three enzymes, creatine kinase, malate dehydrogenase and pyruvate kinase, were targeted for comparison with previously published studies. Creatine kinase (CK) catalyzes the conversion of creatine and uses adenosine triphosphate to create phosphocreatine and adenosine diphosphate. Malate dehydrogenase (MDH) is an enzyme participating in many metabolic pathways, including the citric acid cycle. It reversibly catalyzes the oxidation of malate to oxaloacetate using the reduction of NAD to

NADH. Pyruvate kinase (PK) displays anaerobic metabolic potential and is involved in the final step of glycolysis, the breakdown of glucose (Worthington Biochemical Corporation 2020). PK activity relative to CK activity is distinct for individual species; this can be reflective of morphology, behavior, and differential habitat constraints, such as oxygen availability, temperature and pressure. The factors vary between species; thus, it will be used as the parameter for measuring functional biodiversity.

2.5 Enzyme assays under hydrostatic pressure

Previously frozen ctenophore samples were weighed on a Metler Toledo analytical balance, then promptly homogenized in a buffer of 0.1 mM Tris-HCL at a ratio of 1:1 before assaying. Hand-held glass homogenizers (15 ml or 40 ml) were employed on ice to homogenate whole animal samples. A Baby Bullet™ commercial blender was used for larger specimens. The homogenate was centrifuged for ten minutes at 6600 x *g* at 4˚C. Whole animal samples from each ctenophore were assayed in duplicate. All assays were conducted within 90 minutes of homogenization and at 5˚C to allow for comparison with other published values, though the habitat temperature for ctenophore species used in this study is varied. Prior to each pressure assay, an atmospheric pressure check was conducted using deionized water to ensure the spectrophotometer was functioning correctly.

Maximum activities of CK, MDH, and PK were measured using standard spectrophotometric methods described by and Yancy and Somero (1978) using a Hewett-Packard diode array spectrophotometer with a temperature-controlled cuvette measured at 340 nm (zero order). A 5ml stainless steel cuvette chamber (Mustafa et al., 1971) was employed to measure enzyme activities at pressure. The chamber was equipped with sapphire windows to allow light from the spectrophotometer to beam through the cuvette. To reduced condensation on the cell windows, a constant stream of nitrogen gas was applied to the panes' surface. All reactions were measured to 5.1 ml to prevent the introduction of air to the chamber. If there was negative space present in the chamber, mineral oil was pumped through a line to the chamber to prevent air from entering the reaction. Each assay ran for 500 seconds. Data was only collected between 50-450s to minimize error from mixing effects. The pressure of the chamber was incrementally increased by an Enerpac hydraulic handpump (690 bar-110 cm^3) during the assay. After approximately 100s at atmospheric pressure, the pressure was increased to 200 bar. After another 100s, the pressure

was increased to 400 bar then 600 bar. Four-hundred seconds into the assay, the pressure was released back to 1 bar. Enzymatic reaction rates for each pressure treatment were determined by converting to units of activity (μmoles of substrate converted to product per minute) per g wet weight of the whole animal. Between assays, the cuvette was rinsed and aspirated, once with 70% ethanol and twice with deionized water.

Creatine Kinase (CK) activity is measured as the production of NADPH. The reaction is measured using a coupled enzyme system utilizing pyruvate kinase (PK) and lactate dehydrogenase (LDH). The procedure is described by Tanzer and Gilvarg (1959). One Unit is defined as the conversion of one micromole of creatine to creatine phosphate per minute at 25°C and pH 8.9 under the specified conditions (Tanzer et al., 1959). The final concentrations of the cocktail ingredients were 100 mM Imidazole Buffer (pH 7.1), 10 mM MgCl2, 20 mM glucose, 1.8 mM ADP, 3.0 mM phosphocreatine, 1.3 mM NADP, 1600 Units L^{-1} G-6-PDH, and 2500 U L^{-1} hexokinase.

Malate dehydrogenase (MDH) reaction was initiated with the mixing of oxaloacetic acid. The reaction velocity is established by measuring the decrease in absorbance at 340 nm resulting from the oxidation of NADH. One unit oxidizes one micromole of NADH per minute at 25°C and pH 7.4 under the specified conditions (Worthington Biochemical Corporation 2020). Activity was measured at a final concentration of 50 mM Imidazole-HCl (pH 7.0 @ 20°C), 20 mM $MgCl_2$, 150 μM NADH, 0.4 mM oxaloacetate.

The enzymatic activity of PK was measured using a coupled assay system where the reaction velocity is determined by measuring the decrease in absorbance of lactate dehydrogenase at 340 nm resulting from the oxidation of NADH (Worthington Biochemical Corporation 2020). Pyruvate Kinase (PK) was measured at the following concentrations: 100 mM Imidazole (pH 7.83 @ 20°C), 200 mM KCl, 20 mM $MgSO_4$, 0.2 mM D-Fructose 1 6-bisphosphate, 0.15 mM NADH, 1.0 mM PEP, 5.0 mM ADP, LDH dilution (1:40 dilution of LDH in 100 mM Imidazole, pH 7.83 @ 20°C). All biochemical reagents for enzymatic assays were obtained from Sigma Aldrich, except NADH (AcrosOrganics).

2.6 Statistics

Trends with enzyme activity and pressure were investigated using analysis of variance (ANOVA) constructed assuming normal distributions in the statistical programming platform (R Core Development Team, 2020). Plots of residuals were examined to check assumptions. Initial and recovery enzyme activities were analyzed using a paired t-test, assuming normal distributions (R Core Development Team, 2020). Figures were constructed using the R package ggplot2 (Wickam, 2016).

3. Results

One hundred seventy-seven individuals from 27 species in the phylum Ctenophora were collected during this study (Table 1). Collections ranged from surface to 4000 meters and 20-4˚C (Figure 1). Of the 177 individuals collected and assayed, enzymatic activities were successfully measured on 91 whole specimens. Eleven of the species are undescribed and were given operational names. Results show three enzyme activities measured in 27 different species, belonging to five different orders, Beroida, Cydippida, Lobata, Cestida, and Platyctenida, (Table 2). Three metabolic enzymes, creatine kinase, malate dehydrogenase, and pyruvate kinase, were measured for the phylum.

3.1. Enzymatic activities at atmospheric pressure

Beroe gracilis exhibited the highest CK activity (0.4002 units g^{-1}), whereas Lobate sp. V displayed the lowest, (0.00517 units g^{-1}) (Table 2). Cydippid sp. C displayed the highest MDH activity (0.99 units g^{-1}), whereas Lobate sp. A displayed the lowest (0.0158 units g^{-1}) (Table 2). When examining PK activities, Cydippid sp. N showed the highest activity (0.2144 units g^{-1}), and Lobate sp. V displayed the lowest activity (0.009 units g^{-1}) (Table 2).

3.2. Enzymatic activities in relation to depth

The activities of the three enzymes examined, CK, PK and MDH, did not decline with minimum depth of occurrence in ctenophores (Figure 2).

3.3. Enzyme activities as a function of pressure

All three enzymes exhibited changes in maximal reaction rate with pressure. The shallow species *P. bachei* had enhanced CK activity under high pressure (ANOVA, $p< 0.04$). Deep ctenophore species did not display differences in activity between pressures. Peak enzymatic activity occurred near habitat pressures for all species (Figure 3).

Malate dehydrogenase (MDH) activity increased with pressure for all species (Figure 4). However, the difference in pressure activation from initial activity was not great enough to make assumptions regarding enzyme performance. This was true for all the species tested. It should be noted that while evaluating pressure tolerances of MDH, all species displayed scatter in repeat assays.

Pyruvate kinase (PK) activity was inhibited by pressure in all species except *P. bachei* and Cydippida sp. RLL. Three of the species tested showed a decline in activity indicating conformational damage to the enzyme. (ANOVA, *B. fosteri*, $p < 0.035$; *B. chuni*, $p < 0.001$; *B. abyssicola*, $p < 0.001$). The rate of recovery after decompression varied by enzyme (Figure 5). The activity measured post decompression for all enzymes and species were likely affected by uneven changes in system optics with the release of pressure.

A paired-samples t-test was conducted to compare the species initial and recovery activities from all three enzymes. There were no differences found between the initial and recovery activities across the species and enzymes tested except for MDH of *B. fosteri* and CK of *L. cruentiventer* ($t(1) = 20.551$, $p = 0.03095$), CK of *L. cruentiventer* ($t(1) = 21.036$, $p = 0.03024$).

3.4. Change in activation volume with pressure

Changes in the volume of the enzymes' active site was influenced by pressure. This is seen in all three enzymes (Figure 7). Positive activation, resulting in enzyme inhibition was displayed in CK and PK. While evaluating CK , positive activation was indicated inhibition in five species (ANOVA, *B. fosteri* $F(3, 19) = 10.6$, $p< 0.0003$; *B. chuni* $F(3, 24) = 6.186$, $p< 0.003$, *B. forskalii* $F(3, 4) = 0.231$, $p< 0.008$; *Lampea* sp. B $F(3, 8) = 7.477$, $p< 0.01$*; P. bachei* $F(3, 6) = 5.082$, $p< 0.05$). This pressure inhibition was also displayed in *Lampea* sp. B for PK (ANOVA, *Lampea* sp. B $F(3, 11) = 3.742$, $p < 0.05$). MDH displayed negative activation, resulting in acceleration of enzyme activity, in two species (ANOVA, *B. abyssicola* $F(3, 10) = 6.943$, $p< 0.01$; Undescribed platyctene P. $F(3, 33) = 14.02$, $p< 4.65e-06$).

The change in activation volume for each pressure across the species tested were also analyzed. From this examination, it was unclear whether positive or negative activation was acting on the enzyme. Differences in volume change were seen during the change from 400 to 600 bar and 600 bar to recovery (1 bar) in CK and the change from 1 bar to 200 bar in PK (ANOVA, CK: 400-600 bar, $F(7, 21) = 4.99$, $p<0.002$; CK: 600 bar- recovery (1 bar), $F(7, 21) = 5.01$, $p<0.002$; PK: 1- 200 bar, $F(8, 33) = 2.383$, $p<0.004$).

4. Discussion

Enzymatic pressure adaptation has been displayed in many deep-sea taxa. However, the physiological effect of hydrostatic pressure on metabolic enzymes is not consistent across groups (Seibel et al., 2007). Some enzymes are accelerated by hydrostatic pressure, others decelerated, and yet some are unaffected in activity. From this we can deduce that pressure effects are unidirectional. Thus, the solution is to understand pressure adaptation to metabolic enzymes that are pressure independent. Early enzyme-pressure studies (often using mammalian or non- marine bacterial enzymes) have measured pressure effects on catalysts under conditions of co-factors and/or co-enzymes, stabilizing reactions (Siebenaller, 1984). The formal analogies between the effects of temperature and pressure are only valid under these conditions. In the case pf pressure, catalytic efficiencies change in both directions based upon volume change in the system (Gerringer et al., 2017a). This result suggests that low substrate concentrations controlling catalysts are pressure independent, irrespective of what pressure does to maximum velocity.

4.1 *Enzymatic activities at atmospheric pressure*

Enzymatic activities measured at atmospheric pressure are comparable to those measured in previous studies. The activities measured in all three of the enzymes tested (CK, PK, and MDH) were lower than the activities measured in abyssal fish (Drazen et al., 2015; Gerringer et al., 2017a). Differences in activity were seen across the three enzymes analyzed. The activities of CK and PK were similar, however MDH activities were heighted in all the species tested (Table 2). There were no patterns detected when examining the enzymatic activities of the different orders of Ctenophora. It seems that Lobata displayed the lowest enzymatic activities for each enzyme (Figure 6). Lobate ctenophore species sampled for this study had lower sample sizes, so it could be ill-advised to assume too much from this result.

4.2 *Residual enzymatic activities at pressure*

Changes in activities due to pressure related circumstances are just one other variable to consider in the use of enzymatic activities as proxies for whole animal metabolism. Pressure can be added to a long list of influential factors such as temperature, feeding strategies, locomotion, body mass or phylogeny (Childress et al., 1992; Gerringer et al., 2017a; Seibel et al., 2007; Somero et al., 2017). It must be taken into to account that pressure effects can confound results, making interpretation difficult. For example, in the results, higher CK activities in the shallow ctenophore species *P. bachei* was likely due to pressure confounding effects (Figure 3).

Results indicate that species inhabiting similar vertical ranges can display unique pressure tolerance characteristics. Some ctenophore species displayed broad pressure tolerances, while others were constrained to their respective habitat depth. The decreased residual rate followed by a spike at recovery in CK and PK activities indicate permanent conformational damage to the enzyme after the pressure has been introduced. The enzymatic rates of CK were variable across species and pressures. Of the three enzymes studied, PK was the most affected by increasing pressures. Malate dehydrogenase activity remained relatively stable as pressure was introduced to the system.

4.3 *Pressure influence on enzyme activation volume*

Changes in enzyme activation volume with hydrostatic pressure suggests that enzyme conformations contract and expand with pressure (Basilevsky et al., 1985; Gerringer et al., 2017b; Schuabb et al., 2014). Pressure induced changes in activation volume can be explained counter- intuitively: a negative change in activation volume results in pressure acceleration and positive volume change indicates pressure inhibition (Schuabb et al., 2014). The negative activation volume exhibited in MDH (Figure 7) across species suggests then enzyme may be evolutionarily adapted to high pressures. Contrarily, negative activation displayed in CK during the increase from 400 to 600 may indicate an optimal conformation for enzyme performance under pressure.

5. Conclusion

Despite the expanse and biomass associated with the deep sea, little is known about species' physiological functioning. Much of the data assembled from deep environments was procured

within the last half century, when emerging technology allowed for collection and study of live deep-sea specimens (Dunn et al., 2015; Haddock, 2004; Robison, 2004). These advances, however, have furthered many questions regarding the functional diversity and physiology of deep-sea taxa. The effect of hydrostatic pressure on the metabolic enzymes of ctenophores re-opens questions about pressure adaptation in the deep sea: What amino acid sequences are structurally adapted to tolerate pressure? How many solutions have evolved to solve the same biophysical problem of pressure? Are these adaptive evolution solutions parallel or convergent? Phylogenetically, results indicate that adaptations to moderate depth (100 m) are not necessarily convergent at the scale of a single enzyme. Further assessing functional diversity of Ctenophore metabolism will indicate parallel or convergent protein adaptation in the deep sea.

The effect of temperature on metabolic functioning is widely accepted, however, the effect of hydrostatic pressure can seem negligible when considering most of life on earth. For environments at extremely high pressures, pressure effects can be noteworthy. Identifying pressure adaptation could have implications for reconstructing metabolic theory of ecology, which explains biochemical processes in terms of temperature and body size. In the future, it would be interesting to uncover if enzymatic pressure tolerance could be used as a biochemical indicator for the phylum.

To better inform the effect of hydrostatic pressure on the metabolic enzymes of ctenophores, a more complete knowledge of ctenophore physiology is necessary. Establishing baselines for the phylum across the various depths and temperatures on a global scale can not only provide evolutionary insights but can also inform oceanic climate change models. Prediction and understanding the dynamics that cause ctenophores to aggregate will be key in understanding ctenophores may be impacted by both anthropogenic-driven climate change, and natural environmental fluxes (Childress & Thuesen, 1992; Mills, 2001).

CHAPTER THREE: CLOSING REMARKS

Conclusion

Ctenophore species sampled in this study represent five of the eight orders currently assigned in the phylum. When all specimens sampled were evaluated for enzymatic activities by minimum depth of occurrence, depth did not seem to have an effect on enzymatic activity. While there were no correlations between depth and the rate of activity, the lowest activities were seen in the order Lobata across the three enzymes examined.

Pressure-related changes in maximal reaction rate were displayed while evaluating activities from the three metabolic enzymes examined. Pressure induced changes in activity did not seem to follow a trend for the enzyme creatine kinase. However, some enzymes were accelerated by pressure while others seemed to be inhibited by pressure. Malate dehydrogenase was least affected by pressure. For enzymes like malate dehydrogenase, the stabilizing effects of extrinsic adaptations may be better established. Pyruvate kinase was the most affected by increasing pressure, with reduced activity seen across all species.

High pressure adaptation compliments the mode of reaction volume changes. Enzymes with decreased activation volume (e.g., malate dehydrogenase) may be more equipped to operate under increased pressure. Heightened values for changes in volume indicated that the enzyme may have more room to "flail" within its conformation. Despite differences in pressure adaptive volume changes seen for each enzyme, their respective protein structure is unclear.

The effect of pressure induced conformational change cannot be addressed using whole animal homogenates due to methodological limitations. Structural and mechanical aspects of proteins will have to be addressed using purified recombinant proteins from ctenophores. Recombinant protein is a manipulated form of protein encoded by a gene. They can be produced in large quantities and allow for modification of gene sequences. By using recombinant proteins, mutant ctenophore proteins can be altered to investigate which sequences are optimal for pressure. Further exploration of pressure dependencies of metabolic enzyme activities would provide insight into the structural interpretation of observed modes of adaptation.

Figures

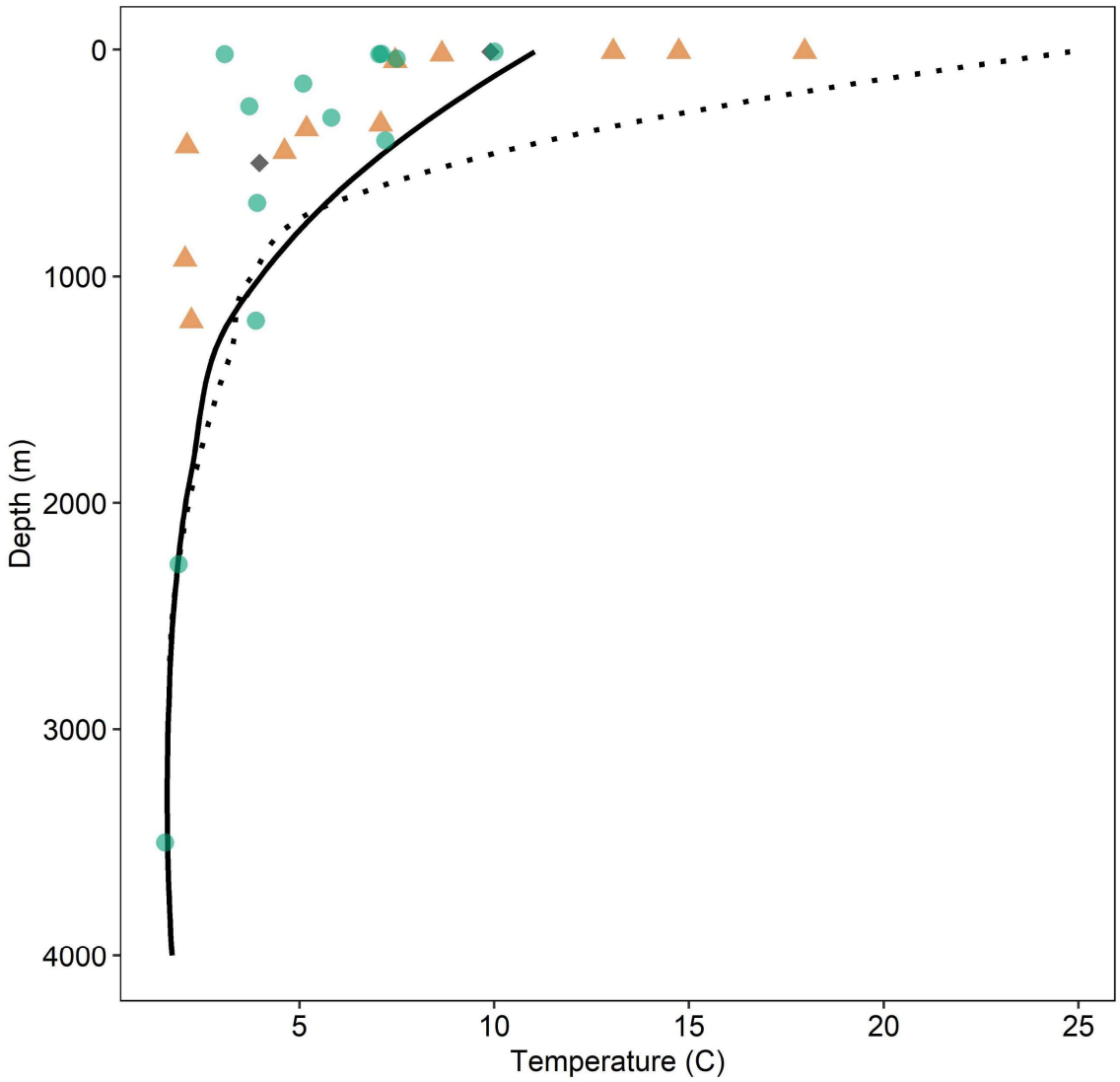

Fig. 1. Habitat depth and temperature distributions of the 27 ctenophore species collected. Species are organized by order; beroid ctenophores (◆), cydippid ctenophores (●), and lobate ctenophores (▲). Temperature profiles are shown for the two sampling locations: Monterey Bay (solid line), and Hawaii (dotted line). Samples collected at the Puget Sound location were collected at 1 m depth and 10˚C.

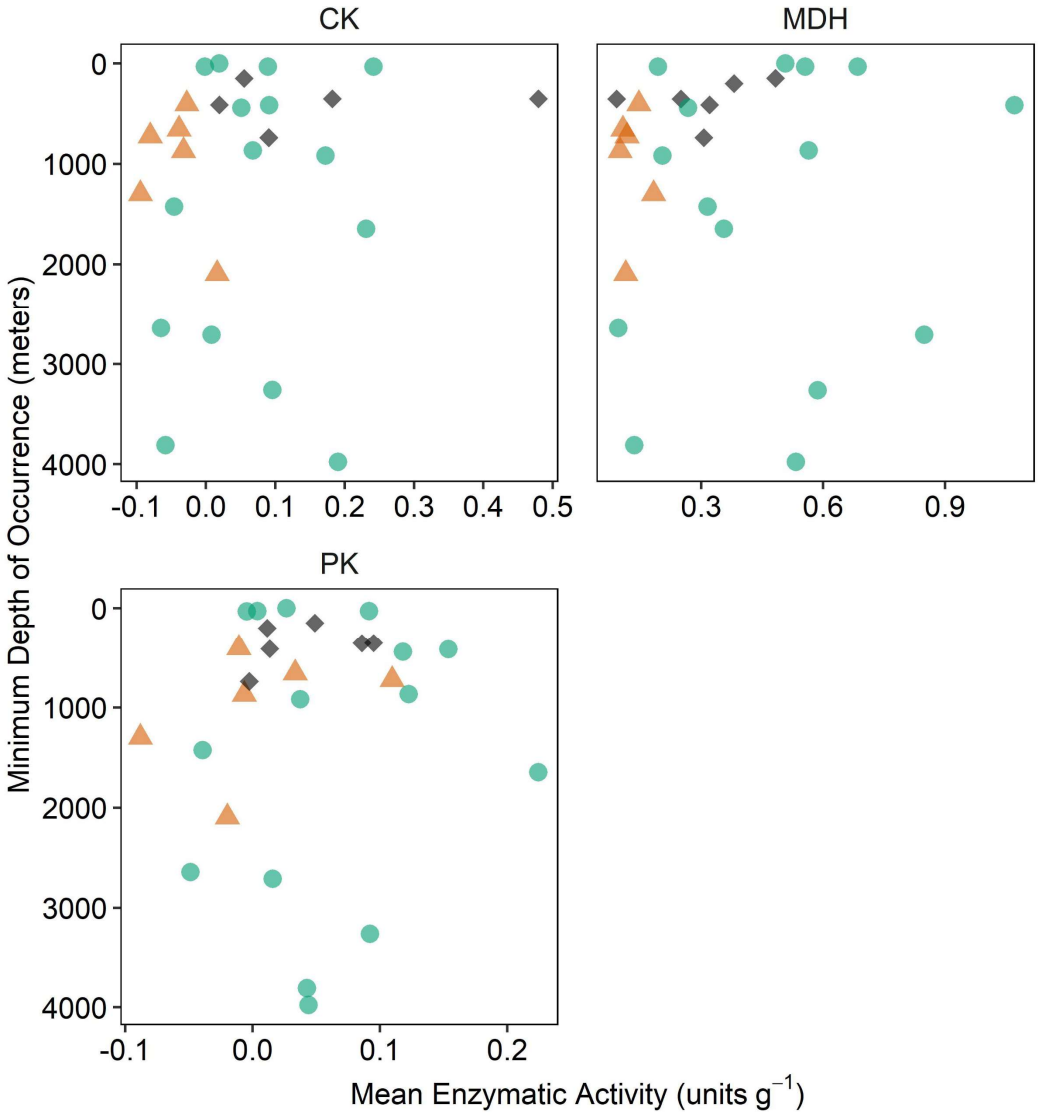

Fig. 2. Creatine kinase, malate dehydrogenase, and pyruvate kinase activities (units g^{-1} wet mass) at atmospheric pressure (1 bar) and 5˚C of 27 ctenophore species as a function of minimum depth of occurrence. Species are organized by order; beroid ctenophores (◆), cydippid ctenophores (●), and lobate ctenophores (▲).

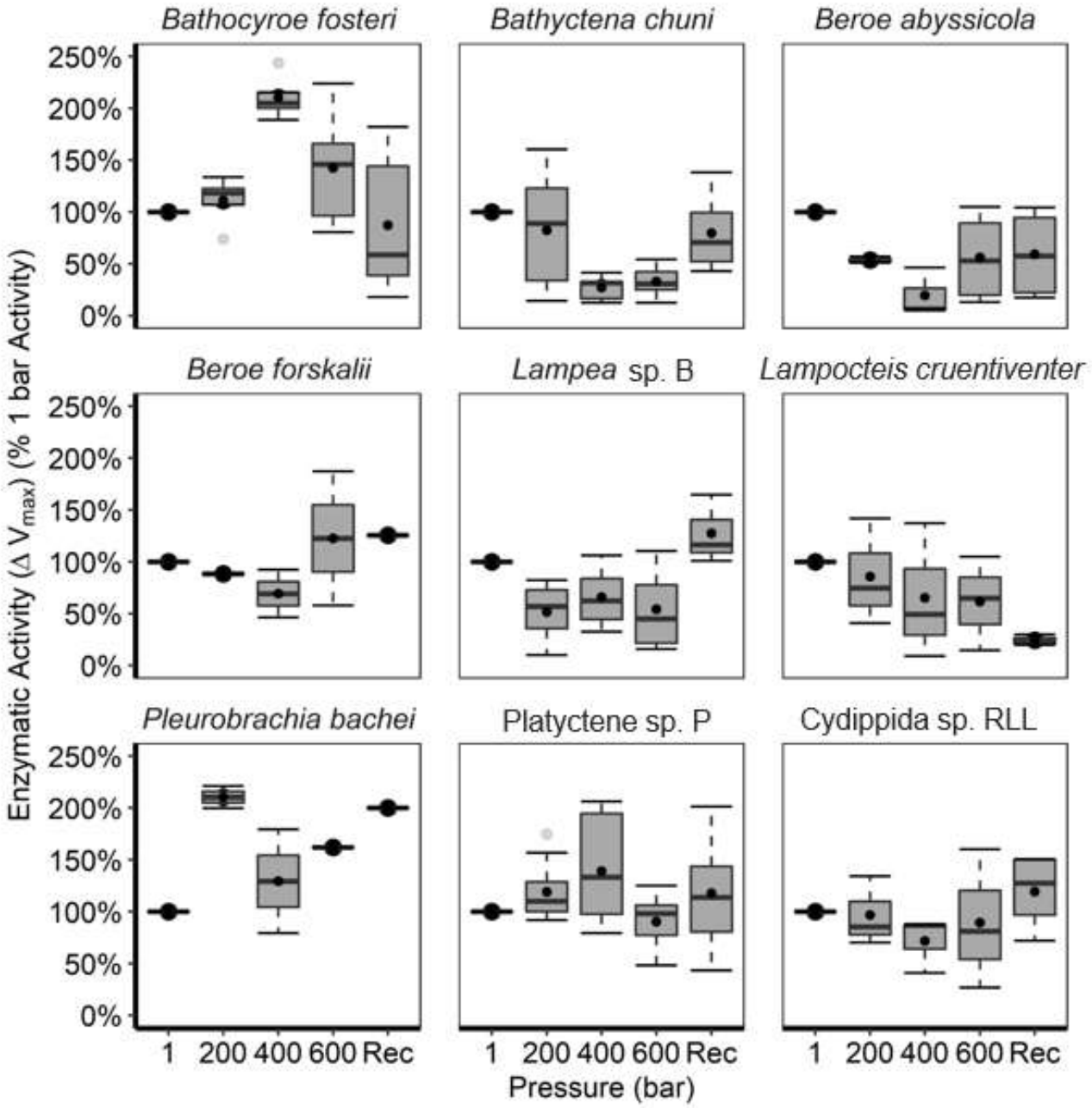

Fig. 3. Creatine kinase activity from nine ctenophore species at different pressures and at 5˚C. Results are shown in percent of activity at atmospheric pressure for each assay. Error bars show standard errors between assays. Recovery (Rec) shows the relative rate from decompression to 1 bar pressure.

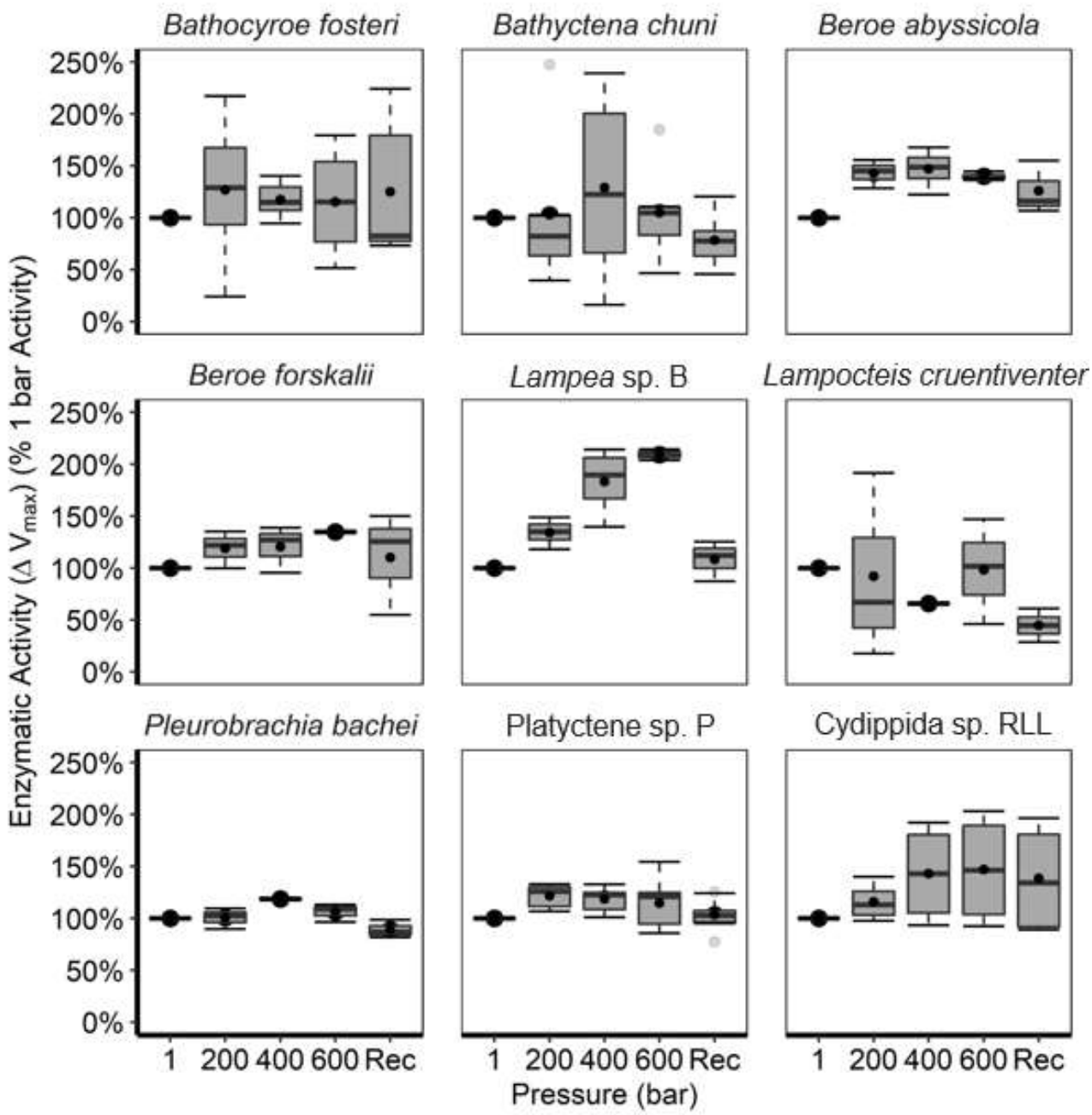

Fig. 4. Malate dehydrogenase activity from nine ctenophore species at different pressures and at 5˚C. Results are shown in percent of activity at atmospheric pressure for each assay. Error bars show standard errors between assays. Recovery (Rec) shows the relative rate from decompression to 1 bar pressure.

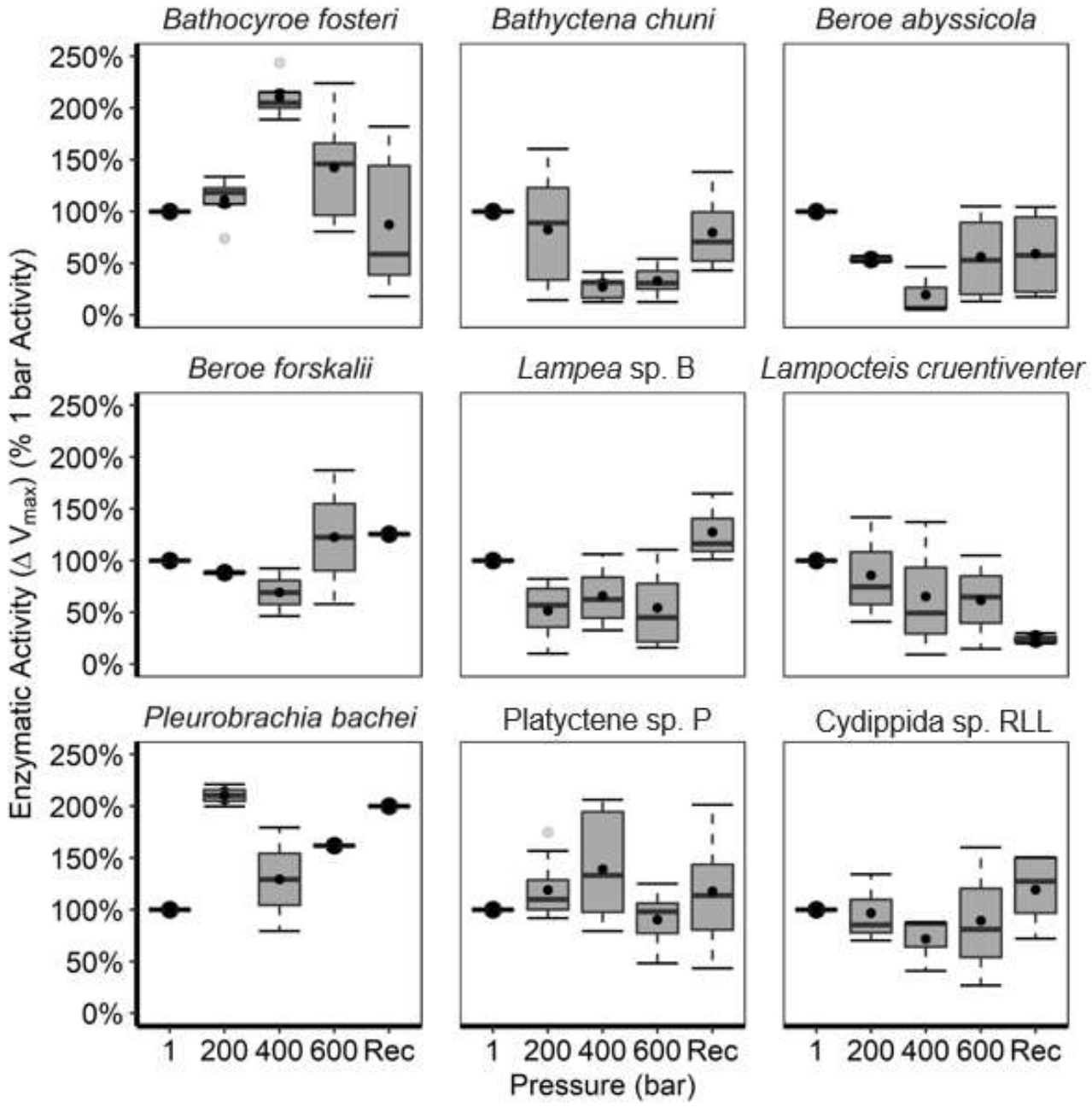

Fig. 5. Pyruvate kinase activity from nine ctenophore species at different pressures and at 5˚C. Results are shown in percent of activity at atmospheric pressure for each assay. Error bars show standard errors between assays. Recovery (Rec) shows the relative rate from decompression to 1 bar pressure.

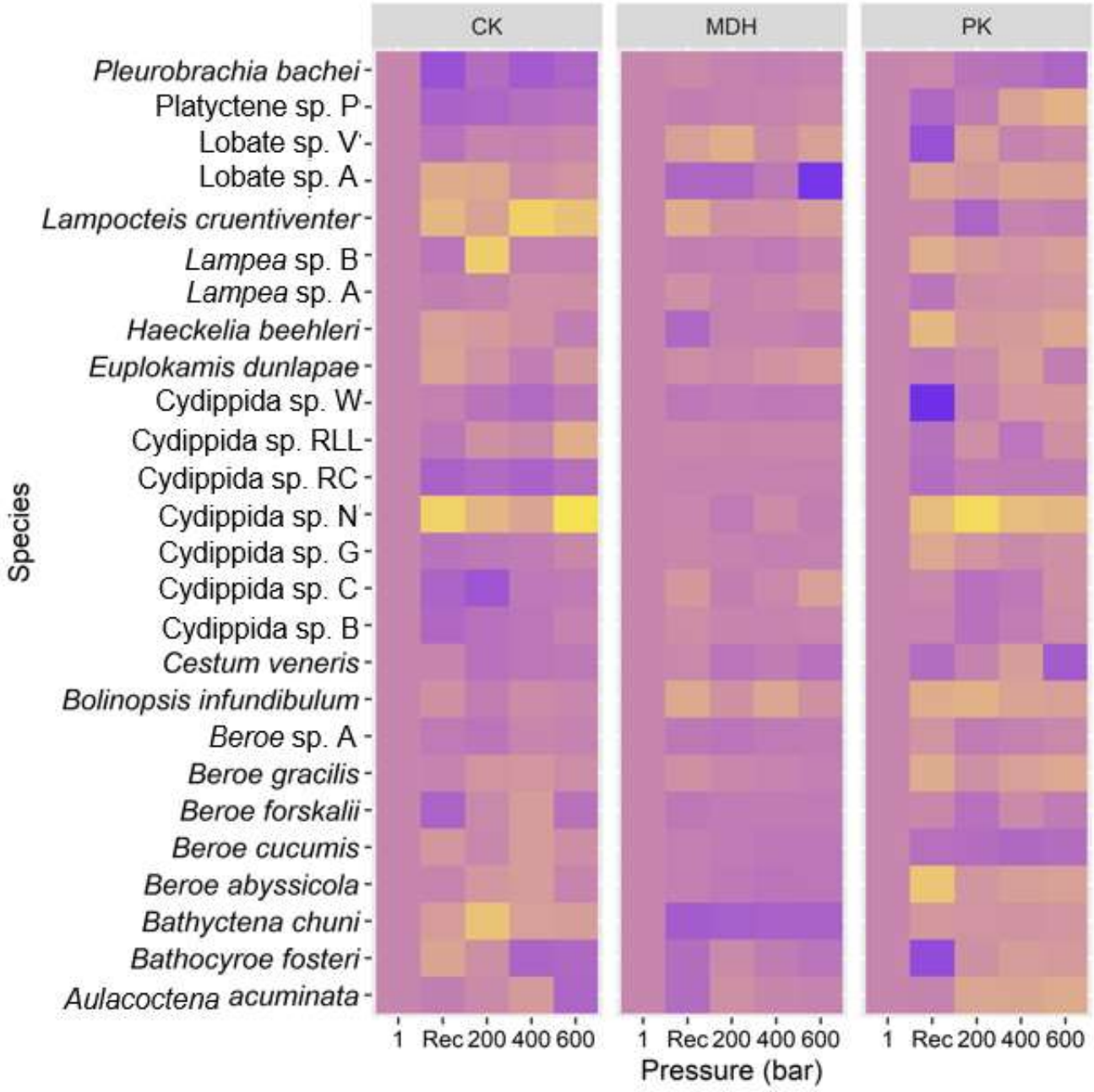

Fig. 6. High (—) , medium (—), and low (—) enzymatic activities for 27 ctenophore species at five pressures and at 5˚C. Enzymatic activities at pressure are determined as high, medium or low activity based on interquartile ranges caculated for each speceies and enzyme in relation to initial pressure (1 bar). Recovery (Rec) shows the relative rate from decompression to 1 bar pressure.

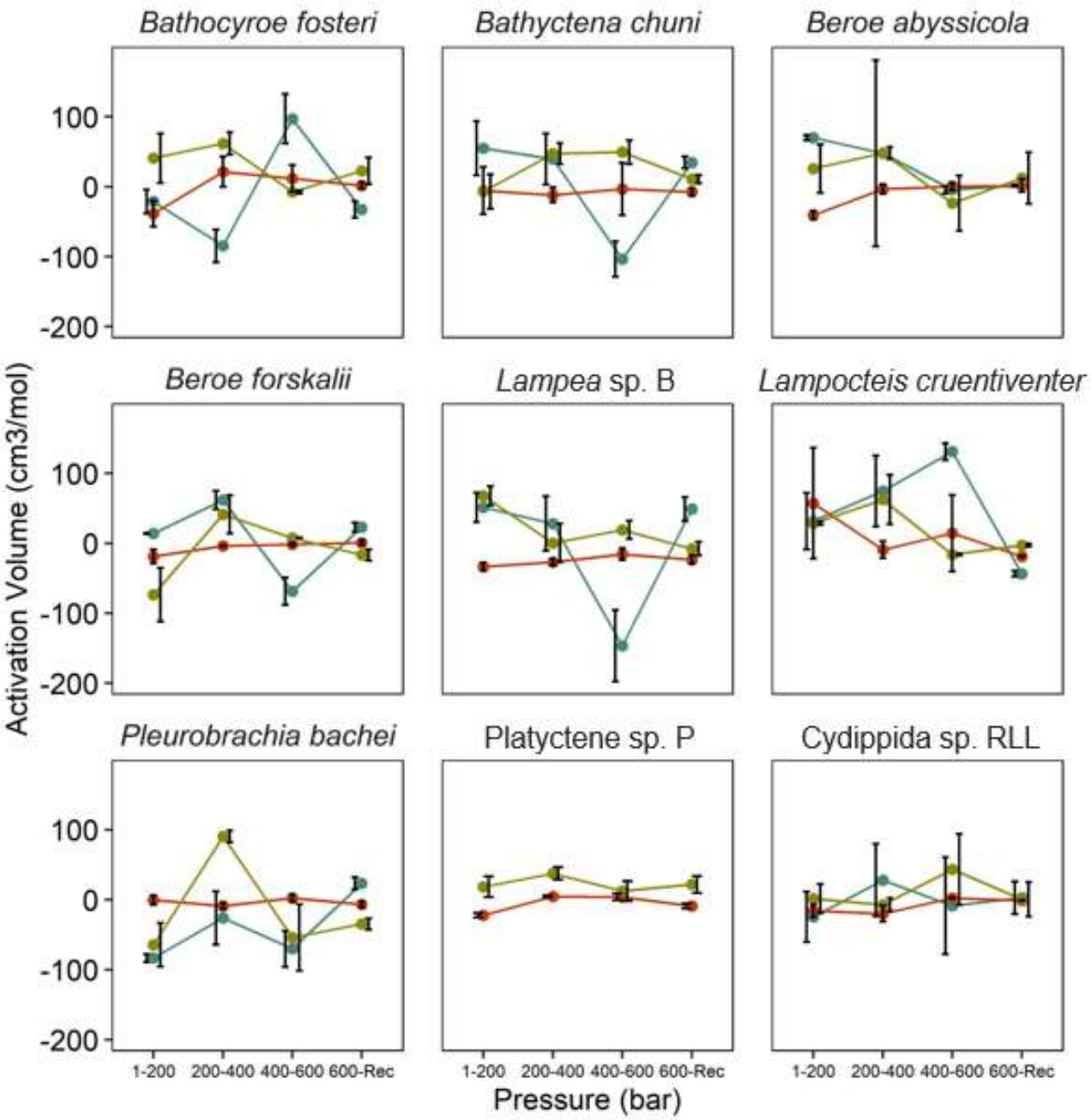

Fig. 7. Change in activation volume between pressures for creatine kinase (●), malate dehydrogenase (●), and pyruvate kinase (●) of nine ctenophore species. The change in volume is calculated using the enzymatic rate at each pressure.

Tables

Table 1. Collection information for the 27 species of ctenophores sampled. Species minimum depth of occurrence (MDO) is taken from MBARI's VARS database. Standard mass is taken from frozen ctenophore samples. N indicates the number of individuals with measured CK, MDH, and PK activities.

Species	MDO (m)	n	Mass (g)	Location
Beroida				
Beroidae				
Beroe abyssicola	500	6	0.93-23.648	Hawaii, Monterey Bay
Beroe cucumis	200	2	0.491-11.117	Hawaii, Monterey Bay
Beroe forskalii	15	7	1.139-42.727	Monterey Bay
Beroe gracilis	11.5	2	0.048-3.08	Hawaii, Monterey Bay
Beroe sp. A	411	2	1.83-6.77	Monterey Bay
Cydippida				
Bathyctenidae				
Aulacoctena acuminata	1200	2	202.8-424.67	Monterey Bay
Bathyctena chuni	925	7	0.89-3.823	Hawaii
Euplokamididae				
Euplokamis dunlapae	250	2	0.07-0.16	Hawaii, Monterey Bay
Haeckeliidae				
Haeckelia beehleri	20	1	0.266-0.968	Monterey Bay
Lampeidae				
Lampea sp. A	150	3	0.944-3.9	Monterey Bay
Lampea sp. B	2270	4	0.61-5.218	Monterey Bay
Pleurobrachiidae				
Pleurobrachia bachei	10	3	0.604-0.847	Puget Sound

Undescribed Cydippida				
Cydippida sp. B	2500	2	2.317-6.791	Monterey Bay
Cydippida sp. C	350	6	0.064-0.212	Monterey Bay
Cydippida sp. G	2710	1	4.27	Monterey Bay
Cydippida sp. N	300	1	.08	Monterey Bay
Cydippida sp. RC	676	1	6.14	Monterey Bay
Cydippida sp. RLL	1690	4	0.248-4.356	Monterey Bay
Cydippida sp. W	300	2	0.048-2.395	Monterey Bay
Platyctenida				
Tjalfiellidae				
Platyctene sp. P	3500	11	3.55-24.99	Monterey Bay
Cestida				
Cestidae				
Cestum veneris	10	2	0.172-82.13	Hawaii, Monterey Bay
Lobata				
Bathocyroidae				
Bathocyroe fosteri	425	6	1.88-51.03	Hawaii, Monterey Bay
Bolinopsidae				
Bolinopsis infundibulum	50	6	0.666-50.62	Hawaii, Monterey Bay, Puget Sound
Eurhamphaeidae				
Kiyohimea usagi	330	2	147.539-258.771	Monterey Bay
Lampoctenidae				
Lampocteis cruentiventer	450	3	15.32-34.93	Monterey Bay
Lobate sp. A	869	1	57.38	Monterey Bay
Lobate sp. V	1200	2	14.97-42.8	Hawaii, Monterey Bay

Table 2

Maximal activities of the metabolic enzymes creatine kinase, malate dehydrogenase, and pyruvate kinase from ctenophore specimens in whole animal wet weight. Enzymatic activities shown here were measured using the pressure cuvette system at atmospheric pressure and 5°C. Errors are presented as standard error. Activities are presented in Units per gram^{-1} wet weight.

	Enzymatic activity (1 bar)								
	CK			MDH			PK		
	mean	±SE	n	mean	±SE	n	mean	±SE	n
Beroida									
Beroidae									
Beroe abyssicola	0.04	±0.013	4	0.279	±0.109	4	0.044	±0.004	4
Beroe Cucumis	*na*		2	0.393	±0.006	2	0.054	±0.012	2
Beroe forskalii	0.03	±0.027	3	0.401	±0.089	3	0.032	±0.01	
Beroe gracilis	0.248		1	0.053		1	0.051		1
Beroe sp. A	0.025	±0.008	2	0.394	±0.109	2	0.032	±0.006	2
Cydippida									
Bathyctenidae									
Aulacoctena acuminata	0.018	±0.012	2	0.034	±0.005	2	0.0114	±0.010	2
Bathyctena chuni	0.075	±0.023	7	0.107	±0.019	7	0.088	±0.023	7
Euplokamididae									
Euplokamis dunlapae	0.139	±0.042	2	0.450	±0.142	2	0.125	±0.052	2
Haeckeliidae									
Haeckelia beehleri	0.032		1	0.66		1	0.065		1
Lampeidae									
Lampea sp. A	0.014	±0.005	2	0.550	±0.037	2	0.118	±0.051	2
Lampea sp. B	0.015	±0.038	4	0.473	±0.092	4	0.027	±0.011	4
Pleurobrachiidae									

Pleurobrachia bachei	0.018	±0.009	3	0.47	±0.102	3	0.025	±0.0007	3
Undescribed Cydippida									
Cydippida sp. B	0.017		1	0.546		1	0.031		1
Cydippida sp. C	0.123	±0.059	3	0.992	±0.386	3	0.098	±0.048	3
Cydippida sp. G	0.052		1	0.852		1	0.02		1
Cydippida sp. N	0.135		1	0.637		1	0.214		1
Cydippida sp. RC	0.009		1	0.175		1	0.023		1
Cydippida sp. RLL	0.025	±0.011	4	0.401	±0.13	4	0.016	±0.005	4
Cydippida sp. W	0.011	±0.004	2	0.271	±0.05	2	0.076	±0.033	2
Platyctenida									
Tjalfiellidae									
Platyctene sp. P	0.127	±0.054	11	0.522	±0.056	11	0.103	±0.017	11
Cestida									
Cestidae									
Cestum veneris	0.155	±0.138	2	0.11	±0.019	2	0.02	±0.004	2
Lobata									
Bathocyroidae									
Bathocyroe fosteri	0.006	±0.002	6	0.032	±0.007	6	0.014	±0.004	6
Bolinopsidae									
Bolinopsis infundibulum	0.023	±0.004	2	0.229	±0.048	2	0.05	±0.002	2
Lampoctenidae									
Lampocteis cruentiventer	0.021	±0.008	2	0.03	±0.012	3	0.029	±0.013	3
Lobate sp. A	0.008		1	0.015		1	0.011		1
Lobate sp. V	0.005	±0.003	2	0.161	±0.111	2	0.009	±0.0008	2

Table 3

Maximal activities of creatine kinase (CK) from whole ctenophore specimens at increasing pressures (200-600 bar) and recovery (atmospheric pressure (1 bar)) and 5˚C. Activities are presented as residual rates relative to the initial activity. SE: standard error; n: number of individuals measured.

	Enzymatic activity											
	CK200			CK400			CK600			Recovery		
	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n
Beroida												
Beroidae												
Beroe abyssicola	0.536	±0.017	3	0.195	±0.134	3	0.559	±0.227	4	0.59	±0.224	4
Beroe cucumis	0.809	±0.146	2	3.022	±2.526	2	1.432	±0.697	2	2.154	±1.599	2
Beroe forskalii	0.884	±0.007	2	0.692	±0.229	2	1.225	±0.646	2	2.065	±0.809	2
Beroe gracilis	1.623		1	1.836		1	1.766		1	2.138		1
Beroe sp. A	1.219	±0.418	2	0.876	±0.118	2	0.819	±0.275	2	1.144	±0.266	2
Cydippida												
Bathyctenidae												
Aulacoctena acuminata	0.835	±0.011	2	0.96	±0.444	2	2.067	±0.715	2	2.03	±0.813	2
Bathyctena chuni	0.824	±0.221	7	0.269	±0.054	5	0.328	±0.061	6	0.795	±0.149	6

Euplokamididae												
Euplokamis dunlapae	0.643	±0.012	2	1.087	±0.156	2	0.411	±0.145	2	0.725	±0.343	2
Haeckeliidae												
Haeckelia beehleri	0.517		1	0.72		1	1.244		1	0.416		1
Lampeidae												
Lampea sp. A	1.274	±0.173	2	1.312	±0.583	2	1.404	±0.66	2	7.268	±6.035	2
Lampea sp. B	0.515	±0.159	4	0.658	±0.161	4	0.541	±0.218	4	1.273	±0.191	3
Pleurobrachiidae												
Pleurobrachia bachei	2.104	±0.107	2	2.156	±0.91	2	2.233	±0.613	3	3.706	±1.704	2
Undescribed Cydippida												
Cydippida sp. B	1.953		1	1.596		1	1.105		1	2.571		1
Cydippida sp. C	0.4	±0.131	2	0.857	±0.371	3	0.764	±0.342	3	0.414	±0.295	2
Cydippida sp. G	1.451		1	1.35		1	0.919		1	1.776		1
Cydippida sp. N	0.21		1	0.362		1	0.053		1	0.084		1
Cydippida sp. RC	2.333		1	3.025		1	1.897		1	3.165		1
Cydippida sp. RLL	0.965	±0.193	3	0.717	±0.154	3	0.893	±±0.386	3	1.194	±0.19	4
Cydippida sp. W	1.247	±0.55	2	1.82	±0.532	2	1.244	±0.211	2	0.721	±0.404	2
Platyctenida												
Tjalfiellidae												

Platyctene sp. P	1.188	±0.097	9	1.39	±0.152	11	0.903	±0.092	9	1.177	±0.158	10
Cestida												
Cestidae												
Cestum veneris	1.16	±0.701	2	1.029	±0.576	2	0.897	±0.545	2	1.10	±0.101	2
Lobata												
Bathocyroidae												
Bathocyroe fosteri	0.129	±0.0052	4	2.104	±0.117	4	1.62	±0.286	6	0.87	±0.288	6
Bolinopsidae												
Bolinopsis infundibulum	0.998	±0.272	2	0.657	±0.157	2	0.598	±0.353	2	0.935	±0.244	2
Lampoctenidae												
Lampocteis cruentiventer	0.856	±0.296	3	0.651	±0.378	3	0.614	±0.261	3	0.241	±0.03	3
Lobate sp. A	0.317		1	0.818		1	0.64		1	0.289		1
Lobate sp. V	1.829	±0.794	2	12.18	±11.052	2	1.115	±0.19	2	1.721	±0.116	1

Table 4

Maximal activities of malate dehydrogenase (MDH) from whole ctenophore specimens at increasing pressures (200-600 bar) and recovery (atmospheric pressure (1 bar)) and 5˚C. Activities are presented as residual rates relative to the initial activity. SE: standard error; n: number of individuals measured.

	Enzymatic activity											
	MDH200			MDH400			MDH600			Recovery		
	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n
Beroida												
Beroidae												
Beroe abyssicola	1.43	±0.078	3	1.468	±0.096	3	1.398	±0.023	4	1.258	±0.147	3
Beroe cucumis	1.227	±0.13	2	1.304	±0.231	2	1.204	±0.333	2	1.118	±0.057	2
Beroe forskalii	1.188	±0.103	3	1.203	±0.129	3	1.349	±0.008	2	1.101	±0.284	3
Beroe gracilis	0.891		1	0.894		1	0.64		1	0.463		1
Beroe sp. A	1.351	±0.384	2	1.226	±0.204	2	1.212	±0.136	2	1.398	±0.159	2
Cydippida												
Bathyctenidae												
Aulacoctena acuminata	0.888	±0.174	2	1.264	±0.299	2	0.927	±0.153	2	2.319	±0.078	2
Bathyctena chuni	1.027	±0.305	6	1.55	±0.402	6	1.047	±0.188	7	0.783	±0.105	6
Euplokamididae												

Euplokamis dunlapae	1.397	±0.513	2	0.688	±0.033	2	0.533	±0.037	2	0.752	±0.032	2
Haeckeliidae												
Haeckelia beehleri	1.06		1	1.086		1	1.245		1	2.528		1
Lampeidae												
Lampea sp. A	1.189	±0.128	2	1.093	±0.157	2	0.93	±0.232	2	0.796	±0.09	2
Lampea sp. B	1.341	±0.066	4	1.832	±0.166	4	2.274	±0.185	3	1.082	±0.111	3
Pleurobrachiidae												
Pleurobrachia bachei	1.005	±0.058	2	1.186	±0.009	2	1.061	±0.05	3	0.89	±0.049	2
Undescribed Cydippida												
Cydippida sp. B	1.002		1	1.019		1	0.954		1	0.762		1
Cydippida sp. C	1.013	±0.111	3	0.905	±0.171	3	0.915	±0.253	3	0.616	±0.074	2
Cydippida sp. G	1.087		1	1.161		1	1.113		1	0.927		1
Cydippida sp. N	1.405		1	0.805		1	1.23		1	0.958		1
Cydippida sp. RC	1.073		1	1.067		1	1.144		1	1.058		1
Cydippida sp. RLL	1.159	±0.094	4	1.429	±0.243	4	1.47	±0.275	4	1.384	±0.277	4
Cydippida sp. W	1.139	±0.24	2	1.278	±0.249	2	1.261	±0.147	2	1.135	±0.37	2
Platyctenida												
Tjalfiellidae												
Platyctene sp. P	1.216	±0.032	10	1.187	±0.036	10	1.146	±0.073	9	1.033	±0.049	9

Cestida												
Cestidae												
Cestum veneris	1.339	±0.402	2	1.081	±0.272	2	1.393	±0.512	2	0.813	±0.084	2
Lobata												
Bathocyroidae												
Bathocyroe fosteri	1.267	±0.277	6	1.172	±0.07	6	1.152	±0.208	6	1.248	±0.294	6
Bolinopsidae												
Bolinopsis infundibulum	0.977	±0.311	2	0.628	±0.282	2	0.914	±0.221	2	0.732	±0.424	2
Lampoctenidae												
Lampocteis cruentiventer	0.92	±0.517	3	0.658	±0.005	2	0.982	±0.292	3	0.446	±0.161	2
Lobate sp. A	2.755		1	1.459		1	12.095		1	2.646		1
Lobate sp. V	0.291	±0.019	2	0.758	±0.057	2	0.465	±0.047	2	0.346	±0.086	2

Table 5

Maximal activities of pyruvate kinase (PK) from whole ctenophore specimens at increasing pressures (200-600 bar) and recovery (atmospheric pressure (1 bar)) and 5˚C. Activities are presented as residual rates relative to the initial activity. SE: standard error; n: number of individuals measured.

	Enzymatic activity											
	PK200			PK400			PK600			Recovery		
	mean	±SE	n	mean	±SE	n	mean	±SE	n	mean	±SE	n
Beroida												
Beroidae												
Beroe abyssicola	0.639	±0.131	3	0.602	±0.253	3	0.502	±0.185	4	0.206	±0.073	3
Beroe cucumis	1.368	±0.742	2	1.403	±1.091	2	1.589	±0.647	2	1.243	±0.741	2
Beroe forskalii	2.118	±0.676	3	0.817	±0.042	2	1.04	±0.314	2	0.999	±0.224	3
Beroe gracilis	0.46		1	2.607		1	3.148		1	3.452		1
Beroe sp. A	1.585	±0.224	2	1.507	±0.384	2	1.278	±0.388	2	1.539	±0.907	2
Cydippida												
Bathyctenidae												
Aulacoctena acuminata	2.143	±1.791	2	1.156	±0.796	2	3.122	±2.82	2	2.714	±1.658	2
Bathyctena chuni	0.839	±0.189	6	0.749	±0.099	5	0.494	±0.097	7	0.587	±0.122	5
Euplokamididae												

Euplokamis dunlapae	0.611	±0.256	2	0.643	±0.195	2	1.171	±0.047	2	0.901	±0.35	2
Haeckeliidae												
Haeckelia beehleri	0.533		1	0.508		1	0.35		1	0.2		1
Lampeidae												
Lampea sp. A	0.939	±0.227	2	0.808	±0.159	2	0.571	±0.008	2	1.067	±0.584	2
Lampea sp. B	0.5	±0.038	3	0.473	±0.092	4	0.659	±0.149	4	0.574	±0.18	4
Pleurobrachiidae												
Pleurobrachia bachei	1.89	±0.514	3	1.219	±0.395	3	1.877	±0.503	3	0.915	±0.016	2
Undescribed Cydippida												
Cydippida sp. B	1.891		1	1.313		1	0.801		1	1.033		1
Cydippida sp. C	1.77	±0.497	3	1.246	±0.17	3	1.239	±0.323	3	0.882	±0.026	2
Cydippida sp. G	0.589		1	0.858		1	0.707		1	0.328		1
Cydippida sp. N	0.065		1	0.165		1	0.196		1	0.166		1
Cydippida sp. RC	1.311		1	1.337		1	1.386		1	2.205		1
Cydippida sp. RLL	1.035	±0.185	3	1.463	±0.088	3	1.052	±0.35	3	1.021	±0.328	4
Cydippida sp. W	0.6	±0.509	2	0.331	±0.247	2	0.336	±0.196	2	7.004	±6.983	2
Platyctenida												
Tjalfiellidae												
Platyctene sp. P	0.92	±0.107	11	0.677	±0.11	10	0.597	±0.114	11	0.861	±0.151	9

Cestida												
Cestidae												
Cestum veneris	1.075	±0.001	2	1.02	±0.557	2	3.191	±0.67	2	3.112	±0.96	2
Lobata												
Bathocyroidae												
Bathocyroe fosteri	0.523	±0.153	5	0.386	±0.101	6	0.524	±0.011	4	0.575	±0.164	5
Bolinopsidae												
Bolinopsis infundibulum	0.452	±0.21	2	0.359	±0.03	2	0.341	±0.083	2	0.295	±0.011	2
Lampoctenidae												
Lampocteis cruentiventer	0.779	±0.016	2	0.747	±0.182	3	0.967	±0.11	3	0.978	±0.003	2
Lobate sp. A	0.549		1	0.386		1	0.4		1	0.391		1
Lobate sp. V	0.465	±0.054	2	0.771	±0.3	2	0.902	±0.047	2	2.719	±2.426	2

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