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Seasonal proteome variation in intertidal shrimps under a natural setting: connecting molecular networks with environmental fluctuations

Running title: Seasonal proteome variation in shrimp

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This manuscript includes **supplementary material**: Fig. S1 Map; Fig. S2 Abiotic data; Fig. S3 Same Spots report; Table S1 Proteomic methods; Table S2 Protein levels *P. elegans*; Table S3 Mass sequences; Table S4 Percent CV

Abstract

The ability of intertidal organisms to maintain their performance via molecular and physiological adjustments under low tide, seasonal fluctuations and extreme events ultimately determines population viability. Analyzing this capacity in the wild is extremely relevant since intertidal communities are under increased climate variability owing to global changes. We addressed the seasonal proteome signatures of a key intertidal species, the shrimp *Palaemon elegans*, in a natural setting. Shrimps were collected during spring and summer seasons at low tides and were euthanized *in situ*. Environmental variability was also assessed using hand-held devices and data loggers. Muscle samples were taken for 2D gel electrophoresis and protein identification through mass spectrometry. Proteome data revealed that 55 proteins (10.6% of the proteome) significantly changed between spring and summer collected shrimps, 24 of which were identified. These proteins were mostly involved in cytoskeleton remodeling, energy metabolism and transcription regulation. Overall, shrimps modulate gene expression leading to metabolic and structural adjustments related to seasonal differences in the wild (i.e. abiotic variation and possibly intrinsic cycles of reproduction and growth). This potentially promotes performance and fitness as suggested by the higher condition index in summer-collected shrimps. However, inter-individual variation (% coefficient of variation) in protein levels was quite low (min-max ranges were 0.6 to 8.3% in spring and 1.2 to 4.8% in summer), possibly suggesting reduced genetic diversity or physiological canalization. Protein plasticity is relevant to cope with present and upcoming environmental variation related to anthropogenic forcing (e.g. global change,

pollution) but low inter-individual variation may limit evolutionary potential of shrimp populations.

1. Introduction

Intertidal communities live in a harsh habitat, often enduring challenging environmental conditions (Helmuth et al. 2006). In these habitats, biotic (predation, competition) and abiotic factors (temperature, UV, salinity, oxygen, wave exposure) rule the distribution and abundance of species (Gaston, 2003; Helmuth et al., 2006; Walther et al., 2002). In temperate regions, abiotic factors vary widely, both daily and seasonally, exposing intertidal communities to extreme conditions. Such factors are known to influence phenotypic variation in intertidal species, regulating several processes related to cellular metabolism and defense, growth, morphology, tolerance to environmental conditions, foraging and reproduction (Hochachka and Somero, 2002; Madeira et al., 2019; Miura et al., 2007; Mueller et al., 2015; Sorte et al., 2011; Trussell, 2000). As such intertidal communities have been proposed as models to study environmental change (Mieszkowska, 2009) and their tolerance limits have been widely assessed under a climate change context (Vinagre et al., 2019). However, the molecular underpinning of phenotypic variation in intertidal organisms is still understudied. Consequently, there is a need to link molecular knowledge with ecologically relevant gradients especially given the growing impacts of anthropogenic forcing (global change, pollution, overfishing) on wild populations.

Field studies are crucial in this context because they allow us to detect molecular and cellular changes in organisms under natural conditions, capturing multifaceted environmental frameworks (Dowd, 2012). Most eco-physiological studies in intertidal

organisms have focused on experiments that do not take into account the environmental complexity of the intertidal (Drake et al., 2017) and use only a set of specific biomarkers (heat shock proteins, anti-oxidants, ubiquitin) to study physiological responses. Thus, the application of 'omics' technology under a natural context can be regarded as a more 'ecological approach' to address fundamental questions in many areas of expertise (Johnson et al., 2007).

Proteomics allows the study of a great set of proteins (functional units) that change between cell types and over time. Thus, the proteome can be viewed as a measure of the cell's phenotype (Dupont et al., 2007) and can be directly related to fitness (Feder and Walser, 2005). Proteomic studies enable the detection of molecular markers that play a role in processes such as adaptation and detoxification of contaminants, providing insights into the success of a species (Dalziel and Schulte, 2012; Hollywood et al., 2006; Joyce and Palsson, 2006). To date, field studies using physiological or biomarker approaches (e.g. Hofmann and Somero, 1995; Madeira et al., 2017; Padmini and Geetha, 2009; Shaw et al., 2004) and experiments using transcriptomics and proteomics (e.g. Fields et al., 2012; Gardeström et al., 2007; López et al., 2002; Stillman and Tagmount, 2009; Tomanek, 2011; Tomanek, 2014; Tomanek and Zuzow, 2010) have shown that the thermal limits and metabolic rates of intertidal, oceanic and freshwater species vary according to fluctuations in abiotic factors (e.g. temperature, salinity, oxygen). Transcripts and proteins related to protein folding, proteolysis, cytoskeletal dynamics, ribin, extension-like genes, immune response, cell growth, gene expression, rRNA synthesis, protein synthesis, energy metabolism, oxidative stress metabolism, extracellular matrix dynamics and structural and muscle proteins have been shown to change with environmental variation. Regarding intertidal organisms, omic studies have

been performed mostly in mollusks (e.g. Diz et al., 2017; Fields et al., 2012; Gardeström et al., 2007; Helmholz et al., 2015; Tomanek, 2014; Zhang et al., 2015), fish (e.g. Jayasundara et al., 2015), crabs (e.g. Garland et al., 2015; Stillman and Tagmount, 2009) and seaweeds (e.g. Collén et al., 2007). However, proteomic studies that address changes *in situ* are still scarce and information on phenotypic changes occurring in the wild is still unknown for many species.

As a follow-up on laboratory work (Madeira et al., 2015, 2012), the aim of this study was to assess phenotypic variation in a natural setting by addressing, *in situ,* the seasonal molecular signatures associated with low tide in the commercial intertidal shrimp, *Palaemon elegans* (Rathke, 1837). This species is a hyper-hypo-osmoregulator shrimp, sub tropically distributed that inhabits tidal pools in rocky and muddy bottoms. Our previous biomarker studies showed that *P. elegans* regulates ubiquitin, anti-oxidant enzyme activity and K^+ in response to temperature and season, maintaining Hsp70 levels stable (Madeira et al., 2016a; Madeira et al., 2015, 2012; Vinagre et al., 2014). Here, we hypothesize that proteome adjustments related to seasonal variation may rely on the up-accumulation of homeostasis-related proteins (cytoskeletal elements, anti-oxidants, ion transporters, metabolic enzymes and immune system proteins). In addition, shrimps can either maintain or down-regulate proteins and pathways that are energetically costly (protein synthesis, growth processes), depending on their capacity to adjust metabolic processes. We hypothesize that these changes allow shrimps to survive and maintain physical condition under low tides in the summer. To test these hypotheses, we sampled field acclimatized shrimps during low tides in spring and summer and compared proteome profiles and condition indices. Differentially accumulated proteins were then categorized into biological functions to detect which

pathways were up or down-regulated. Information on protein response networks and individual health is crucial to understand acclimatization capacity or damage to tissues and predict the vulnerability of species towards environmental extremes that will tend to increase due to global change.

2. Materials & Methods

2.0 Ethics statement

This study was approved by Direcção Geral de Alimentação e Veterinária and carried out in accordance with European legislation (Directive2010/63/EU).

2.1 Collection of the organisms

Shrimps were collected on the 29th of April (spring, n=6, TL 3.98±0.63 cm, wt 0.86±0.55 g) and on the 27th of June (summer, n=6, TL 4.75 ± 0.63 cm, wt 1.53 ± 0.52 g) 2013, in a rocky intertidal shore (n_{total}=12, Cabo Raso, Cascais, 38°42'38"N and 9°29'08"W; Fig. S1). Health condition was assessed (absence of parasites, wounds, spots, recent molting) and all sampled specimens were considered in good condition. To reduce the impact of intrinsic factors on the proteome analysis, all specimens were collected within the time frame for reproduction (April to September) to reduce the influence of reproductive status. No egg carrying females were sampled. No distinction between sexes was made to enable population representativity. The organisms were collected from 4-5 tide pools (mean ± SD depth of 0.26±0.10 m) located in middle to high shore (~100 m^2 sampled) during low tide peaks that occurred during the hottest hours of the day (around 14:00 h), with the aim of unraveling molecular mechanisms that occurred during the low tide period from one season (spring) to another (summer). These seasons were chosen because they best represent temperatures already tested in the laboratory (Madeira et al., 2015, 2012; Vinagre et al., 2014). Standardizing

collection time also prevented biased results due to circadian changes in protein content. The shrimps were collected using hand nets and were euthanized by splitting and frozen, *in situ*, in dry ice. Then they were transported to the laboratory and frozen at -80°C until further analyses.

2.2 Field data acquisition

Environmental parameters (air temperature, water temperature, salinity and water pH) were measured at least 3 times in each intertidal pool where organisms were collected (using a glass thermometer Wolf X-ray USA, a hand-held refractometer Atago Japan and a digital pH probe model HI9025 Hanna Instruments USA, respectively). Photoperiod data was obtained from the website http://www.golden-hour.com. Long-term data and daily variations were also assessed in the sampling area using multi-parameter probes (i) water temperature from 2011-2012 was measured monthly at spring tides in the middle tide zone in three adjacent rocky beaches (two pools in each beach) using a YSI 556 probe (adapted from Madeira et al., 2015), (ii) water temperature measurements were taken in one mid-littoral and one supra-littoral pool in July 2014 using HOBO U22-001 data-loggers (data were registered every hour during day and night), (iii) measurements of abiotic factors (air temperature, water temperature, pH, dissolved oxygen and salinity) were carried out in 14 pools (n=4 lower littoral pools; n=5 mid-littoral pools; n=5 supra-littoral pools) throughout a tidal cycle in July 2016 to infer on daily variation (using refractometer Atago, Japan; digital pH probe model HI9025, Hanna Instruments, USA; digital O_2 and temperature meter model HI 9146, Hanna Instruments, USA), (iv) long-term air temperature data (from January 2011 to February 2014) was obtained from Mohid database, in which temperature is registered every half hour during day and night (see supplementary Fig. S2).

2.3 Protein extraction

Samples of muscle from the abdomen (200 mg) were homogenized in 1 mL of phosphate buffered saline (PBS, pH 7.4) using Tissue Master 125 homogenizer (Omni International, Kennesaw, USA), on ice. Six individuals collected in spring and six individuals collected in summer were analysed. The crude homogenates were then centrifuged for 15 min at 10,000 × *g*. The supernatant was then collected and frozen immediately (-80 ºC). Muscle was chosen due to 1) its commercial interest; 2) muscle activity accounts for a great part of the organism's energy consumption, 3) muscular performance determines locomotion ability and thus the capacity to escape unfavorable conditions, determining organismal survival, 4) muscle is a common target tissue in environmental monitoring, allowing comparisons with other studies, 5) muscle is easily collected during field sampling.

2.4 Proteomic analysis

2.4.1 Sample preparation

Samples were precipitated through the DOC/TCA (Na-deoxycholate/trichloroacetic acid) method (Madeira et al., 2016b). Subsequently, pellets were re-suspended in rehydration buffer and protein content was determined through the Bradford method (Bradford, 1976). See supplementary Table S1 for buffer details.

2.4.2 Two-Dimensional Gel Electrophoresis (2-DE)

Samples containing 200 µg of protein were loaded onto IPG strips (pH 4-7, 7cm, Bio-Rad) and isoelectric focusing was carried out in a Protean® IEF Cell (Bio-Rad), according to the manufacturer's instructions for 7 cm strips: 250V for 20 min (linear

mode), 4000 V for 2 h (linear mode) and 4000 V for 10,000 V-h (rapid mode). Strips were incubated in equilibration buffer I and II (15min each) and then placed on top of 12.5% polyacrylamide gels (7cm) and covered with an agarose sealing solution. Gels were run in Mini-Protean® 3 Cell (Bio-Rad) at 200 V for 45 min and were then stained for 48 h with a solution of colloidal Coomassie Blue G-250. Following, gels were de-stained with milli-Q water in several washes. See supplementary Table S1 for buffer details.

2.4.3 Gel image analysis

Gel imaging was carried out with the PropicII-robot (Genomic Solutions™, Cambridgeshire, UK) and digitalized images of the gels were analysed with Progenesis SameSpots software (version 4.0, NonLinear Dynamics, Totallab, UK). Spot quantification was carried out automatically by the software's algorithm and the incorporated statistical package was used to compare treatments – spring *versus* summer group (via a one-way analysis of variance – ANOVA, α=0.05).

2.4.4 Protein digestion

The spots of interest were manually excised and trypsin digested (0.02 μg/μL of trypsin in Ambic 12.5 mM/2% acetonitrile). Supernatants were collected to new tubes, solution was dried-down in SpeedVac (Thermo Fisher Scientific Waltham, MA, USA) and the dried peptides stored at -20 ºC until MS and MS/MS analyses.

2.4.5. Mass spectrometry

Peptides were re-suspended and mixed (1:1) with a matrix of α-Cyano-4-hydroxycinnamic acid. Three aliquots of samples (0.5μL) were spotted onto the MALDI sample target plate. Peptide mass spectra were obtained on a MALDI-TOF/TOF mass

spectrometer (4800 Proteomics Analyzer, ABSCIEX, Europe) in the positive ion reflector mode. Spectra were obtained in the mass range between 900 and 4500 Da with ca. 1500 laser shots. For each sample spot, a data dependent acquisition method was created to select the six most intense peaks, excluding those from the matrix, or acrylamide peaks, for subsequent MS/MS data acquisition (S/N threshold of 50). A fragmentation voltage of 2kV was used. MS/MS spectra were randomly selected for manual inspection to confirm the presence of six successive amino acids covered by b or y fragmentations. Trypsin autolysis peaks (m/z 842.5 and m/z 2211.1) were used for internal calibration of the mass spectra.

2.4.6 Database Search

Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems), which uses internal MASCOT software (v2.1.0 Matrix Science, London, UK) on searching the peptide mass fingerprints and MS/MS data following the parameters (i) taxonomy Chordata, since there is little information for marine invertebrate species (ii) carbamidomethylation and propionamide of cysteine as a variable modification as well as oxidation of methionine (iii) allowance for up to two missed tryptic cleavages (iv) peptide mass tolerance 40 ppm, (v) fragment ion mass tolerance 0.3 Da. Positive identifications were accepted up to 95% of confidence level. Protein identifications were considered reliable when a minimum sequence tag of five amino acids (six consecutive peaks in the MS/MS spectrum) occurred.

2.4.7 Protein level analysis

A cluster analysis was carried out to visualize level changes of proteins of interest using Cluster 3.0 plus Java TreeView.

2.4.8 Categorization of identified proteins into functional classes

Biological function of identified proteins was assessed using the tools UniProt, GeneCards, neXtprot beta and InterPro. A protein network analysis was also performed using ClueGo v2.5.4 + CluePedia v1.5.4 plugin (from Cytoscape v3.5.1 platform).

2.4.9 Validation of proteomic analysis

Glycogen phosphorylase (PYG) was chosen as target protein for validation via Enzyme Linked Immunosorbent Assay using anti-PYG (produced in rabbit, Sigma Aldrich #SAB21011921) as primary antibody (1 $\mu g.mL^{-1}$, reactivity previously tested via western blot see Supplementary Table S1), anti-rabbit IgG conjugated to alkaline phosphatase (whole molecule, produced in goat, Sigma-Aldrich, #A3687) as secondary antibody (2 $\mu g.mL^{-1}$) and a p-nitrophenyl phosphate solution as substrate. Results were normalized using total protein content, determined through the Bradford method. As no standards were available, a relative quantification was carried out and results were expressed as absorbance $units.mg^{-1}$ of total protein. To compare PYG levels between spring and summer acclimatized shrimps, a *t*-test was applied (the data met the assumptions of normality and homescedasticity, previously tested via Shapiro-Wilk and Levene's test). Statistics were carried out in STATISTICA (StatSoft, v10).

For methodological details concerning proteomic analysis, bioinformatics and validation see supplementary Table S1.

2.5 Fulton's K condition index

Fulton's K condition index was calculated using the formula:

$K = 100\ M_t/L_t^3$ (1)

Where M_t is the total wet mass (g) and L_t is the total length (cm) (Ricker, 1975). This index is suitable to compare different individuals of the same species, indicating differences in condition (heavier individuals at a given length are assumed to be in better condition) that may be related to season, sex, place of capture (Ricker, 1975). Data was checked for normality and homoscedasticity through Shapiro Wilk's and Levene's test, respectively. As the data met these assumptions, a t-test was used to compare the condition of spring- *vs* summer-acclimatized shrimps (α=0.05 for all analyses) (Statistica v10, StatSoft Inc, USA).

2.6 Inter-individual variation analyses

Differentially accumulated proteins may have an adaptive value, carrying out functions that are significant under ecologically relevant stresses. In order to evaluate their inter-individual variation, the log normalized spot volumes ± SD were used to calculate the coefficient of variation (CV, in %) for each differentially accumulated protein in each season using the formula:

% CV = mean/standard deviation *100 (2)

A Mann-Whitney U test was applied to detect if the % CV of proteins varied between seasons. Afterwards, the same test was applied to detect if % CV was different between seasons when proteins were grouped by functional categories (zinc finger proteins, energetic pathway proteins and cytoskeletal proteins). A Kruskal-Wallis ANOVA was also applied to test if % CV of functional groups differed within each season (α=0.05 for all analyses) (Statistica v10, StatSoft Inc, USA).

3. Results

3.1 Field data

During the spring collection, air temperature was 13.90±0.32 ºC and water temperature in the intertidal pools was 18.50±0.71 ºC. During this low tide, pool temperatures remained fairly constant. Salinity values remained constant (38 ‰). The pH was 8.91±0.38. During the summer collection, air temperature was 27.5±2.08 ºC. The mean ± SD of water temperature was 28.30±1.30. Salinity and pH were 36.47±1.87 ‰ and 8.47±0.43, respectively. Photoperiod was assessed for both sampling months and the mean ± SD daylight hours were 13.22±0.35 h in April and 14.83±0.06 h in June. Long-term data showed that seasonal variation in tidal pool water temperature is in the range of 15-20 ºC from winter to summer and approximately 8-10 ºC from spring to summer. Daily variation is in the range of 10 ºC or higher. Daily variation in oxygen saturation is on the order of 20 ppm whereas salinity is in the order of 3 ‰ and pH in the order of 0.52-1.37 units (see supplementary Fig. S2).

3.2 Proteomic analysis

The analysis of variance ($p<0.05$) showed that among the 520 detected spots, 55 were differentially accumulated between the two seasons (15 were up-accumulated and 40 were down-accumulated from spring to summer, Fig. 1, Table S2, and see Fig. S3 for SameSpots report). Of the 55 spots, 24 were successfully identified (Table 1). Eighteen spots were identified as actin, corresponding to different forms of the protein, mainly involved in cytoskeleton dynamics, muscle functioning and cell motility (76% of the proteins, Fig. 2, 3 for protein level changes and pathways). In addition, three spots were identified as zinc finger proteins, which are transcription regulation factors (12% of the proteins, Fig. 2). Two other spots were identified as enzymes important in the energetic

metabolism i.e. glycolysis and glycogenolysis (phosphoglycerate mutase and glycogen phosphorylase, 8% of the proteins, Fig. 2, 3) and one spot was identified as albumin (Table 1). The network analysis (Fig. 3) showed the non-redundant biological terms associated with the genes that code the proteins detected in this study. Actin genes have functions in cellular structure (cytoskeleton organization, filament assembly, muscle adaptation and contraction), inter-cellular interactions (formation and degradation of gap junctions, and stress induction of heat shock proteins regulation). Moreover, actins have functions in cellular proliferation, transformation, survival and motility. PYG (glycogen phosphorylase) gene was associated with the use of glycogen reserves (glycogen breakdown) and ALB (albumin) was associated with mitochondrion location (Fig. 3). Regarding the validation of proteomic results, PYG levels were significantly different between spring and summer acclimatized shrimps (t-test=2.331; df=10; p=0.0419, Fig. 4).

Mass and sequence results can be consulted in Table S3.

Table 1 Proteins differentially accumulated between spring and summer conditions in *Palaemon elegans* muscle collected in Cabo Raso (Cascais, Portugal, 38°42'38"N and 9°29'08"W). Protein fold-changes were automatically calculated by SameSpots software. These proteins were identified using MASCOT under the taxonomy Chordata.

Spot no.	Fold-change	p value	Protein Name	Species	Accession number	Protein MW (theoretical)	Protein PI (theoretical)	Peptide Count	Protein Score	Protein Score CI %	Ion Score	Ion Score CI %	Sequence
869	↑1.4	0.041	Phosphoglycerate mutase 2	*Bos taurus*	PGAM2_BOVIN	28666.78	8.99	3	64.9	97.29	53.69	99.911	HGEEQVKIWR
887	↓2.9	0.046	Actin, muscle	*Styela plicata*	ACTM_STYPL	42327.06	5.23	3	87.9	99.98	69.29	99.997	SYELPDGQVITIGNER
815	↑1.7	0.015	Serum albumin	*Bos taurus*	ALBU_BOVIN	69248.44	5.82	6	179	100	58.61	99.957	LGEYGFQNALIVR
											90.85	100	DAFLGSFLYEYSR
1046	↓1.4	0.021	Actin, cytoplasmatic 1	*Oryctolagus cuniculus*	ACTB_RABIT	41728.77	5.3	2	107	100	95.91	100	SYELPDGQVITIGNER
588	↓1.7	0.040	Actin, cytoplasmatic 2	*Xenopus tropicalis*	ACTG_XENTR	41737.76	5.3	9	236	100	64.4	99.998	GYSFTTTAER
											138.05	100	SYELPDGQVITIGNER
437	↓1.7	0.043	Actin, cytoplasmatic 2	*Xenopus tropicalis*	ACTG_XENTR	41737.76	5.3	3	68.3	98.76	50.16	99.646	SYELPDGQVITIGNER
949	↓2.0	0.007	Glycogen phosphorylase, muscle form	*Oryctolagus*	PYGM_RABIT	97227.85	6.77	17	115	100	56.82	99.976	DYYFALAHTVR
793	↓2.2	0.006	Actin, alpha muscle	*Bos taurus*	ACTS_BOVIN	42023.85	5.23	3	90.5	99.99	74.98	100	SYELPDGQVITIGNER
1039	↑1.7	0.007	Actin, cytoplasmatic 2	*Xenopus tropicalis*	ACTG_XENTR	41737.76	5.3	4	100	100	67.85	99.992	SYELPDGQVITIGNER
1025	↓4.1	0.045	Actin, cytoplasmatic	*Branchiostoma lanceolatum*	ACTC_BRALA	41685.73	5.22	15	210	100	46.9	99.902	GYSFTTTAER
											96.83	100	SYELPDGQVITIGNER
576	↓2.0	9.693e-005	Zinc finger protein 728	*Mus musculus*	ZN728_MOUSE	86365.49	9.39	19	63.8	96.52			
794	↓2.1	0.027	Actin, alpha	*Carassius auratus*	ACTS_CARAU	41943.89	5.23	8	134	100	96.12	100	SYELPDGQVITIGNER

810	↓2.0	0.017	muscle Actin, alpha muscle	*Carassius auratus*	ACTS_CARAU	41943.89	5.23	8	156	100	124.77	100	SYELPDGQVITIGNER
792	↓2.0	0.035	Beta-actin like protein 2	*Homo sapiens*	ACTBL_HUMAN	41975.96	5.39	10	139	100	95.01	100	SYELPDGQVITIGNER
651	↓1.4	0.028	Actin, alpha cardiac	*Takifugu rubripes*	ACTC_TAKRU	41947.85	5.22	3	131	100	113.35	100	SYELPDGQVITIGNER
481	↑1.5	0.012	Zinc finger protein 227	*Bos taurus*	ZN227_BOVIN	89302.72	8.88	11	64.2	96.82			
1004	↓1.5	0.005	Actin, alpha muscle	*Takifugu rubripes*	ACTSB_TAKRU	41949.82	5.22	3	66.3	98.04	44.73	98.889	SYELPDGQVITIGNER
809	↓1.8	0.026	Actin cytoplasmatic	*Branchiostoma lanceolatum*	ACTC_BRALA	41685.73	5.22	11	239	100	71.4	100	GYSFTTTAER
											129.3	100	SYELPDGQVITIGNER
865	↑1.6	0.035	Actin, cytoplasmatic 1	*Camelus dromedarius*	ACTB_CAMDR	41774.76	5.3	3	73.6	99.63	51.59	99.678	SYELPDGQVITIGNER
888	↓2.1	0.033	Beta-actin like protein 2	*Mus musculus*	ACTBL_MOUSE	41976.99	5.3	9	146	100	116.86	100	SYELPDGQVITIGNER
825	↓1.6	0.001	Actin, cytoplasmatic 2	*Xenopus tropicalis*	ACTG_XENTR	41737.76	5.3	3	97.8	99.99	30.24	88.413	GYSFTTTAER
											56.11	99.97	SYELPDGQVITIGNER
468	↓1.6	0.049	Putative beta-actin like protein 3	*Homo sapiens*	ACTBM_HUMAN	41988.83	5.91	2	96.2	99.99	80.14	100	SYELPDGQVITIGNER
572	↓1.6	0.014	Zinc finger protein 823	*Homo sapiens*	ZN823_HUMAN	70224.50	9.04	19	71.1	99.35			
883	↓2.0	0.040	Actin, cytoplasmatic type 8	*Xenopus laevis*	ACT8_XENLA	41820.80	5.31	13	150	100	89.07	100	SYELPDGQVITIGNER

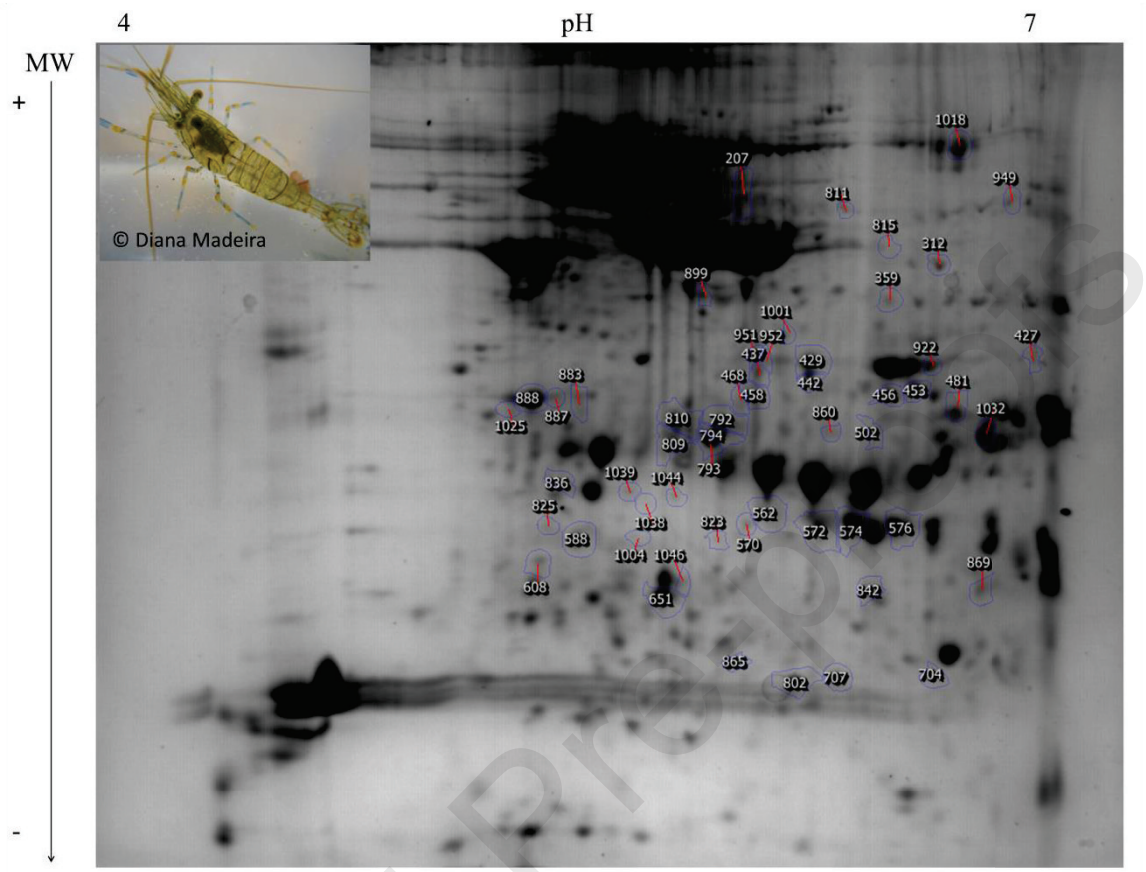

Fig. 1 Image of the master gel depicting the protein spots detected in *Palaemon elegans* muscle (total of 520 spots). The master gel is a representative gel automatically chosen by the software (SameSpots) among all sample gels. This gel summarizes spot identifications and serves as the basis for comparisons of different spot patterns between groups. Individual gel replicates can be accessed in supplementary material (Fig. S3). Annotated spots were those that were differentially accumulated (ANOVA, $p<0.05$) between spring (n=6) and summer (n=6) acclimatized shrimps.

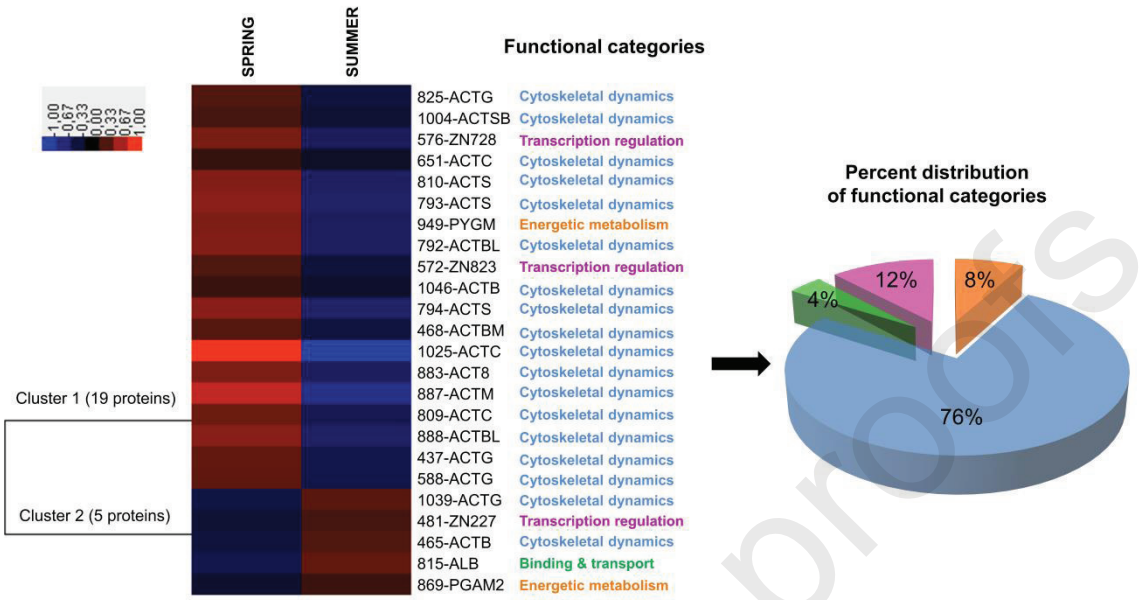

Fig. 2 Heat map representation of the clustered data matrix in which cells represent the $\log_2$ values of protein normalized volumes. The color scale ranges from blue (lower than the mean protein level, thus down-accumulated) to orange (higher than mean protein level, thus up-accumulated). Columns represent different seasons while rows represent different proteins (identified by spot number and the correspondent identification by mass spectrometry). Biological functions were listed for identified proteins in each cluster. Cluster one represents proteins that were down-accumulated in summer-acclimatized shrimps and cluster two represents proteins that were up-accumulated in summer acclimatized shrimps (*Palaemon elegans*) collected in Cabo Raso (Portugal).

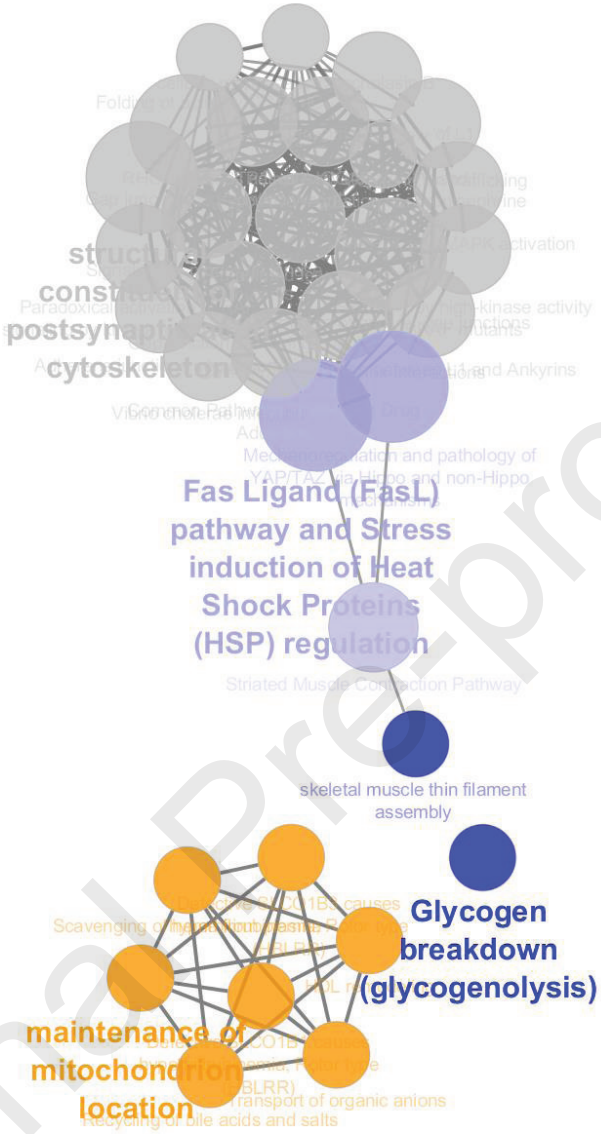

Fig. 3 Protein network analysis carried out using ClueGo v2.5.4 + CluePedia v1.5.4 plugin (from Cytoscape v3.5.1 platform, using the human database), to depict pathways associated with the differentially accumulated proteins identified from the muscle of *Palaemon elegans* collected in spring and summer (Cabo Raso, Portugal). Blue nodes indicate down-regulated pathways; orange nodes indicate up-regulated pathways; gray nodes indicate up- and down-regulated pathways. Node size relates to significance and number of genes associated to that biological process. The most significant gene ontology terms for each gene are shown in bold.

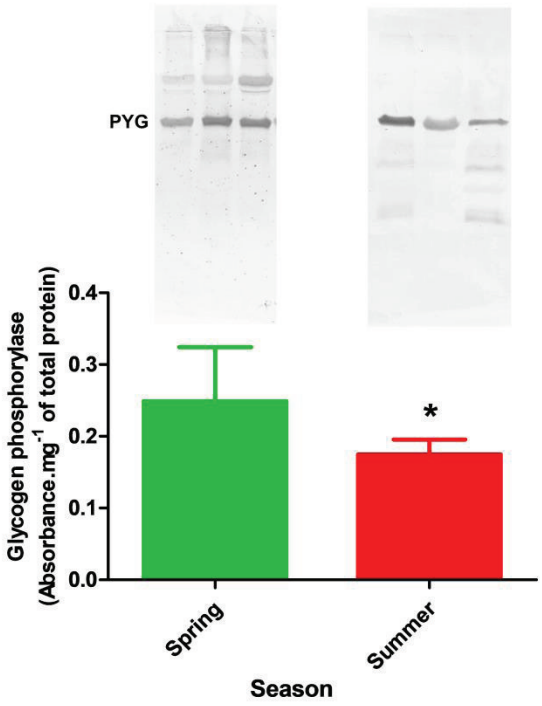

Fig 4. Relative quantification of glycogen phosphorylase (PYG) levels in *Palaemon elegans* via Enzyme Linked Immunosorbent Assay (n=6 spring acclimatized shrimps and n=6 summer acclimatized shrimps). Antibody reactivity was tested via western blot for n=3 shrimps in each season. PYG levels were significantly different between seasons (t-test=2.331; df=10; p=0.0419).

3.3 Fulton's K condition index

The Fulton's K (mean±SD) was significantly different between seasons (t=-2.48, df=13, p=0.027), being 1.13±0.20 and 1.35±0.09 for spring and summer collected shrimps, respectively.

3.4 Inter-individual variation in differentially accumulated proteins

The overall coefficient of variation (% CV) for differentially accumulated proteins was 2.77±1.44 (mean±SD) in spring (ranging from a minimum of 0.64% for actin cytoplasmatic 2 proteoform to a maximum of 8.29% for actin cytoplasmatic proteoform) and 2.48±0.82

(mean±SD) in summer (ranging from a minimum of 1.16% to a maximum of 4.81% for unknown proteins) (see Table S4). Within identified spots, the % CV of cytoskeletal proteins was significantly different between spring and summer (higher % CV in spring, U=110, p=0.04). There were no significant differences between % CV of functional groups within each season (spring H=3.9, p=0.14; summer H=4.05, p=0.13).

4. Discussion

The ability of organisms to cope with environmental variability depends on their capacity to modify gene expression (Logan and Somero, 2011) which is especially important in variable habitats like the intertidal zone. Proteomic analysis revealed significant changes in the muscle of shrimp exposed to low tide depending on sampling season (spring *vs* summer). Differentially accumulated proteins were mainly related to cytoskeleton dynamics (76%). Other major functional categories include energetic metabolism (8%) and regulation of transcription (12%), confirming our hypothesis that shrimps would regulate proteins involved in homeostasis (e.g. cytoskeletal proteins) and metabolic function as a mechanism to endure low tide conditions during summer. Proteomic changes in this study could be interpreted in two ways (i) solely a response to acute stress during low tide, or (ii) a mechanism of seasonal acclimatization. Both processes are very likely and are probably occurring at the same time. However, there is no evidence of a response to acute stress as an induction of cellular repair mechanisms was not observed (e.g. proteins involved in redox regulation, DNA damage and repair, molecular chaperones, protein degradation). Still, some intertidal animals seem able to maintain protein levels unaltered even under exposure to stress (Diz et al., 2017).

Zinc finger protein (ZFP, transcription regulators) regulation across seasons indicates that shrimps are able to modulate gene expression probably contributing to a shift in physiological parameters from spring to summer. However, knowing which genes are being regulated by ZFPs is not possible. Storey and Storey (2011) mention that cells can shut down gene expression, as well as protein turnover and cell cycle under great stress. However, this does not seem to be the case in *P. elegans*, suggesting that shrimps were not under life-threatening stress during summer low tides. Moreover, Fulton's K was higher in summer-collected shrimps, indicating that molecular adjustments probably coupled to higher food availability contributed to a remodelling of metabolism and a higher condition index in summer. Still, an up-accumulation of ubiquitin in warmer seasons has been previously detected in *Palaemon* (Madeira et al., 2016a) indicating irreversible protein damage. It should be noted that ubiquitin increases may in fact contribute to maintain health by preventing the cytotoxic effects of accumulated unfolded proteins. However, ubiquitin changes were not detected in this study, possibly due to limitations in protein identification. Non-model organisms often lack sequencing data, requiring homology-based and ultra-tolerant searches, relying on cross-species identification which is less successful when compared to standard approaches using model organisms with available genomes and proteomes. Nonetheless, regulated proteins detected in this study should be conserved among taxa, as many proteins that change with seasonal fluctuations are part of the ubiquitous minimal stress proteome (Kultz, 2003; Kültz, 2005). Also, the classic 2D-gel proteomic approach has some shortcomings that could prevent the detection of several proteins (i) low load ability, (ii) poor separation of hydrophobic, acidic and basic proteins, (iii) narrow dynamic range: only a limited number of highly abundant and soluble proteins are resolved while low abundant proteins are much harder to detect (Magdeldin et al., 2014;

Rabilloud and Lelong, 2011). Some of these problems can be resolved by prefractionation of the samples which decreases complexity, depletion of highly abundant proteins, using large gels or choosing narrow pH gradients to separate closely arrayed spots. Still, 2D-gel workflows are considered robust (Magdeldin et al., 2014; Rabilloud and Lelong, 2011).

No changes were detected in anti-oxidant enzymes as opposed to other studies in intertidal and oceanic organisms exposed to changing environmental conditions (Abele et al., 1998; Machado et al., 2014; Madeira et al., 2016a; Pöhlmann et al., 2011; Vinagre et al., 2014). Enzymes can be regulated by two main mechanisms (1) control the synthesis/degradation of the enzyme and/or (2) control the activity of the enzyme. The fastest and energetically advantageous mechanism is to regulate the activity of a preexisting enzyme; this way, enzyme activity increases when substrate concentration is high. Following this rationale, if an organism is under fluctuating environmental conditions, one would first see changes in enzyme activity and not enzyme levels. The aforementioned studies analysed enzyme specific activities (that depend on substrate affinity, thermal stability, thermal optimum for activity), whereas this study evaluates enzyme levels and thus these may remain stable despite variation in enzyme activity.

P. elegans inhabits intertidal zones where the maximum registered water temperature in tidal pools is 30 ºC during summer (Madeira et al., 2015) and the minimum is 7 ºC during winter. Seasonal variation (15-20 ºC from winter to summer; 8-10 ºC from spring to summer) and daily variation (≥10 ºC) in water temperature (supplementary Fig S2) are elevated. Thus, to be able to cope with such a fluctuating regime, in which extremes are very frequent, shrimps must possess adaptations and enough phenotypic plasticity to

survive, grow and reproduce. Even though temperature is one of the most variable factors between seasons, there are other abiotic and biotic factors that may influence protein expression such as photoperiod, pH, salinity, food abundance and reproductive status. Some of these variables were quite similar between seasons (e.g. pH, salinity). However, such factors can vary widely throughout the day (due to sea water flow through the pools, photosynthesis, tidal pool location sun *vs* shadow - see supplementary Fig S2), hence the need to standardize collection time. Food supply and composition is also known to vary across season in intertidal habitats (Barry and Ehret, 1993) potentially influencing energy reserves and growth of *P. elegans*. Water oxygenation also varies seasonally, as temperature and photoperiod increase from spring to summer. Thus, there could be higher oxygen levels during summer days, especially due to increased photosynthesis. During a tidal cycle, variation can be in the range of 20 ppm, with very elevated levels during the day/low tide and lower levels during the night (due to coupled plant and animal respiration)/high tide. All of these factors are considered relevant and may play a part in the mechanism of seasonal acclimatization and responses to acute stress.

Previous physiological studies have shown that *P. elegans* has some capacity to acclimate to changes in temperature (Vinagre et al., 2016), but may incur high metabolic costs when compared to subtidal congeners, which inhabit more stable environments (Magozzi and Calosi, 2015). Given that biological variation is more important under stressful regimes (e.g. intertidal habitat) than under benign conditions (Forsman and Wennersten, 2016), the inter-individual variation in protein expression may be crucial for the viability of intertidal organisms and may have impacts on the ecological and evolutionary processes of populations (Forsman and Wennersten, 2016). However, inter-individual variation of

proteins in this study was quite moderate (maximum of 8.3%) which is highly relevant in the context of fluctuating habitats like the intertidal area. Forsman and Wennersten (Forsman and Wennersten, 2016) state that (i) variation promotes population performance, and (ii) higher biological diversity decreases the population vulnerability to environmental changes. Thus, lower levels of inter-individual variation could promote the vulnerability of intertidal shrimps to further stressful conditions (e.g. global change, pollution). Interestingly however, there is another hypothesis, proposed by (Oleksiak and Crawford, 2012) which states that under highly variable environments, evolution should favor physiological canalization. This means that the induction of physiological mechanisms to maintain homeostasis will minimize phenotypic differences created by genetic polymorphisms (Oleksiak and Crawford, 2012), which could also explain the low coefficient of variation in protein levels between individuals. Moreover, other studies report a limited acclimation capacity in intertidal organisms (Hopkin et al., 2006; Somero, 2010) as they are already living close to their physiological limits, potentially limiting the viability of such populations under further anthropogenic changes. Noticeably, the inter-individual variation of cytoskeletal proteins was significantly higher in spring when compared to summer. Inter-individual variation may be related not only to genetic profiles but also to differences in age, gonad maturation stages, nutritional status and recent thermal history (Madeira et al., 2012). Such differences could be more accentuated in spring, during which shrimps start to mature and food resources are more variable.

Cytoskeletal proteins were overrepresented (75% of identified proteins) in proteomic data suggesting that structural properties are important to cope with low tides across seasons. Abundance of cytoskeletal proteins (actin proteoforms) generally decreased from spring to

summer. Actins are structural proteins regulating cellular processes related to motility, structure and integrity. We identified 18 actin proteoforms, including alpha and beta proteoforms, cardiac-like and muscle-like proteoforms and cytoplasmatic proteoforms. Isoform diversity in crustaceans is known to be related with tissue and organelle-specific localization (Kim et al., 2009). Both increases and decreases in cytoskeletal proteins in organisms acclimated to different environmental conditions (mainly temperature) have been detected in laboratory studies (Cottin et al., 2010; Fields et al., 2012; Jayasundara et al., 2015; Tomanek, 2011; Tomanek and Zuzow, 2010), indicating a reorganization of cytoskeleton with acclimation possibly to maintain cellular function and performance. Thus, this cytoskeleton remodeling may be an important mechanism that allows shrimps to adjust their thermal tolerance across seasons (i.e. with acclimation temperature), as reported for marine crustaceans (Hopkin et al., 2006; Madeira et al., 2015). Cytoskeleton is known to be a major target of oxidative stress and cytoskeletal proteins may be the 'weak elements' that trigger the expression of molecular chaperones (Tomanek et al., 2011; Tomanek and Zuzow, 2010). Furthermore cytoskeleton stability seems to be of crucial importance in thermal protection. Such hypothesis was tested in locusts exposed to heat stress and the authors found that the stabilization of cytoskeleton was crucial in increasing time to failure and decreasing time to recovery. Disruption of cytoskeleton stability terminated thermal protection, confirming the importance of cytoskeletal homeostasis in preserving cellular function under thermal stress (Garlick and Robertson, 2007). The authors further suggest that this may be related with the consequent stabilization of ion channels and integral membrane proteins and their interactions with heat shock proteins. Moreover, cytoskeleton is important in the co-localization of metabolic enzymes and muscle fiber restructuring, facilitating acclimatization by switching between fast twitch,

slow twitch and slow phasic under challenging conditions (Garland et al., 2015). In this study, no molecular chaperones were detected among the differentially accumulated proteins. This result is in accordance with laboratory studies performed in *P. elegans* exposed to thermal stress (Madeira et al., 2015, 2012) which did not detect changes in molecular chaperones. Moreover, Jayasundara et al. (Jayasundara et al., 2015) reported the same result in the intertidal fish *Gillichthys mirabilis*, detecting changes in cytoskeletal proteins but not in molecular chaperones. The authors state that this fish may have attained a steady-state cytoskeletal structure with acclimation, a phenomenon that could also be occurring in *P. elegans*.

The maintenance of homeostasis is crucial in highly variable environments. However, it may be energetically costly, potentially leading to a hypometabolic state or anaerobic metabolism in order to maintain energy production (Boutet et al., 2009; Lagerspetz and Vainio, 2006; Pörtner and Farrell, 2008; Storey and Storey, 2011; Thebault and Raffin, 1991). In this study, only two metabolic enzymes showed abundance changes according to season. Phosphoglycerate mutase increased whereas glycogen phosphorylase decreased from spring to summer low tide conditions. The former enzyme is known to play a role in glycolysis, catalyzing the interconversion of 3-phospho-D-glycerate into 2-phospho-D-glycerate. However, as the reaction is reversible and the enzyme catalyzes it in both directions, it is not exactly clear if the increase in this enzyme is favouring the products or the reactants of glycolysis. Concerning glycogen phosphorylase, it plays a role in glycogenolysis, breaking glycogen into glucose-1-phosphate subunits, to be further used in glycolysis as glucose-6-phosphate. Thus, it seems that glycogenolysis is down-regulated, suggesting that shrimps could be reducing the substrate for glycolysis and maintaining

glycogen stores in summer low tides. This is quite unexpected because glycogen content is usually higher in colder seasons/conditions when compared to warmer ones as observed in limpets and fish (Bjelde and Todgham, 2013; Vornanen et al., 2011; Yang et al., 2015). However, an increase in glycogen content during chronic warming has been detected in the thermally tolerant freshwater mussel *Villosa lienosa* (Payton et al., 2016) suggesting that the accumulation of glycogen may be a metabolic strategy of tolerant species. Nonetheless, the hypothesis that elevated summer temperatures could denature the enzyme and lower its activity should not be discarded, even though this is unlikely since the metabolic enzymes of intertidal species are more thermally stable when compared to those of subtidal species and can function close to the thermal limits (Sokolova and Pörtner, 2001) (34.08 ºC in summer-acclimatized *P. elegans* (Madeira et al., 2015). Still, previous thermal history, present acclimation temperature and intertidal height could also account for metabolic enzyme concentration and activity (Sokolova and Pörtner, 2001). For instance, intertidal animals may have lower enzyme activity than subtidal ones, thus showing metabolic compensation (Sokolova and Pörtner, 2001). A similar phenomenon could be occurring across seasons.

5. Conclusions

There is a need for integrating proteomic methods in ecology and population studies (Biron et al., 2006) and these should not be restricted to model organisms because new hypothesis and questions can arise from research in non-model organisms (Diz et al., 2012). Here, we showed that proteome changes in the muscle of *P. elegans* associated with seasonal variation in low tide pools were mostly related to cytoskeleton dynamics, energy metabolism and transcription, highlighting that seasonal physiological adjustment overlaps

with the typical response to elevated temperature detected in marine organisms in laboratory experiments. Shrimps modulate these proteins as a mechanism to cope with seasonal variation possibly to promote performance and homeostasis, as suggested by the higher condition index in summer-collected shrimps. Thus, seasonal adjustments may corroborate the beneficial acclimation hypothesis, even though such an assumption needs further testing. However, the ability to cope with further extremes imposed by anthropogenic forcing (e.g. global change) remains an issue to be explored, especially considering the low inter-individual variation in protein levels detected in this study and the limited acclimation capacity reported for intertidal species. This study has implications for the research of phenotypic plasticity in intertidal species under a natural framework since differentially accumulated proteins may have an adaptive value and could be further used as indicators of environmental variation *in situ*.

Data Accessibility

Accession numbers of the MASCOT search:
Q32KV0, Q00214, P02769, P29751, Q6P378, Q6P378, P00489, P68138, Q6P378, O17503, Q6P5C7, P49055, P49055, Q6P378, Q562R1, P53480, A0JNB1, P53482, O17503, P84336, Q8BFZ3, Q6P378, Q9BYX7, P16415, P53506.

Accession numbers of human homologues used to construct the cytoscape network:

P15259, P68133, P02768, P60709, P63261, P63261, P11217, P68133, P63261, P68133, P0DKX0, P68133, P68133, Q562R1, P68133, Q86WZ6, P68133, P68133, P60709, Q562R1, P63261, Q9BYX7, P16415, P68133

Author contributions

MSD and CV designed the study; DM, VM, MSD and CV did the field work; DM and VM prepared the samples; JEA carried out 2D electrophoresis and image analysis; RV did the mass spectrometry; RV, DM and JEA carried out bioinformatics analyses; CM carried out

the validation of proteomic analysis; DM wrote the manuscript with relevant inputs from all authors.

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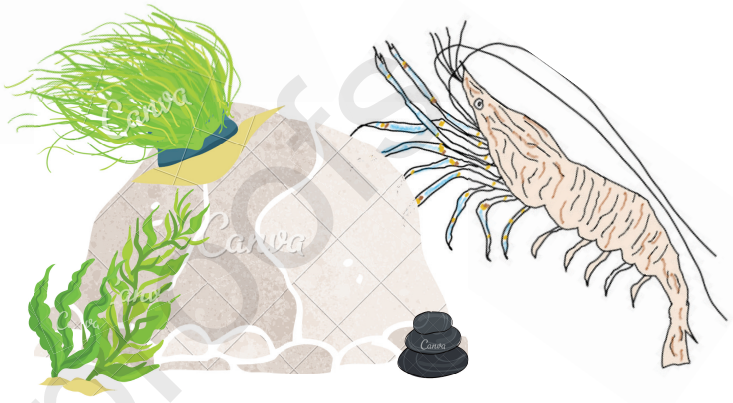
Seasonal acclimatization

in situ



SPRING **SUMMER**

Proteome variation

Cytoskeletal dynamics
Energy metabolism
Transcription regulation

Protein coefficient of variation: low

Condition index

Highlights

1. Seasonal proteome variation in intertidal shrimps was analyzed in situ
2. Shrimps were collected during spring and summer low tides
3. Cytoskeleton, energy metabolism and transcription were modulated by season
4. Inter-individual variation in protein levels was low
5. Molecular plasticity is crucial to cope with environmental fluctuations

Declarations of interest

None.