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Metabolic enzyme activities of abyssal and hadal fishes: pressure effects and a re-evaluation of depth-related changes

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ABSTRACT

Metabolic enzyme activities of muscle tissue have been useful and widely-applied indicators of whole animal metabolic capacity, particularly in inaccessible systems such as the deep sea. Previous studies have been conducted at atmospheric pressure, regardless of organism habitat depth. However, maximum reaction rates of some of these enzymes are pressure dependent, complicating the use of metabolic enzyme activities as proxies of metabolic rates. Here, we show pressure-related rate changes in lactate and malate dehydrogenase (LDH, MDH) and pyruvate kinase (PK) in six fish species (2 hadal, 2 abyssal, 2 shallow). LDH maximal reaction rates decreased with pressure for the two shallow species, but, in contrast to previous findings, it increased for the four deep species, suggesting evolutionary changes in LDH reaction volumes. MDH maximal reaction rates increased with pressure in all species (up to 51 ± 10% at 60 MPa), including the tide pool snailfish, *Liparis florae* (activity increase at 60 MPa 44 ± 9%), suggesting an inherent negative volume change of the reaction. PK was inhibited by pressure in all species tested, including the hadal liparids (up to 34 ± 3% at 60 MPa), suggesting a positive volume change during the reaction. The addition of 400 mM TMAO counteracted this inhibition at both 0.5 and 2.0 mM ADP concentrations for the hadal liparid, *Notoliparis kermadecensis*. We revisit depth-related trends in metabolic enzyme activities according to these pressure-related rate changes and new data from seven abyssal and hadal species from the Kermadec and Mariana trenches. Results show that, with abyssal and hadal species, pressure-related rate changes are another variable to be considered in the use of enzyme activities as proxies for metabolic rate, in addition to factors such as temperature and body mass. Intraspecific increases in tricarboxylic acid cycle enzymes with depth of capture, independent of body mass, in two hadal snailfishes suggest improved nutritional condition for individuals deeper in the hadal zone, likely related to food availability. These new data inform the discussion of factors controlling metabolism in the deep sea, including the visual interactions hypothesis and extend published trends to the planet's deepest-living fishes.

1. Introduction

Certain citric acid cycle and glycolysis enzymes have been commonly used as proxies for whole-animal metabolic capacity and activity (Childress and Somero, 1979; Sullivan and Smith, 1982; Dickson et al., 1993; Vetter and Lynn, 1997; Hickey and Clements, 2003; Dahlhoff, 2004; Friedman et al., 2012; Torres et al., 2012; Ombres et al., 2011; Condon et al., 2012; Drazen et al., 2015; Saavedra et al., 2015). This technique has been particularly valuable in deep-sea systems, due to the logistical constraints of traditional measurements of metabolic rate, such as the monitoring of oxygen consumption, although a few of these data exist at great depths (e.g. Smith et al., 1978; Hughes et al., 2011; Drazen and Yeh, 2012). Four major metabolic enzyme activities (maximal reaction rate) are typically used to estimate metabolic rate, to estimate relative metabolic capacity, or as indices of metabolic capacity—lactate dehydrogenase (LDH), pyruvate kinase (PK), citrate synthase (CS), and malate dehydrogenase (MDH). LDH, which catalyzes the conversion of pyruvate to lactate during anaerobic glycolysis followed by fermentation and the reverse reaction of lactate to pyruvate during recovery from anaerobiosis, and PK, which catalyzes an ATP-yielding step in glycolysis, are used as proxies to indicate burst locomotory capability and anaerobic capacity (Childress and Somero, 1979; Dahlhoff, 2004). The activities of the tricarboxylic acid (TCA) cycle enzymes, CS and MDH, are applied as indicators of routine metabolic rate and aerobic activity (Somero and Childress, 1980; Childress and Thuesen, 1992; Thuesen and Childress, 1993).

The most common use of enzyme activities in deep sea animals has been to evaluate changes in metabolic capacity with depth (e.g.

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Childress and Somero, 1979; Sullivan and Somero, 1980; Siebenaller et al., 1982). Many taxa such as pelagic cephalopods, shrimps, and fishes, as well as benthic fishes, show declines in both measured respiration rates and metabolic enzyme activities in white and red muscle (e.g. Childress and Thuesen, 1992; Thuesen and Childress, 1993; Drazen et al., 2015). These declines are hypothesized to reflect a decrease in metabolic rate, which has been attributed to a reduction in food supply with depth (Smith et al., 1978; Siebenaller and Yancey, 1984) and/or reduced predator-prey interaction distances with declining light levels, known as the visual interactions hypothesis (Childress, 1995; Seibel and Drazen, 2007). The latter hypothesis suggests that in dark environments, where interaction distances are short, there is limited selective pressure for high locomotory capacities, explaining the declines in metabolic activities with depth that are not otherwise accounted for by temperature and body mass. This hypothesis was recently supported by an analysis of 61 species of benthic and benthopelagic fishes ranging from 50 to 3180 m depth, using a standardized methodology of measuring metabolic enzyme activities (Drazen et al., 2015).

Conclusions of these studies rely on the assumptions not only that metabolic enzyme activities are indeed indicators of metabolic capacity, but also that rates of these metabolic enzymes at atmospheric hydrostatic pressure reflect those at *in situ* pressures. However, the effects of pressure on enzyme catalysis can be non-linear and complex (reviewed by Mozhaev et al., 1996), calling the assumption that maximum reaction rates would not change with pressure into question. Half-saturation constants (K_m) for NADH of A_4-lactate dehydrogenase (originally termed M_4), which catalyzes the conversion of pyruvate to lactate to convert NADH to NAD^+ in anaerobic glycolysis and fermentation, have been shown in a number of deep-sea fish species to be either insensitive or less sensitive to pressure than orthologs from shallow species (Siebenaller and Somero, 1979; Somero and Siebenaller, 1979; Siebenaller, 1984; Dahlhoff et al., 1990; Brindley et al., 2008). A similar insensitivity was discovered in other important metabolic enzymes of deep-sea fishes—MDH (Dahlhoff and Somero, 1991) and phosphofructokinase (PFK; Moon et al., 1971a). These types of studies have suggested that pressure insensitivity in deep-sea species comes at the cost of a reduced catalytic efficiency (Somero and Siebenaller, 1979; Hennessey and Siebenaller, 1985). Enzyme concentration can be increased to offset the effects of lower catalytic efficiencies (capacity adaptations), so tissue-specific maximum reaction rate has not been hypothesized to change with pressure in fishes. However, at least 25 enzymes are known to exhibit increased maximum activity under pressure. Most of these have been isolated from piezophilic microbes (Eisenmenger and Reyes-De-Corcuera, 2009; Luong and Winter, 2015), but at least one animal enzyme has this property: a cellulase from the hadal amphipod *Hirondellea gigas* reportedly increased activity at 100 MPa (their habitat pressure in the Mariana Trench) relative to atmospheric pressure (Kobayashi et al., 2012).

In other contrasting studies, other enzymes appear to lack intrinsic pressure adaptations or are only partially adapted, and so may require protection from pressure by factors extrinsic to the protein, i.e., other cellular molecules. For example, K_m of ADP (but not maximum reaction rate, V_{max}) for PK in both shallow and deep-sea fish and anemones was found to be equally, and greatly, inhibited by pressure, such that higher ADP concentrations than in routine assay buffers are needed to achieve V_{max} (Yancey et al., 2001, 2004). However, in the presence of the osmolyte trimethylamine oxide (TMAO)—which is high in the deep-sea animals from which PK was tested (Kelly and Yancey 1999)—K_m of ADP was largely restored under pressure. TMAO was designated a 'piezolyte' ('pressure solute') for this property (Martin et al., 2002), which arises from TMAO's enhancing effects on water structure (reviewed by Yancey and Siebenaller. 2015). Unlike PK, LDHs appear to rely on both intrinsic and extrinsic adaptations. As noted earlier, K_m of NADH for LDH from many deep-sea fishes is more resistant to pressure than for shallow orthologs, but is still somewhat sensitive. However, full counteraction of this residual pressure inhibition was found with TMAO at *in situ* concentrations (Gillett et al., 1994; Yancey et al., 2004). Despite these and findings for other taxa, the effects of pressure on enzyme maximum reaction rates (as opposed to K_m) have been considered negligible in studies of metabolic rate. Moreover, enzyme kinetic responses to pressure in fishes at *in situ* habitat pressures greater than 40 MPa have not been explored.

To inform the discussion of metabolic capacity declines with depth, we use recent collections from the Mariana and Kermadec trenches to extend the published depth range of metabolic enzyme activities for fishes from ~3000 to almost 8000 m (*in situ* pressure ~80 MPa), approaching the likely depth limit for bony fishes (Yancey et al., 2014; Linley et al., 2016). The inclusion of hadal species in this analysis also allows the exploration of two additional factors that may affect metabolic rates besides light levels: food availability and hydrostatic pressure. In terms of food supply, although the deep sea is generally considered a food-limited environment, the topography of hadal trenches is hypothesized to facilitate the accumulation of organic matter (George and Higgins, 1979; Danovaro et al., 2003; Jamieson et al., 2011; Ichino et al., 2015). This is comparable to submarine canyons, which channel organic material, resulting in high faunal abundance, biomass and diversity (e.g. De Leo et al., 2010). In subducting trenches, downslope transport is enhanced by seismic activity and internal tides, resulting in the deposition of material into the trench (Itou et al., 2000; Oguri et al., 2013; Turnewitsch et al., 2014). The depositional characteristics of the hadal zone likely allow trenches to support higher biomass than the surrounding abyss (Wolff, 1970; Beliaev, 1989; Jamieson et al., 2010), as seen in increased amphipod (Jamieson, 2015) and meiofaunal (Danovaro et al., 2002; Itoh et al., 2011) abundances with depth and high rates of sediment community oxygen consumption (Glud et al., 2013; Wenzhöfer et al., 2016). This increased food availability may be a strong evolutionary driver to inhabit greater depths for a number of animals, particularly for the amphipod-feeding hadal snailfishes (Linley et al., 2017; Gerringer et al., 2017). According to previous analyses, neither food availability nor pressure is expected to affect metabolic rate in the deep sea interspecifically (reviewed by Seibel and Drazen, 2007). The hadal zone offers an ideal site to test the previously proposed hypotheses that neither pressure nor food availability will affect metabolic rates using a standardized protocol.

Here, we investigate pressure-related rate changes in maximal reaction rates of three metabolic enzymes from fast-glycolytic myotomal (white) muscle of deep- and shallow-adapted fishes. We then apply the pressure-related rate changes in metabolic enzyme activities to published and new results measured at atmospheric pressure, allowing a re-evaluation of the depth trends for metabolic proxies. This study extends a large existing dataset of metabolic enzyme activities to much greater depths with new data on abyssal and hadal species and elucidates depth-related trends in metabolic capacities in fishes in light of pressure-related changes in maximum enzyme reaction rates.

2. Materials and methods

2.1. Sample collection

Abyssal and hadal fishes were collected by free-vehicle trap baited with mackerel near and from the Kermadec (Apr–May, 2014) and Mariana trenches (Nov–Dec, 2014). Further details on collection sites and traps are provided by Linley et al. (2016). *Liparis florae*, the tidepool snailfish, was collected from Puget Sound near Friday Harbor, WA, by trawl and hand net (July 2014). A shallow-living, cold adapted species was also included, *Paraliparis devriesi,* collected by trawl from Antarctica (Andvord Bay, FjordEco Cruise), where it lives at a habitat temperature of ~−1°C, comparable to the hadal environment. Collected whole fish were kept on ice or in a cold room and processed as quickly as possible. White muscle samples were dissected from the

Table 1
Collection information. Standard lengths and mass are taken from fresh fish. N indicates the number of individuals with measured CS, LDH, MDH, and PK activities. Sex indicates the number of male, female, and immature individuals. Others not sexed due to damage. Sex was determined visually.

Species	Location	Depth (m)	n	SL (cm)	Mass (g)	Sex
Liparidae						
Liparis florae	Puget Sound	~1–30	5	8.2–14.6	6.7–35.3	0, 0, 0
Liparidae sp.nov.	Mariana	6961–7929	16	13.7–26	8–160	4, 8, 2
Notoliparis kermadecensis	Kermadec	6500–7500	20	13.9–31.5	26–230	5, 11, 1
Paraliparis devriesi	Andvord Bay	550	3	~12–18	–	–
Macrouridae						
Coryphaenoides armatus	Kermadec	3500–4000	4	51.9–84.6	576–3130	2, 0, 0
Coryphaenoides yaquinae	Kermadec	5000	1	70.5	1344	0, 0, 0
Coryphaenoides yaquinae	Mariana	4441–6081	4	23.8–78.4	40–2200	0, 2, 2
Ophidiidae						
Spectrunculus grandis	Kermadec	3500–4000	5	29–73.8	106–2128	0, 1, 4
Synaphobranchidae						
Diastobranchus capensis	Kermadec	1500	1	91	608	0, 1, 0
Zoarcidae						
Pachycara sp.	Kermadec	5000	2	44.2–47.4	460–660	1, 0, 0

anterior portion of the epaxial muscle. Red muscle and gelatinous tissues were carefully avoided. Tissues were frozen immediately in liquid nitrogen and stored at −80°C prior to analysis in the lab.

2.2. Enzyme activities at atmospheric pressure

For comparison to published studies, maximum activities of four metabolic enzymes—citrate synthase (CS), lactate dehydrogenase (LDH), malate dehydrogenase (MDH) and pyruvate kinase (PK)—were measured using a standard unpressurized spectrophotometric method described by Condon et al. (2012) and Drazen et al. (2015), updated from Srere (1969) and Yancey and Somero (1978). Assays were conducted on white muscle homogenates ground in 10 mM tris(hydrozymethyl)aminomethane hydrochloride (Tris-HCl) buffer (pH 7.55 at 10°C) at a ratio of 1:10. Two tissue samples from each fish were assayed in duplicate. Although habitat temperature for these species is colder (down to ~−1°C), assays were conducted at 10°C to allow for comparison to published values (e.g. Drazen et al., 2015). Chemicals for all assays were sourced from Sigma-Aldrich. Collection information for the samples analyzed at atmospheric pressure are presented in Table 1. Activities are presented in international enzyme units (Units) per gram tissue wet weight.

2.3. Enzyme activities as a function of hydrostatic pressure

In addition to standard unpressurized assays, enzymes from 6 species (all Liparidae and Macrouridae in Table 1) were tested under pressure. White muscle samples (~0.1 g) were homogenized in a 1 ml buffer of 50 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM dithiothreitol (DTT) (pH = 7.5 at 5°C). Homogenates were centrifuged for 10 min at 2000 × *g* at 4°C. All chemicals were sourced from Sigma-Aldrich (St. Louis, MO, USA). To minimize variation due to pipetting error and slightly differing amounts of enzyme in different parts of the tissue, pressures were varied incrementally on one proceeding reaction. Before each pressure test, an atmospheric pressure check determined that the reaction proceeded at saturating substrate concentrations and maintained a linear rate over the time of the assay. All pressure assays were conducted at 5°C. Individual samples used for experiments were randomly selected.

A stainless steel cuvette chamber (Mustafa et al., 1971) was used for pressure assays with a Jasco V550 UV/Vis spectrophotometer (Easton, MD, USA). Cell volume was 5 ml, though all reactions were added to 5.1 ml to prevent any air in the chamber. To minimize condensation on the cell windows, trays of desiccant (silica gel) and a steady stream of nitrogen gas were added to the closed chamber. Each assay lasted 300 s. To minimize error from mixing effects, only data from the last 250 s were used. After 100 s at atmospheric pressure, pressure was increased to 20 MPa by hand pump for 50 s, then 40 and 60 MPa. The pressure was then released back to 0.1 MPa to measure enzyme recovery for the final 50 s. Reaction rates were determined from the last 40 s of slope at each pressure and converted to units of activity (μmoles of substrate converted to product per minute) per g wet weight of tissue. The cell was rinsed and aspirated once with isopropyl alcohol and twice with distilled water between assays.

Lactate dehydrogenase (LDH) activities were measured as follows. The reaction buffer, 80 mM Tris-HCl (pH = 7.55 at 5°C), was added to the cell in the spectrophotometer first. 150 μM nicotinamide adenine dinucleotide (NADH) was then added to determine initial absorbance. Enough homogenate (between 3 and 10 μl) was added to achieve a linear reaction rate for 300 s. Finally, 512 μl of 40 mM sodium pyruvate was added to start the reaction. The chamber was closed and sealed quickly and the extinction of NADH measured. Final concentrations of NADH and pyruvate were 0.15 mM and 4 mM. To measure pyruvate kinase activity, a buffer of 80 mM Tris-HCl, 100 mM potassium chloride (KCl), 10 mM magnesium sulfate ($MgSO_4$), and 0.1 mM fructose-1,6-biphosphate was added to the chamber. 512 μl of 150 μM NADH was added, followed by 5 μl rabbit LDH (Type II, ammonium sulfate suspension, 800–1200 units/mg protein), the homogenate (5–15 μl for linear rate) and 100 mM phosphoenolpyruvic acid (PEP). The reaction was initiated by the addition of 512 μl 20 mM adenosine diphosphate (ADP). Final concentrations of NADH, PEP, and ADP were 0.15 mM, 1.0 mM, and 2.0 mM respectively. Activity of rabbit LDH was found to be slightly inhibited at pressures near 40–60 MPa, however the magnitude of this change was smaller than the response seen in pyruvate kinase and was considered negligible in this assay. Malate dehydrogenase assays were run in an 80 mM Tris buffer with 150 μM NADH. Homogenate was added (4–5 microliters to achieve linear reaction rate across 300 s). Reaction was initiated with mixing of 0.5 mM oxaloacetic acid. Final concentrations of NADH and oxaloacetate were 0.15 mM and 0.05 mM.

The increase in pressure results in a slight expansion of the reaction chamber as the windows of the cell seat in their rubber O-rings, increasing the path length across which the extinction of NADH is measured. A blank with buffer and NADH solution was measured at each pressure and the slight increase in absorbance was recorded and subtracted as a correction factor: 1.18%, 2.26%, and 3.35% for 20, 40, and 60 MPa, respectively.

To investigate pressure-related changes in reaction rate in the presence of the osmolyte TMAO, the pyruvate kinase assay was selected for the hadal snailfish, *Notoliparis kermadecensis*. Protocol followed that

listed above, with the addition of 400 mM TMAO, levels found in these fish (Yancey et al., 2014). Assays were conducted with 0.5 mM and 2.0 mM ADP levels, and activities measured at 0.1 MPa and high pressure (65.5 MPa).

2.4. Re-evaluation of depth trends

Atmospheric pressure results were compared to activities at *in situ* pressures found using the same collection. For families that were tested under pressure (Macrouridae, Liparidae), enzyme activities measured at atmospheric pressure were adjusted to reflect the percent reaction rate changes seen at *in situ* pressure. Results were compared to depth trends shown in the literature using the same method of enzyme analysis (Drazen et al., 2015).

2.5. Statistics

Trends with body mass and depth were investigated using generalized linear models (GLM) constructed based on the log link function assuming normal distributions in the statistical programming platform, (R Core Development Team, 2015). Normal quantile-quantile plots and plots of residuals were examined to check these assumptions. Best fit GLM models were chosen according to lowest Akaike Information Criterion. Figures were constructed using the R package ggplot2 (Wickam, 2009).

3. Results

3.1. Enzyme activities at atmospheric pressure

Results of four metabolic enzyme activities measured in the white muscle of nine species are presented in Table 2. LDH and PK were significantly correlated (linear model, $R^2=0.63$, $F(1, 65)=105.6$, $p<0.001$), as were MDH and CS ($R^2=0.82$, $F(1, 65)=5.81$, $p=0.019$), suggesting consistency of activity results. Fish family was a significant predictor of activity for all enzymes (ANOVA, LDH: $F(4, 62)=11.99$, $p<0.001$; PK: $F(4, 62)=25.54$, $p<0.001$; MDH: $F(4, 62)=5.34$, $p<0.001$; CS: $F(4, 62)=6.60$, $p<0.001$). Post hoc testing (Tukey HSD multiple comparisons of means, 95% confidence interval) revealed LDH activity was lowest in liparids, compared to macrourids ($p<0.01$), ophidiids ($p<0.001$), synaphobranchids ($p<0.01$), and zoarcids ($p<0.05$). PK activities were also lowest in liparids, relative to macrourids ($p<0.01$), ophidiids ($p<0.01$), synaphobranchids ($p<0.001$), and zoarcids ($p<0.001$). Citrate synthase activity was significantly higher in liparids than in macrourids ($p<0.01$) and ophidiids ($p<0.01$). Activity of MDH in liparids was lower than in macrourids ($p<0.05$), but higher than in ophidiids ($p<0.05$).

No significant trends were found between mass and enzyme activity across all families; however, there were some intraspecific trends with body mass. Larger individuals had significantly lower citrate synthase activities in hadal liparids (log-transformed linear model, *N. kermadecensis* $R^2=0.23$, $F(1, 17)=4.97$, $p<0.05$; Mariana liparid $R^2=0.55$, $F(1, 14)=17.28$, $p<0.001$) and abyssal macrourids (*C. yaquinae* $R^2=0.86$, $F(1, 3)=18.17$, $p<0.05$; *C. armatus* $R^2=0.34$, $F(1, 2)=1.05$, $p<0.05$). LDH activity was higher in larger *C. yaquinae* individuals ($R^2=0.81$, $F(1, 3)=13.03$, $p<0.05$). In larger individuals of the hadal liparid, *N. kermadecensis*, MDH activities were significantly higher ($R^2=0.41$, $F(1, 17)=11.91$, $p<0.01$). PK did not vary significantly with body mass for any species tested. Sample sizes for *Diastobranchus capensis* and *Pachycara* sp. were not large enough to test mass effects.

At atmospheric pressure, enzyme activities for hadal liparids were largely similar to shallower-living counterparts from this study and published results from Drazen et al. (2015), with a few differences. Lactate dehydrogenase activities were significantly different only between *N. kermadecensis* and the Mariana liparid, the latter being higher (post-hoc Tukey HSD, 95% confidence interval, $p<0.001$). *L. florae* had significantly higher MDH than *C. melanurus* (from Drazen et al., 2015; $p<0.01$), *N. kermadecensis* ($p<0.001$), and the Mariana liparid ($p<0.05$). PK activity was higher in *L. florae* than in *C. melanurus* (Drazen et al., 2015; $p<0.01$), the Mariana liparid ($p<0.05$), and *N. kermadecensis* ($p<0.001$). Citrate synthase activity did not vary significantly across all liparid species (ANOVA, $F(5, 44)=0.47$, $p=0.794$).

In the Mariana liparid, CS activities increased intraspecifically with depth of capture independent of body mass, and LDH decreased, while other intraspecific relationships between enzyme activity and depth were not significant (Fig. 1; GLM, depth, mass, interaction, 15 df, LDH: $t=-2.50, -1.86, 1.85$, $p<0.05$, $=0.088, 0.088$; MDH: $t=-1.06, -0.73, 0.67$, $p=0.308, 0.481, 0.516$; CS: $t=-2.61, -2.77, 2.54$, $p<0.05, 0.05, 0.05$; PK: depth, mass $t=-1.45, -0.01$, $p=0.170, 0.992$). For *N. kermadecensis*, LDH and PK showed no significant depth effects, while CS and MDH increased with depth of capture (GLM, depth, mass, 18 df, LDH: $t=1.78, -0.31$, $p=0.095, 0.758$; MDH: $t=2.68, -3.57$, $p<0.05, 0.01$; CS: $t=2.69, -2.16$; $p<0.05, 0.05$; PK: $t=-0.17, -1.69$, $p=0.870, 0.111$).

3.2. Enzyme activities as a function of pressure

Changes in maximal reaction rate with pressure were seen in all three enzymes. In the case of lactate dehydrogenase (LDH), abyssal (*C. armatus*, *C. yaquinae*) and hadal (*N. kermadecensis*) species showed

Table 2
Maximal activities of four metabolic enzymes in white muscle at atmospheric pressure and 10°C. Errors are presented as standard deviation. Activities are presented in Units per gram tissue wet weight. Capture location indicated: K, Kermadec and M, Mariana trench regions.

	CS	LDH	MDH	PK
Liparidae				
Liparis florae	1.46 ± 0.50	34.40 ± 7.79	32.44 ± 5.71	20.40 ± 2.98
Liparidae sp.nov.(M)	1.05 ± 0.44	40.18 ± 16.71	22.00 ± 6.16	21.82 ± 6.68
Notoliparis kermadecensis	1.16 ± 0.78	21.97 ± 8.08	17.82 ± 6.99	16.15 ± 2.82
Macrouridae				
Coryphaenoides armatus	0.39 ± 0.27	62.93 ± 11.24	38.69 ± 8.19	31.13 ± 5.83
Coryphaenoides yaquinae (K)	0.37	83.65	19.7	26.3
Coryphaenoides yaquinae (M)	0.55 ± 0.18	31.60 ± 4.51	22.03 ± 3.48	22.02 ± 11.25
Ophidiidae				
Spectrunculus grandis	0.29 ± 0.09	66.51 ± 24.16	9.25 ± 2.20	30.16 ± 4.51
Synaphobranchidae				
Diastobranchus capensis	0.34	96.03	21.01	54.3
Zoarcidae				
Pachycara sp.	0.49 ± 0.37	73.55 ± 5.24	18.92 ± 5.42	50.31 ± 14.56

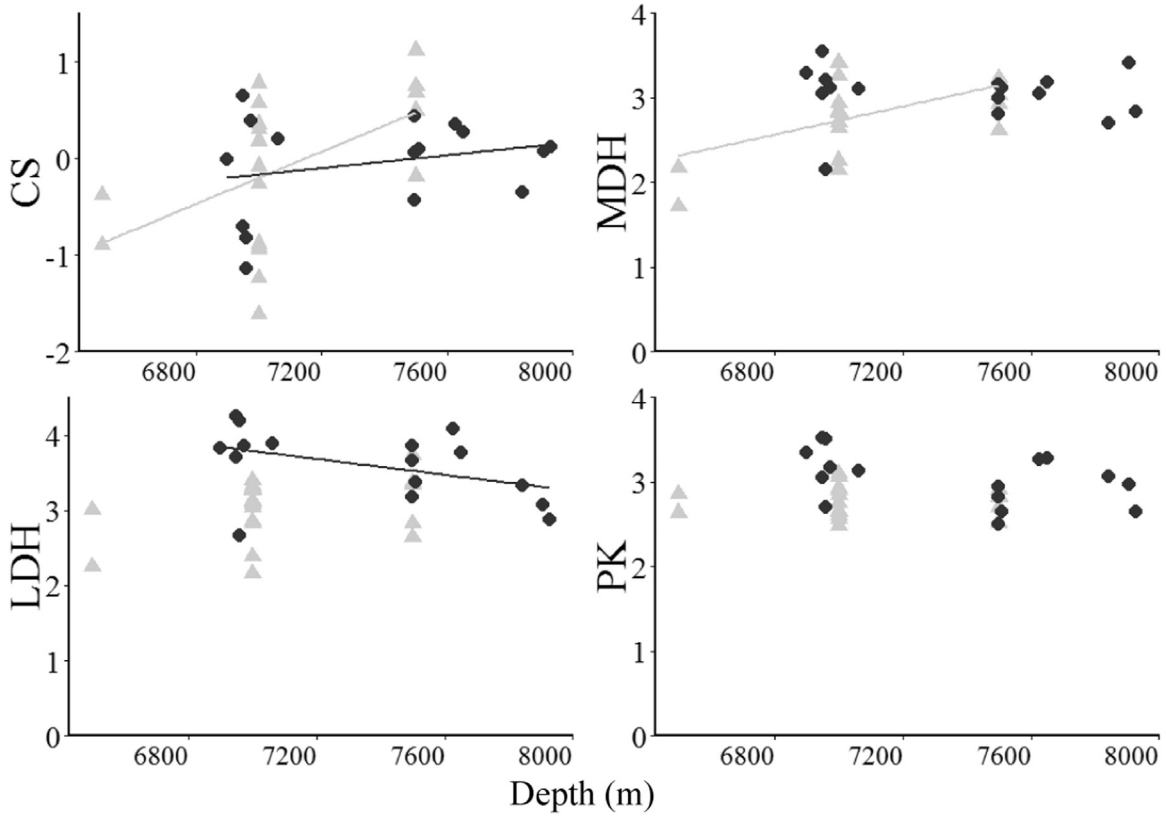

Fig. 1. Hadal liparid enzyme activities (log-transformed, U/g wet mass) as a function of depth of capture (m). Linear regressions are shown for significant depth relationships from GLMs that included mass effects. Mariana liparid (M) shown in circles *Notoliparis kermadecensis* (K) in triangles (R^2 values—CS: M = 0.015, CS: K = 0.339, MDH: K = 0.204, LDH: M = 0.235).

enhanced activity under pressure, while shallow-adapted species (*L. florae*, *P. devriesi*) were inhibited by pressure (ANOVA, *C. armatus* $F(5, 37) = 7.52$, $p < 0.001$; *C. yaquinae* $F(5, 24) = 3.28$, $p < 0.05$; *N. kermadecensis* $F(5, 31) = 4.74$, $p < 0.01$; Mariana liparid not statistically significant $F(5, 10) = 1.63$, $p = 0.24$; *L. florae* $F(4, 15) = 10.47$, $p < 0.001$; *P. devriesi* $F(4, 22) = 9.76$, $p < 0.001$). Peak activities seemed to occur near habitat pressures for all species (Fig. 2).

Malate dehydrogenase (MDH) activity increased with pressure for all species (Fig. 3). This pressure activation from atmospheric pressure rates were statistically significant for all species tested, except *C. armatus* (ANOVA, *L. florae* $F(3, 29) = 13.57$, $p < 0.001$; *C. armatus* $F(3, 34) = 0.53$, $p = 0.66$; *N. kermadecensis* $F(3, 15) = 24.57$; $p < 0.001$; *P. devriesi* $F(3, 20) = 3.50$, $p < 0.05$; *C. yaquinae* $F(3, 31) = 3.54$, $p < 0.05$; Mariana liparid $F(3, 35) = 9.79$, $p < 0.001$). Some species, particularly *C. armatus*, showed more scatter in repeat assays than others.

Pyruvate kinase was inhibited by pressure in all species, from shallow to hadal (Fig. 4) and the effect was statistically significant for *C. armatus* (ANOVA, $F(4, 17) = 18.34$, $p < 0.001$), *C. yaquinae* ($F(4, 20) = 12.98$, $p < 0.001$), *N. kermadecensis* ($F(4, 28) = 4.38$, $p < 0.01$) and the Mariana liparid ($F(4, 20) = 14.22$, $p < 0.001$), near significant for *L. florae* ($F(4, 18) = 2.72$, $p = 0.062$). Recovery after decompression was highly variable for all enzymes and these values were omitted from statistical tests. Recovery rates for all enzymes and all species were likely confounded by uneven changes in system optics with the release of pressure.

The addition of TMAO counteracted this pressure-inhibition of pyruvate kinase, restoring activity of pyruvate kinase at both a low and high ADP concentration (Fig. 5). TMAO had no effect on activity at atmospheric pressure at high substrate concentration (maximal reaction rate), but did increase activity at subsaturating levels (0.5 mM ADP).

3.3. Depth and pressure trends

Fig. 6 shows how the enzyme activities of species studied here compare to the activity-depth relationships shown by Drazen et al. (2015) and how the *in situ* pressure changes documented in the present study would affect these results. For abyssal and hadal species, both citrate synthase and malate dehydrogenase activities at atmospheric pressure were higher than expected according to the Drazen et al. (2015) regression. LDH and PK were somewhat higher than the regression from shallower-living species predicted. Increased activities of LDH at *in situ* pressures increased this difference slightly. PK activities at in situ pressures were similar to the regression prediction, although these did not take into account the TMAO effect, which counteracts pressure inhibition of the enzyme, but only at subsaturating ADP concentrations.

4. Discussion

Deep-sea species are known to display enzymic pressure adaptation. This has been understood as a maintenance of functional stability under increasing pressure, through the pressure-insensitivity of K_m (Siebenaller and Somero, 1979; Somero and Siebenaller, 1979; Siebenaller, 1984; Dahlhoff et al., 1990;). This insensitivity has been hypothesized to come at the cost of reduced catalytic efficiencies, based on experiments with LDH, MDH, and PK. Previous work focused mainly on half-saturation constant, K_m, responses, while enzyme activity was not hypothesized to change with pressure at the level of the tissue because of species' ability to increase enzyme concentration. Further, previous work focused on shallower-living (< 1000 m) deep-sea species and, as a result, pressure typically was not modified during experiments in which tissue maximal enzyme activity was quantified.

Contrary to previous assumptions, we found pressure-related changes in maximal reaction rate per gram of tissue for three enzymes,

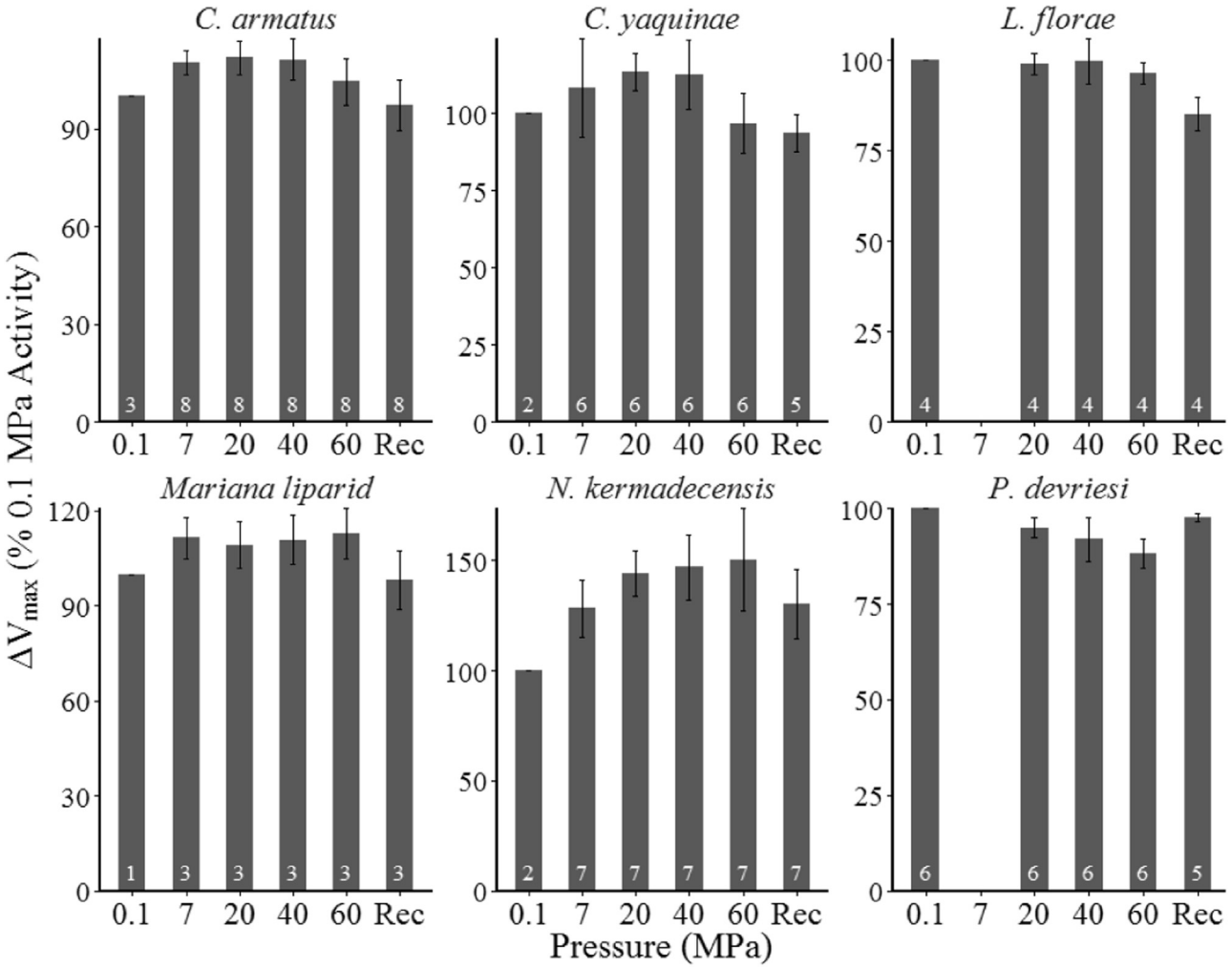

Fig. 2. Lactate dehydrogenase activity at different pressures at 5°C. Results are shown in percent of activity at atmospheric pressure for each assay. Results from repeat assays (n values included at the base of each bar) of one individual per species. Error bars show standard deviations between assays. Recovery (Rec) shows rate after return to atmospheric pressure after pressure trials.

with LDH activity peaking at habitat pressure, MDH being pressure-activated in all tested species, and PK consistently pressure-inhibited. For maximal reaction rate to change in these assays as a function of pressure, one of two things must be changing—either the K_m value or the catalytic efficiency. Although pressure related changes in K_m value are well known (e.g. Siebenaller, 1984), the present assays were conducted at high substrate concentrations that would not have been rate limiting. Therefore, catalytic efficiencies likely change with pressure—in both directions depending upon volume changes in the reaction. These results show that macromolecular evolution (stabilization of K_m) has not completely resulted in stability of maximal reaction rate. According to Le Chatelier's principle, reactions with net positive volume change are inhibited by pressure, whereas reactions which have reduced volume in the products are favored at higher pressure (e.g. Somero, 1992; Macdonald, 1997). Our results suggest negative volume changes for lactate dehydrogenase in the abyssal and hadal species tested, as well as for malate dehydrogenase in all species. This increase in activity with pressure has not, to our knowledge, previously been shown in MDH. There are studies of MDHs capable of adaptation to extreme temperature conditions, such as one that remains active (90%) even after it is boiled for six hours (Gharib et al., 2016). The pressure-enhancement effects may be more pronounced than those seen in LDH due to the dimeric, rather than tetrameric nature of the enzyme.

Pressure-related changes in maximal reaction rate may not have been addressed in earlier work for a number of reasons. Changes in K_m with pressure are perhaps a more intuitive indicator of macromolecular sensitivity to pressure than maximum reaction rates. It was believed that LDHs of deep-sea species were inefficient in catalysis compared to cold-adapted shallow species (Somero and Siebenaller, 1979). However, that conclusion came from catalytic efficiencies measured at atmospheric pressure. Understanding enzymic adaptation in deep-sea fishes as a tradeoff between catalytic efficiency and pressure sensitivity is incomplete when the reduced catalytic efficiency of LDH occurs only at low pressures. Given these new data, this paradigm should be reconsidered. Another study that concluded low catalytic efficiencies of LDH was based on pressure incubations of LDH for an hour in pressure vessels. After decompression, activity was measured at atmospheric pressure (Hennessey and Siebenaller, 1985). When measuring the progress of one reaction, we found recovery of LDH after pressurization from these species to be extremely variable, and often the enzyme did not recover. By measuring at atmospheric pressure after an incubation, that study explored decompression survivability, missing the pressure-activation and change in catalytic efficiency of LDH in these deep-sea fishes. Another previous experiment found that maximum catalytic rate of LDH in *Coryphaenoides* is pressure insensitive until about 80 MPa, when it becomes inhibited (Moon et al., 1971b). We measured reaction rates on one reaction, with a step-wise increase in pressure and with more replicates and greater precision than the spectrophotometer technology allowed at the time of that experiment, where variability or rapid pressurization may have masked the pressure

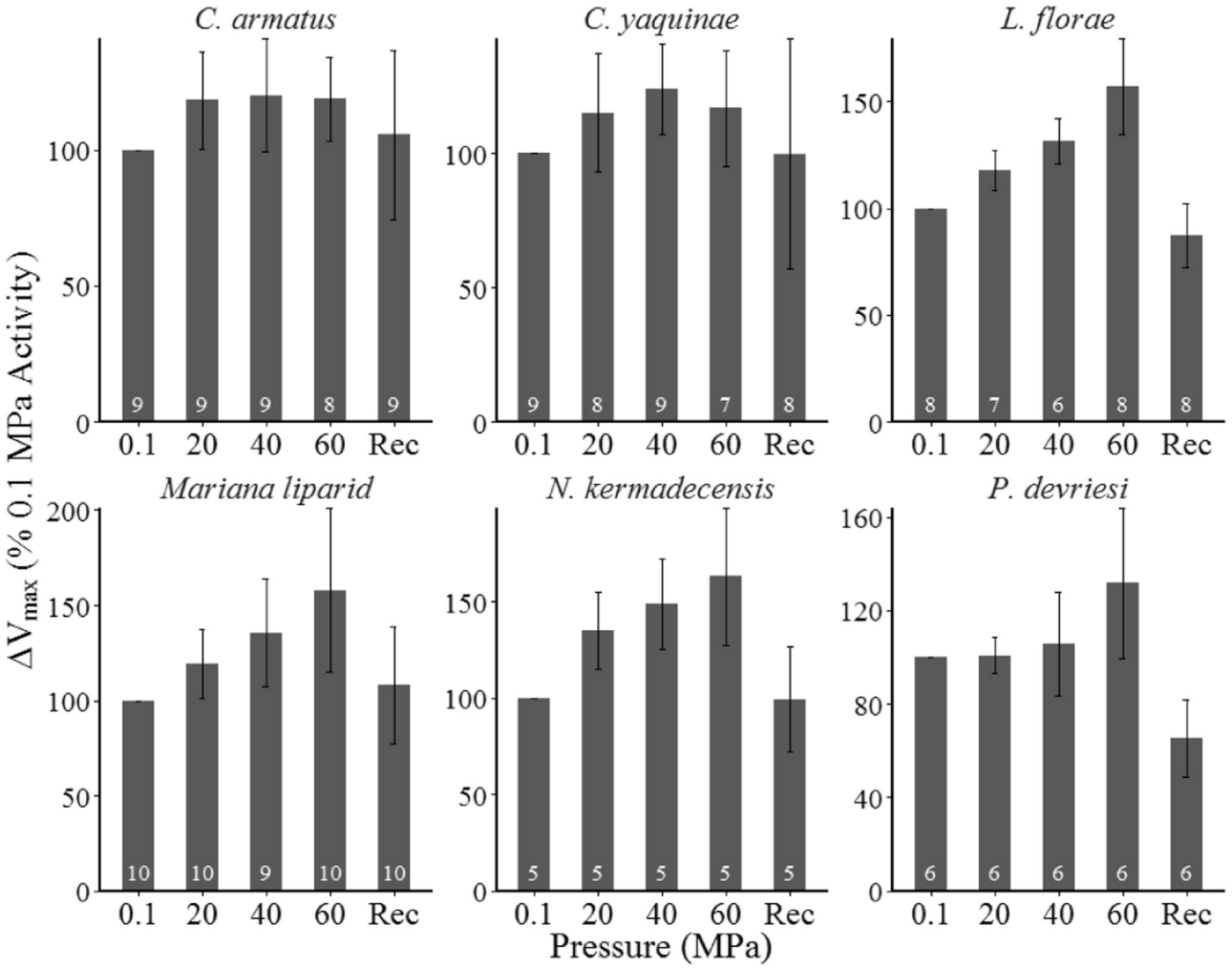

Fig. 3. Malate dehydrogenase activity at different pressures at 5°C. Results are shown in percent of activity at atmospheric pressure for each assay. Results from repeat assays (n values included at the base of each bar) of one individual per species (n = 2 for Mariana Trench liparid). Error bars show standard deviations between assays. Recovery (Rec) shows rate after return to atmospheric pressure after pressure trials.

activation. Also, in shallower-living (bathyal) deep-sea species, this pressure-activation effect may be small. The hadal fishes add a more extreme example to this trend, displaying a nearly 50% increase in maximum catalytic rate at habitat pressure. The pressure-related changes in maximal reaction rate shown here should be considered when studying organisms that live at high hydrostatic pressures.

Since the three enzymes showed very different responses in maximum reaction rate to changing pressures, this suggests that these results are not due to system artifacts such as changes in optics with pressure. We note that even when using a sensitive spectrophotometer and measuring changes over one reaction, results were variable. Some of this variability may have stemmed from tissue degradation. For example, the Mariana liparid showed high variability compared to *N. kermadecensis*, perhaps because it was retrieved through warm surface waters (> 25°C) before trap recovery, although every effort was made to minimize recovery time and samples were dissected on ice and frozen immediately in liquid nitrogen. Further, the early studies on pressure activities of enzymes from deep-sea fishes found that phosphofructokinase from abyssal *Coryphaenoides* reaches peak maximum reaction rate near 30 MPa (Moon et al., 1971c). The authors noted that this is a low-activity, unstable enzyme that is very susceptible to degradation and decompression, and this finding seems to have remained unexplored.

In previous studies, metabolic enzyme activities have been useful and widely-applied indicators of metabolic capacity and condition (reviewed by Dahlhoff, 2004). Studies have shown depth-related declines in enzyme activity (LDH, PK, MDH, CS) from surface waters to depths over 3000 m in benthic and benthopelagic species (e.g. Drazen et al., 2015). As noted earlier, results were interpreted as a decline in metabolic rate due to decreasing predator-prey interaction distances with decreasing light availability (visual interactions hypothesis), while older studies attributed such declines to reduced food supply (reviewed by Childress, 1995). Again, assays have traditionally been conducted only at atmospheric pressure; however, our studies show that for abyssal and hadal species, maximum reaction rates of three of these enzymes (LDH, PK, and MDH) are pressure-dependent, suggesting that pressure-related changes in maximum reaction rate may alter interpretations of these depth-related trends in metabolic rate. This is a complicated question, as the degree to which atmospheric assay results would differ from *in situ* pressure rates depends both on the enzyme, TMAO effects and the habitat depth of the fish. To begin to address this, we first compared the present results measured at atmospheric pressure to those presented by Drazen et al. (2015, same experimental method) and then applied estimates of how these activities would change at *in situ* pressure for the four abyssal and hadal species tested in this study (Fig. 6).

First, for nine species, enzyme activities measured at atmospheric pressure are comparable to those measured in previous studies. *C. armatus* activities were similar to those measured off California (Drazen et al., 2015), although MDH values were significantly higher in the present study, perhaps anomalously high according to the depth trends

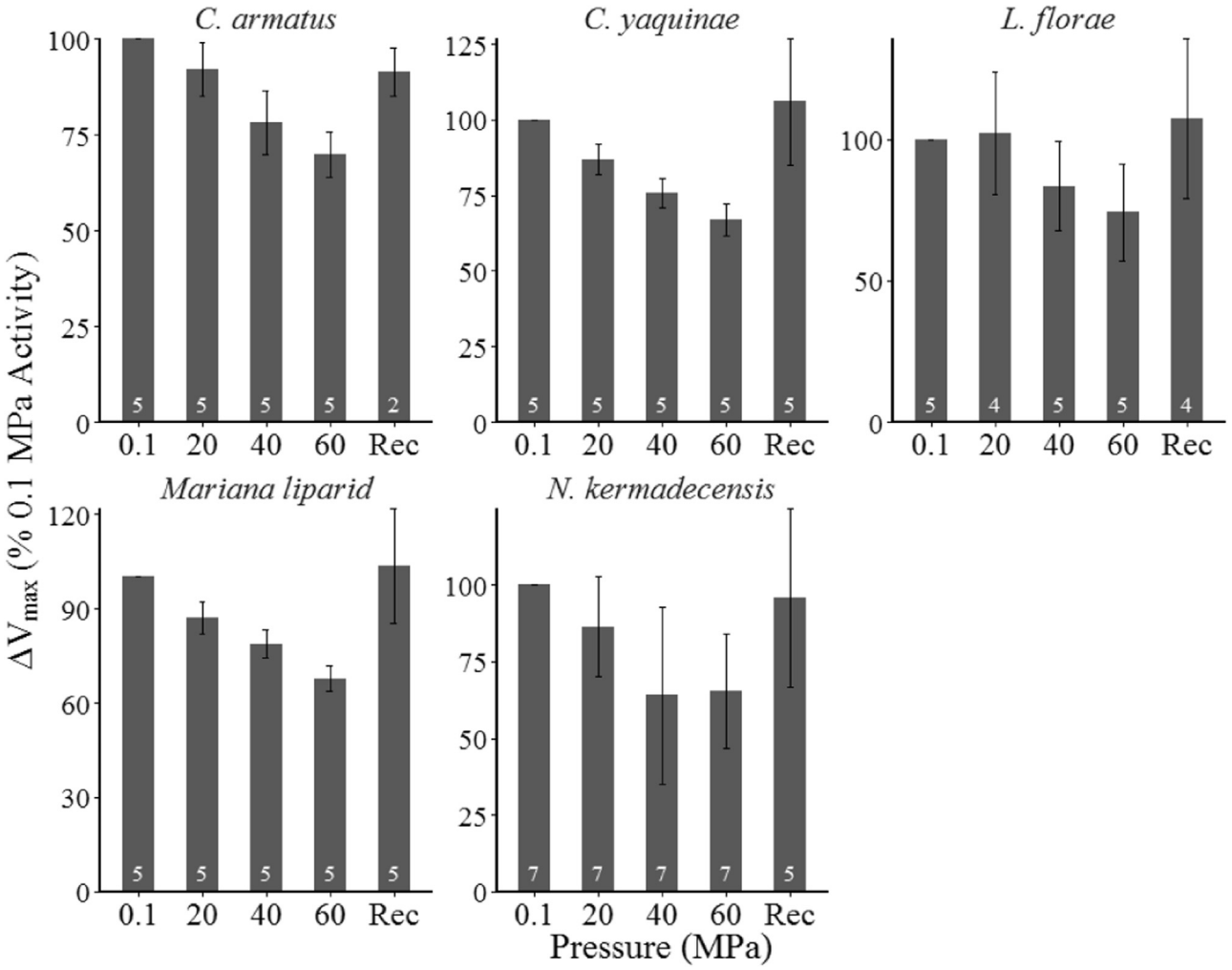

Fig. 4. Pyruvate kinase activity at different pressures at 5°C. Results are shown in percent of activity at atmospheric pressure for each assay. Results from repeat assays (n values included at the base of each bar) of one individual per species. Error bars show standard deviations between assays. Recovery (Rec) shows rate after return to atmospheric pressure after trials.

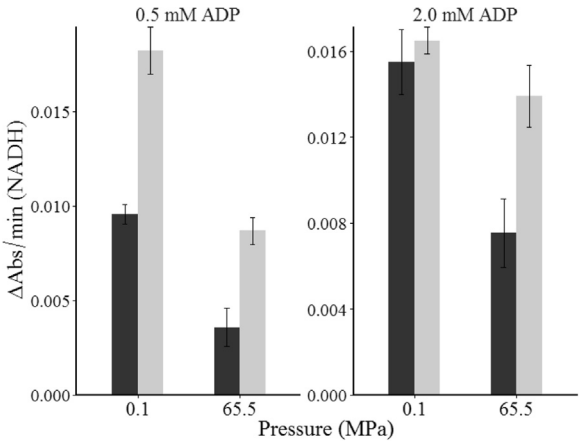

Fig. 5. Pyruvate kinase response to pressure with TMAO. Results are shown for two concentrations of ADP at 5 °C. Assays with the addition of 400 mM TMAO are shown in dark grey, control pressure response without TMAO in light grey. Data are from *N. kermadecensis* (n = 4 for 0.5 mM ADP, n = 3 for 2.0 mM ADP). The effect of TMAO on activity was significant for each treatment (Mann-Whitney-Wilcoxon test; $p < 0.05$), except at 0.1 MPa and 2 mM ADP ($p = 0.35$).

presented in Fig. 6. In another macrourid, *C. yaquinae*, LDH and PK activities were lower than in *C. armatus*, perhaps reflecting differences in trophic strategy (Drazen et al., 2008). *Spectrunculus grandis* activities were near identical to those from near California (Drazen et al., 2015), measured using the same experimental method. *D. capensis* activities were largely similar to activities for *Synaphobranchus kaupii*, another deep-sea eel from the North Atlantic (235–3200 m), with the exception of PK, which was significantly higher in our study (Bailey et al., 2005). With a sample size of one, however, it would be imprudent to infer too much from this difference. As seen in other studies, glycolytic enzymes were lower in liparids than in macrourids, ophidiids, synaphobranchids, and zoarcids, suggesting lower burst locomotory capability. Large differences in body mass between these families might also explain some of these differences (e.g. Childress and Somero, 1990).

Despite pressure-related changes in activity reported here, activity results at atmospheric pressure are still useful as proxies for metabolic rate intraspecifically, as we found in hadal liparids. Citrate synthase values increased with depth of capture in hadal liparids from the Kermadec and Mariana trenches, independent of body mass. This trend may have been weaker for the Mariana liparid due to lack of shallower hadal collections (nearer to 6000 m). Resource availability is hypothesized to be higher in hadal systems, due to a funneling of organic matter into the steep topography of the trench (e.g. George and Higgins, 1979; Danovaro et al., 2003; Itoh et al., 2011; Ichino et al., 2015). Laboratory controlled studies showed intraspecific increases in metabolic enzyme activity in fishes that were better fed (Yang and Somero, 1993; Dutil et al., 1998; Martínez et al., 2003; Ombres et al., 2011). The results of this study suggest greater metabolic capacity and/or better nutritional condition with depth. An increase in number of prey items per gram of fish with depth of capture in the Mariana liparids (Gerringer et al.,

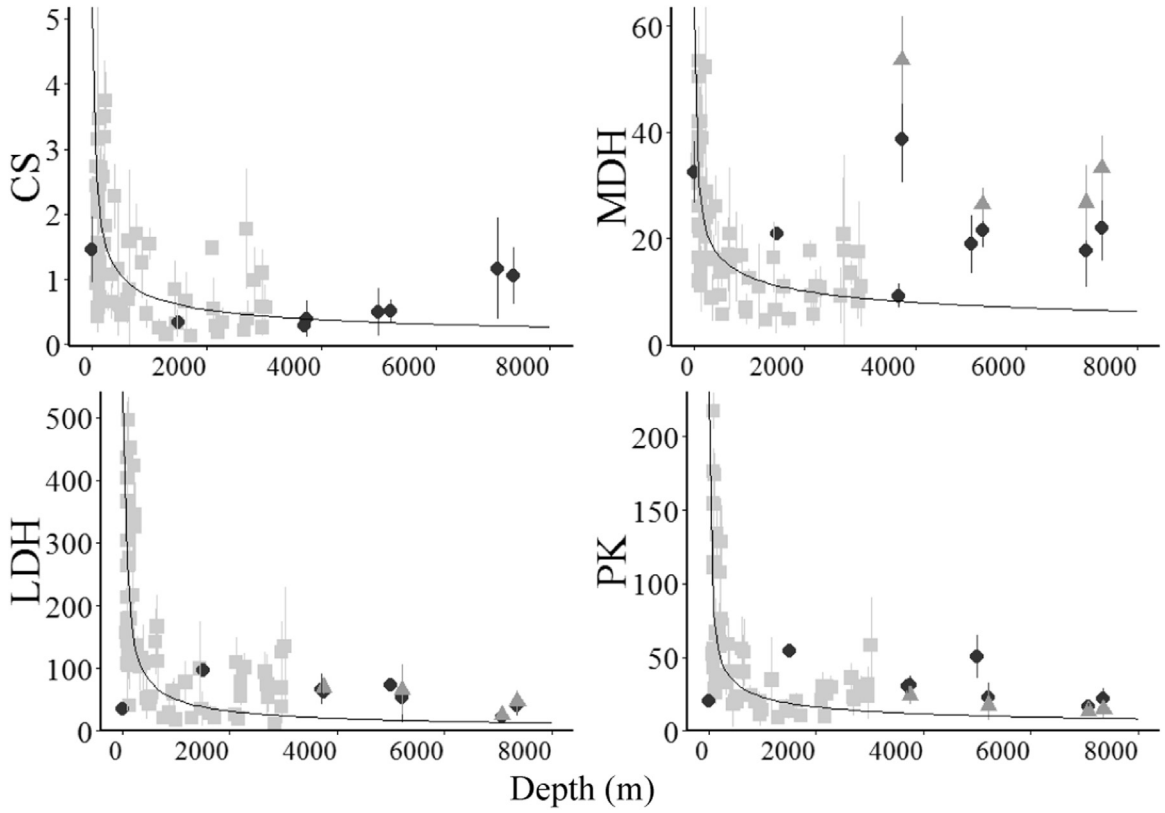

Fig. 6. Average enzyme activity (U/g wet mass) by average depth of capture (m) for 67 species of demersal fishes. Present study results (0.1 MPa, dark grey circles), Drazen et al. (2015) (0.1 MPa, light grey squares), and expected activities at *in situ* pressures for study species (triangles) shown. Error bars show standard deviation between individuals (n = 1–20). Models of depth-related declines in activities for benthopelagic species from Drazen et al. (2015) shown in black curves. Pressure effects on CS not tested.

2017) and a higher abundance of liparids within the trench than at the upper edges (Linley et al., 2016) may support what the citrate synthase results suggest, an increase in nutritive condition of individual fish with depth. Few inter-specific differences were seen between the two groups, though LDH activity was significantly higher in the liparid from the Mariana than in *Notoliparis kermadecensis* from the Kermadec, maybe reflecting greater competition for food under the oligotrophic Mariana surface waters (Longhurst et al., 1995; Watling et al., 2013).

Second, given the present results, the decline in enzyme activity with depth appears to hold true overall, despite being somewhat tempered by pressure effects. With these new data included, the glycolytic enzymes PK and LDH show the most significant declines, suggesting a lower burst locomotory potential in deep-sea species. Although PK showed significant pressure-inhibition of maximum reaction rate for all species tested (Fig. 4), the addition of the counteracting osmolyte TMAO restored reaction rates to those seen at atmospheric pressure in a hadal liparid (Fig. 5). It is probable, then, that the rate reductions presented in Fig. 6, which do not take into account this restoration of activity with added TMAO, do not reflect *in situ* activity for pyruvate kinase. CS and MDH activities are higher than the Drazen et al. (2015) models predicted, particularly at hadal depths, perhaps due to increased food availability, as discussed above. These additional data extend the depth range of previous studies by over 4000 m. Earlier conclusions that there are depth-related declines in enzyme activity still hold. Most of these changes occur over the first thousand meters, where pressure effects as observed here would be minimal, and the declines with depth are of a much greater magnitude than the pressure inhibition or activation observed. The initial decline may be based on light levels as stated by the visual interactions hypothesis (Childress, 1995), while at abyssal and hadal depths, food availability may play a greater role, suggesting that both hypotheses likely apply.

Enzyme activities are already known to be approximate, imperfect, proxies for metabolic rate. Overall, pressure-related rate changes are merely another variable to be considered in the use of enzyme activities as proxies for metabolic rate, adding to an already long list of factors including temperature, body mass (Childress and Somero, 1990; Burness et al., 1999; Martínez et al., 2000; Sullivan and Somero, 1983, present results), locomotory and feeding strategies, phylogeny, nutritional condition, mitochondrial density and specific activity (Moyes et al., 1992), age (Singh and Kanugo, 1969), tissue storage (Nilsson and Ekstrand, 1993), and citrate concentration (for MDH; Gelpi et al., 1992) that can confound results and make interpretation difficult (e.g. Gibb and Dickson, 2002). Pressure effects will need to be taken into account when comparing species across large habitat pressure ranges. For example, in our results measured at atmospheric pressure, the significantly higher MDH values in the intertidal species *L. florae* are likely an example of pressure confounding effects. At *in situ* pressures, LDH activities for both hadal liparid species were significantly higher, up to 150% activity at atmospheric pressure. Swimming speeds in *L. florae* and the hadal liparids are comparable when standardized for temperature (Gerringer and Linley, unpublished data). When using LDH activity as a proxy, previous studies may have underestimated burst capacity for deep-adapted species living at or below ~4000 m by up to 50%. This could provide some explanation for studies that have found higher than expected swimming performance in deep-sea fish species (e.g. Bailey et al., 2003, 2005).

Our results further highlight the interplay of extrinsic and intrinsic adaptation. Of the three enzymes studied, pyruvate kinase was the most affected by increasing pressures, with reduced activity seen in all species. For enzymes like pyruvate kinase, the stabilizing effects of extrinsic adaptations may be particularly important. The piezolyte TMAO is known to stabilize proteins under high pressure, due to the interaction with the water molecules in solution (e.g. Sarma and Paul, 2013), and TMAO is known to increase with depth in fishes and other taxa as an extrinsic pressure adaptation (Kelly and Yancey, 1999; Linley et al., 2016; Yancey et al., 2014). Yancey et al., (2001, 2004) found

significant decreases in K_m of PK from *Antimora microlepis* (bathyal morid cod), as well as from an abyssal sea anemone and from rabbit muscle, with the addition of 250–300 mM TMAO offsetting some or all of the inhibitory increases by pressure. The saturation of the pyruvate kinase mechanism could require a molecule with hydrophilic properties like TMAO to divert water from the active site of the reaction. In the present study, we found that TMAO considerably counteracted the inhibition of pyruvate kinase activity under pressure, suggesting that, while some enzymes such as LDH may be intrinsically adapted to function under high pressure, others may need extrinsic adaptation, such as stabilizing cosolutes.

Pressure-related changes in metabolic enzyme activities at abyssal and hadal depths re-open an old question: Does hydrostatic pressure affect whole animal metabolic rate? Although previous studies have suggested that it does not (Seibel and Drazen, 2007), this may need to be reconsidered for abyssal and hadal organisms. On a biomolecular level, the effects of temperature and pressure are similar with respect to protein stability, which is perturbed by both high temperature and high pressure. For example, *C. armatus* LDH exhibits higher thermostability compared to shallow-water orthologs despite its colder habitat, presumably due to pressure adaptation (Somero, 1992). This comparison is oversimplified; however, volume changes, whether from changes in temperature or changes in pressure, will affect reaction rates. New models are seeking to quantify and predict these effects (Chen and Makhatadze, 2017). The effects of temperature on metabolic rate are accepted. For most of life on Earth, the effects of pressure on metabolic rate may be negligible; however, at extremely high pressures, such as on the abyssal plain and in hadal trenches or the deep subsurface biosphere, these pressure effects may be significant. This would have implications for models such as the metabolic theory of ecology, which seeks to explain biological processes and patterns through temperature and body size (Brown et al., 2004) or the growing degree day, which standardizes time to temperature (e.g. Neuheimer and Taggart, 2007). Perhaps future work considering organisms that live at high pressures will require an additional component in metabolic models, something of a degree-day-MPa, to consider the biological effects of high hydrostatic pressures.

Competing interests

We have no competing interests to declare.

Author contributions

MEG, JCD, and PHY collected samples. MEG ran atmospheric pressure assays. MEG and PHY conducted assays using the pressure system. All authors contributed to the writing and editing of the manuscript.

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