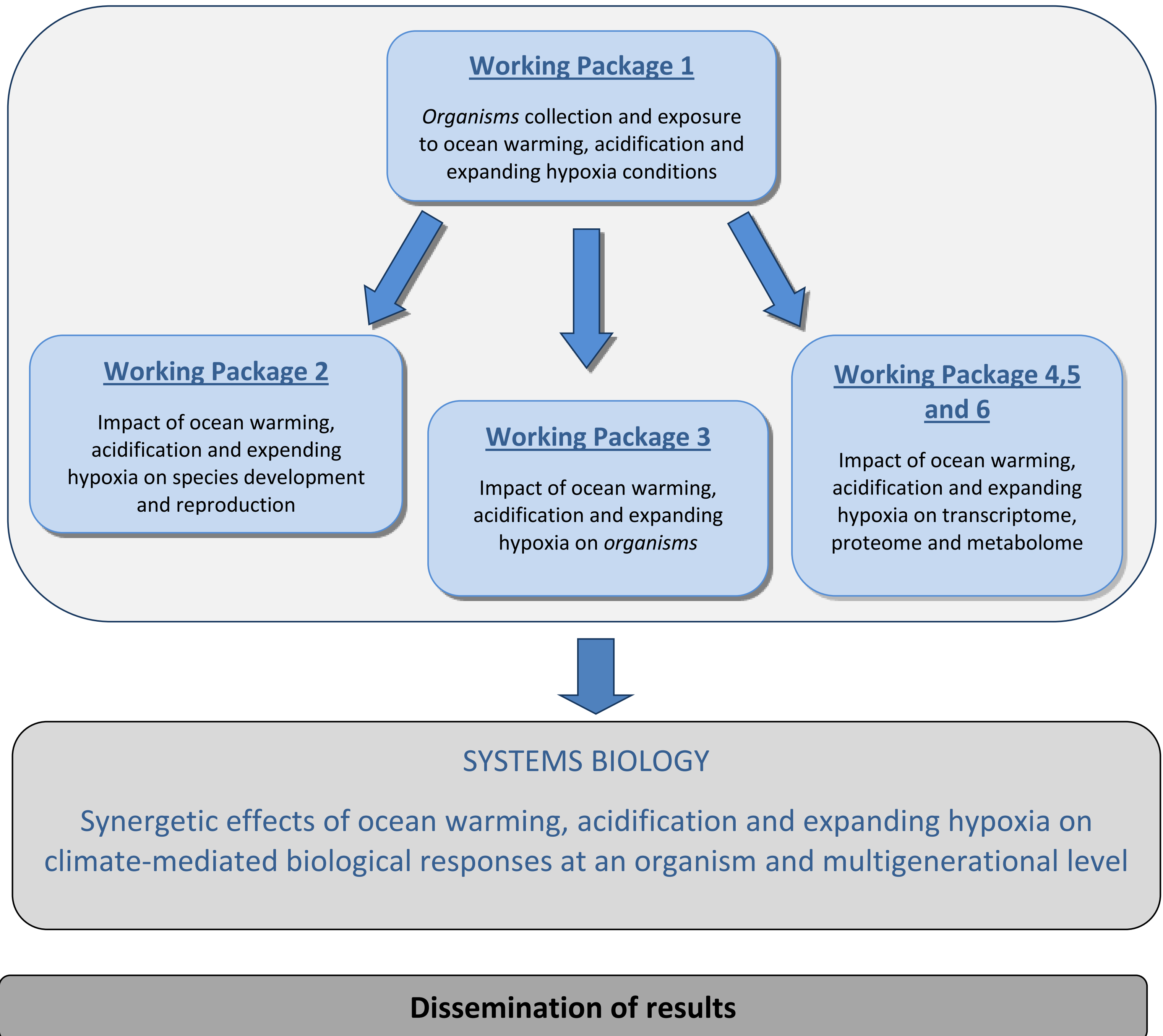
Annex 3 – Flowchart of the Project proposal and WP details



Details for some Working Packages

WP3 Ecophysiology

Task 3.1 Thermal tolerance limits, heat shock response, energy metabolism

Routine Metabolic Rates (RMR)

Oxygen consumption measurements (routine metabolic rates; RMR) will be determined accordingly [1-5]. Individualized organisms, from each experimental condition (control, ocean warming and acidification, hypoxia), will be incubated in sealed water-jacketed respirometry chambers (RC300 Respiration cell, Strathkelvin, North Lanarkshire, Scotland) containing filtered (0,35µm) and UV sterilized seawater (35 PSU), mixed with antibiotics (50 mg L^{-1} streptomycin), in order to avoid bacterial respiration. Water volumes will be adjusted in relation to animal mass (up to 4 mL) in order to minimize locomotion and stress. Concomitantly, bacterial controls will be conducted to correct for possible bacterial respiratory activity. Respiration chambers will be placed in water temperature controlled baths (Lauda, Lauda-Königshofen, Germany). Oxygen concentrations will be recorded with Clarke-type O_2 electrodes (Strathkelvin, North Lanarkshire, Scotland) connected to a multi-channel 929 oxygen interface (Strathkelvin, North Lanarkshire, Scotland). The duration of respiratory runs will vary according to development stage (nauplios, juveniles or adult organisms) from 1 to 12 h.

External critical oxygen partial pressures ($P_{C\ ext}$; the point at which the rate of oxygen consumption is no longer maintained independent of ambient oxygen partial pressure) will be determined by plotting specific rates of oxygen consumption against oxygen partial pressure. Regressions will be calculated for the two distinct sections of the curve: the regulated (higher PO_2) segment and the very sloped (low PO_2) segment. P_C is defined as the point where the two regressions intersect [4].

Respiratory enzymatic analyses (citrate synthase and lactate dehydrogenase)

Respiratory enzymatic analyses will be determined accordingly [1*]. Pooled organism samples, from each experimental condition (control, ocean warming and acidification, hypoxia), will be homogenized in varying dilutions of 0.01 mol l^{-1} Tris buffer (pH 7.5 at 10°C) in a Duall hand-held glass homogenizer kept on ic. Afterwards, homogenates will be centrifuged in an Eppendorf 5402 Centrifuge (Eppendorf, NY, USA), at 6600g for 10 minutes, at 5°C. Samples (25 µl) of supernatant will be placed in 1 ml quartz cuvettes under non-limiting substrate conditions and

enzymatic activities will be measured (at 20°C) using a Shimadzu UV160U spectrophotometer (Shimadzu Scientific Instruments, MD, USA) equipped with a water-jacketed cuvette holder connected to a recirculating water bath. The enzyme citrate synthase (CS, E.C. 4.1.3.7) will be assayed as an indicator of aerobic metabolic potential and lactate dehydrogenase (LDH, E.C. 1.5.1.11) as an indicator of anaerobic metabolic potential.

Thermal tolerance and heat shock response

Thermal tolerance

In every generation, organism's upper thermal tolerance limits will be determined [2]. Organisms, from each experimental condition (control, ocean warming and acidification, hypoxia), will be incubated in small containers with seawater (35 PSU) taken from incubation tanks, corresponding to each of the experimental conditions. Each container, comprising 4 replicates, will be suspended in a temperature regulated water bath, controlled to the nearest 0.1°C. The temperature of the water bath will be set to the acclimation temperature and maintained for 30 minutes. Thereafter, the temperature will be increased at a rate of 1°C/30 minutes. Every 30 min, the water will be aerated with an air stone type injector and the temperature in each container will be checked (with thermocouple probes), and the activity of the organisms will be monitored through 40x stereomicroscope observation (model S6D, Leica). If no responsiveness is noticed, the specimen will be considered dead. The percentage of living individuals at each temperature will be calculated and then transformed by the arcsine square root function and expressed in radians. Linear-regression analysis will then used to find the slope of the line, from which the temperature at which 50% of the organisms had died (0.785 radians) will be calculated. This will be used as the measure for upper thermal tolerance limits and referred to as the LT50.

Thermal sensitivity (Q_{10}) will be assessed from metabolic rates [2*] and determined using the standard equation:

$$Q10 = [R(T2)/R(T1)]*10/(T2-T1)$$

$R(T_2)$: oxygen consumption rate at temperature T_2

$R(T_1)$: oxygen consumption rate at temperature T_1

Heat shock response

Heat shock proteins (HSP70)

Pooled samples homogenates, corresponding from each experimental condition (control, ocean warming and acidification, hypoxia), will be prepared for HSP70 quantification and will be assessed by Enzyme-Linked Immunoabsorbent Assay (ELISA), accordingly [2*, 6*-8*]. Briefly, 10 μL of the homogenate's supernatant will be diluted in 250 μl of PBS (1x), and 50 μL of the diluted sample will be added to a 96-well microplates (Nunc-Roskilde, Denmark) and allowed to incubate overnight at 4°C (dilutions may be subject to further optimization). Afterwards, microplates will be washed (3X) in 0.05% PBS-Tween-20. One hundred microliters of blocking solution (1% bovine serum albumin [BSA], Sigma-Aldrich, USA) will be added to each well and left to incubate at room temperature for 2 h. After washing off the 96-well plates, 50 μL of 5 μg mL^{-1} primary antibody (anti-HSP70/HSC70, Acris, USA), detecting 72 and 73 kDa proteins corresponding to the molecular mass of inducible and constitutive isoforms, respectively, will be added to each well and then incubated at 37°C, for 90 minutes. The primary antibody reactivity for the used species will be validated by Western blot. The non-linked antibody will be removed by washing the microplates again, which will then be incubated for 90 minutes, at 37°C with 50 μL of 1 μg mL^{-1} of the secondary antibody, anti-mouse IgG (SIGMA), Fab specific, alkaline phosphatase conjugate (Sigma-Aldrich, USA). After three additional washes, 100 μL of substrate (SIGMA *FAST*™ p-Nitrophenyl Phosphate Tablets, Sigma-Aldrich, USA) will be added to each well and incubated 10-30 minutes, at room temperature. Stop solution (50 μL; 3N NaOH) will be added to each well and the absorbance will be then read at 405 nm in a 96-well microplate reader (BIO-RAD). The amount of HSP70 in samples will be calculated from a calibration curve, based on serial dilutions of purified HSP70 active protein (Acris, USA) to give a range from 0 to 2000 ng mL^{-1}.

Stress oxidative enzymes

Pooled samples, from each experimental condition (control, ocean warming and acidification, hypoxia), will be treated and determined accordingly [2, 6-8].

Glutathione S-Transferase (GST)

Glutathione-S-transferase (GST) total activity (EC 2.5.1.18) assay uses 1-Chloro-2,4-dinitrobenzene (CDNB) as substrate and, upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance. Enzyme activity will be determined spectrophotometrically by measuring the formation of the conjugate of glutathione (GSH) and

l-chloro-2,4-dinitrobenzene (CDNB). The assay contains 200 mM L-glutathione (reduced), Dulbecco's PBS and 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) solution. Equine liver GST (Sigma-Aldrich, Germany) will be used as positive control to validate the assay. Then, to perform the assay, 180 µL of substrate solution will be added to 20 µL of GST standard or sample in each well of a 96-well microplate (Nunc-Roskilde, Denmark) and the absorbance at 340 nm will be recorded every minute for 6 minutes, using a plate reader (BIO-RAD, California, USA). Thereby the increase in absorbance is directly proportional to GST activity. Afterwards, GST activity will be calculated using a molar extinction coefficient for CDNB of 5.3 ƐmM (Sigma Technical Bulletin, CS0410) and the results expressed as U g-1 ww. Where one unit conjugate 1.0 µmol of CDNB with reduced glutathione per minute (pH=6.5; 25°C).

Catalase (CAT)

CAT activity will be assessed by measuring the rate of H_2O_2 removal. Consequently, the reaction can be followed by a decrease in the absorbance as H_2O_2 is turned into oxygen and water. The total reaction volume of 3 mL contains 50 mM potassium phosphate buffer (pH 7.0), 12.1 mM H_2O_2 as a substrate, and the reaction is started by the addition of the sample into quartz cuvettes. The enzyme activity will be calculated using a molar extinction coefficient (at 240 nm). The consumption of peroxide (extinction coefficient for H2O of 0.04 ƐmM) (Weydert and Cullen, 2010) will be monitored using a Helios spectrophotometer (Unicam, UK) at 240 nm. The absorbance will be measured each 15 second intervals across a 180 second incubation period at 25°C and results will be given as U g^{-1} ww (one unit decompose 1.0 µmol H_2O_2 per minute (pH=7.0; 25°C)). Standard catalase activity will be measured using a bovine catalase solution (1523.6 U mL^{-1}; Sigma-Aldrich, Germany) as a positive control for assay validation.

Superoxide dismutase (SOD)

The SOD enzyme will be performed using a 96-well microplate (Nunc-Roskilde, Denmark), adding to each well, 200 µL of 50 mM phosphate buffer (pH 8.0) (Sigma-Aldrich, Germany), 10 µL of 3 mM EDTA (Riedel-Haën, Germany), 10 µL of 3 mM xanthine (Sigma-Aldrich, Germany), 10 µL of 0.75 mM NBT (Sigma-Aldrich, Germany) and 10 µL of SOD standard or sample. The reaction will start with the addition of 100 mU XOD (Sigma-Aldrich, Germany) and the absorbance at 560 nm will be recorded every minute for 5 minutes, using a plate reader (BIO-RAD, Benchmark, USA). Negative control will include all components except SOD or sample, producing a maximal increase in absorbance at 560 nm, which will allow determining the inhibition percentage per minute, caused by SOD activity. SOD from bovine erythrocytes

(Sigma-Aldrich, Germany) will be used as standard and positive control. The total SOD activity is expressed in U g^{-1} of protein, where one unit is equivalent to the SOD activity that causes 50% inhibition of the reaction rate without SOD.

WP4. Transcriptomics

Task 4.1 RNA extraction, sequencing

Pooled whole organism samples, corresponding from each experimental condition (control, ocean warming and acidification, hypoxia), will be preserved in RNAlater® (Ambion), at -22°C. Total RNA will be extracted, DNase-treated and purified (RNeasy Mini Kit, Qiagen) from pooled samples of experimental organisms, of each experimental condition. RNA quality check and quantification will be performed using a 2100 bioanalyser (Agilent Technologies). Reverse transcription (RT) will be performed in order to obtain complementary DNA (cDNA). After cDNA synthesis, each sample, corresponding to each experimental condition, will be associated with a unique sequence barcode, which will allow an accurate assignment of each transcript to the respective sample. Transcriptome sequencing [9, 10] will be performed through next-generation sequencing (Stabvida, Portugal), using a Hiseq 2000 sequencing system (Illumina).

Obtained sequences (reads), obtained trough next-generation sequencing, will be checked for quality using the sofware FastQC (Bioinformatics Group, Babraham Institute, Cambridge, UK) and Prinseq [11]. Low quality reads will be trimmed, using the Q20 value (base call accuracy of 99%). Consequently, reads will be re-checked for quality, using the software described previously to confirm trimming results. The assembly of cleaned reads will be performed using the software Trinity [12]. Assemblies will be carried out with all libraries, for posterior comparison. Alignment of reads, for each library, will be done using Bowtie software [13], while counts of the number of reads of all contigs present in each library will be calculated using RSEM software [14]. Afterwards, a search for differently expressed (DE) genes between libraries will be performed using Edge software [15]. After DE genes identification, filtration of initial assembly for contigs identified as DE genes will be done using a custom-made python script, developed by the Evolutionary Genetics Group of Professor Manuela Coelho in FCUL (http://cba.fc.ul.pt/members/manuela_coelho.php). Consequently, and with these contigs subset, a search with Blast2GO program [16] will be done in non-redundant public databases for the most similar correspondence in organisms available. Gene ontology will also be performed and functional annotation for the genes of interest will be achieved using the Blast2GO program.

Task 4.2 Bioinformatic data analysis

Genes of interest will be chosen among the most differentially analysed expressed genes. In order to obtain expression profiles of genes of interest, qRT-PCR (Linegene 9640, Bioer) will be performed in order to quantify expression levels of target genes related to proteomic and physiological changes derived from experimental conditions.

All the equipment is already available in DINIZ lab, Rosa's lab and in Professor Manuela Coelho's lab [17-20]

WP5 Proteomics

Task 4.1 Protein extraction, separation and identification

Pooled whole organism samples, corresponding from each experimental condition (control, ocean warming and acidification, hypoxia), will be stabilized using Allprotect Tissue Reagent (Qiagen). Afterwards, samples will be homogenized using a disposable probe, coupled to a tissueruptor (Qiagen). Total protein will be extracted using an AllPrep DNA/RNA/Protein Mini Kit (Qiagen). Subsequently, samples will be submitted to 1 and 2DE PAGE (Mini-Protean III and Protean® IEF Cell, BIO-RAD) [21, 22] to resolve complex protein mixtures into unique spots. Imaging of protein spots (e.g. Apex 1.1; open2Dprot and Image J software) will provide semi-quantitative assessment of alterations that can occur during the exposure to the different experimental conditions (control, ocean warming and acidification, hypoxia). After gel spot excision and protein processing (by in-gel tryptic digestion), samples will be analyzed by: (i) MALDI-TOF/TOF MS (4700 Proteomic Analyzer, Applied Biosystems) and proteins identified by Peptide Mass Fingerprint (PMF) using adequate software (MASCOT), including manual validation by analyzing the generated mass spectra [23]. Whenever necessary/possible the novo sequencing will be performed after tandem MS [24]. Sample treatments for protein depletion will include: Aurum Serum Protein Minikit (BIO-RAD), ProteoExtract Albumin/IgG Removal Kit (Merck); Multiple Affinity Removal Column (Agilent Technologies); POROS Affinity Depletion Cartridges (Applied Biosystems); Albumin and IgG Removal Kit (Amersham); ProteoPrep 20 plasma immunodepletion Kit (Sigma); TCA precipitation; acetone precipitation; chloroform/methanol precipitation; ammonium sulfate precipitation and fractionation with ammonium sulfate. Additionally, data will be treated using Data Explorer software (Applied Biosystems) and then searched in databases using MASCOT search engine (www.matrixscience.com) to identify proteins of interest.

The equipment and analytical techniques are available at FCT-UNL, in Dr. Diniz BIOSCOPE group [25-27]

WP6 Metabolomics

Task 6.1 Metabolites extraction

The main objective of this task is to use metabolomics techniques to assess, detects and quantifies the low molecular weight molecules, known as constituents of the metabolome:

1) Metabolites extraction will be achieved by liquid extraction with one solvent, aqueous or organic, or with a combination of solvents (liquid-liquid extraction) following the usual procedures (Marcinowska et al., 2011). Depending on the target metabolites and type of samples other extraction procedures can be optimized and used such as SPME or MAE (XX). Briefly, tissues (≈100 mg) will be immediately flash-freezed in liquid nitrogen and stored at -80ºC. Then for quenching, cold Chloroform/Methanol/Water (1:3:1 ratio) are added to samples. Then, tissue disruption will be achieved by sonication or homogenization then vortex at 4ºC (1h). For some metabolites a derivatization step may be included. Centrifuge for 3 minutes at 13,000xg at 4ºC and then store (-80ºC) until analysis. When necessary extraction kits can be employed (e.g. AbsoluteIDQ AB SCiex).
2) Detection: will be done using the group Instrumentation for metabolomics which includes LC-MS-MS (Micromass Q-TOF Ultima API; DIONEX ultimate 3000) and GC-MS (Fisons 8000). NMR facilities (http://www.dq.fct.unl.pt/servicos-externos/ressonancia-magnetica-nuclear) and GCT-TOF-MS (Fissons8000) at REQUIMTE can be used for additional analysis.
3) Bioinformatic data analysis: metabolite-specific database matching using accurate mass information will be used to identify compounds; diverse open source software (e.g. MetaboAnalyst 2.0 (www.metaboanalyst.ca); MetabolomeExpress (www.metabolome-express.org)) will be used to process metabolomics data and results acquired from various analytical techniques to support biomarker discovery, finding a biological signature (fingerprint) or mapping pathways.
This task will be undertaken under the Financial support of Proteomass (www.proteomass.org) with which an agreement has been established.
For support and advice on metabolomics collaboration was established with Prof. Tamara Garcia (U. Huelva)

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