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Near future pH conditions severely impact calcification, metabolism and the nervous system in the pteropod *Heliconoides inflatus*

Running head: Effect of low pH on Mediterranean pteropods

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Abstract (300 words)

Shelled pteropods play key roles in the global carbon cycle and food webs of various ecosystems. Their thin external shell is sensitive to small changes in pH and shell dissolution has already been observed in areas where aragonite saturation state is ~ 1. A decline in pteropod abundance has the potential to disrupt trophic networks and directly impact commercial fisheries. Therefore it is crucial to understand how pteropods will be affected by global environmental change, particularly ocean acidification. In the present study, physiological and molecular approaches were used to investigate the response of the Mediterranean pteropod, *Heliconoides inflatus*, to pH values projected for 2100 under a

moderate emissions trajectory (RCP6.0). Pteropods were subjected to pH_T 7.9 for 3 d, and gene expression levels, calcification and respiration rates were measured relative to pH_T 8.1 controls. Gross calcification decreased markedly under low pH conditions, while genes potentially involved in calcification were up-regulated, reflecting the inability of pteropods to maintain calcification rates. Gene expression data imply that under low pH conditions both metabolic processes and protein synthesis may be compromised, while genes involved in acid-base regulation were up-regulated. A large number of genes related to nervous system structure and function were also up-regulated in the low pH treatment, including a $GABA_A$ receptor subunit. This observation is particularly interesting because $GABA_A$ receptor disturbances, leading to altered behaviour, have been documented in several other marine animals after exposure to elevated CO_2. The up-regulation of many genes involved in nervous system function suggests that exposure to low pH could have major effects on pteropod behaviour. This study illustrates the power of combining physiological and molecular approaches. It also reveals the importance of behavioural analyses in studies aimed at understanding the impacts of low pH on marine animals.

INTRODUCTION

Shelled pteropods both play a critical role in the export of calcium carbonate from the sea surface into the deep ocean and contribute approximately 12 % of the global carbonate flux (Bednaršek *et al.*, 2012a). Pteropods deposit $CaCO_3$ as external aragonite shells, which makes them more vulnerable to ambient oceanic conditions that organisms with internal aragonite skeletons such as hard corals. As aragonite is the most soluble form of $CaCO_3$, calcification by pteropods is predicted to be sensitive to subtle changes in ambient pH. The key role of pteropods in the global carbon cycle is thus likely to be compromised by ocean acidification (Ries, 2012). Dissolution of shelled organisms is predicted to occur when the

aragonite saturation state (Ω_a) falls below 1. In the case of pteropods, however, shell dissolution has already been observed in the upper layers of the Southern Ocean where Ω_a levels were around 1 (Bednaršek *et al.*, 2012b) as well as in the North Pacific/California current (Bednaršek *et al.*, 2014; Bednaršek & Ohman, 2015).

Because pteropods are also an important food source for a variety of organisms, ranging from zooplankton to whales and including commercial fish species (e.g. salmon) (Foster & Montgomery, 1993), a decline in pteropod abundance has the potential to disrupt trophic networks and directly impact commercial fisheries. In light of their ecological and economic significance, it is crucial to understand how pteropods will be affected by global environmental change, particularly ocean acidification.

Previous studies have reported declines in calcification rates (Comeau *et al.*, 2012b; Comeau *et al.*, 2009; Comeau *et al.*, 2010b), linear extension rates (Comeau *et al.*, 2012a; Comeau *et al.*, 2010a; Comeau *et al.*, 2009; Lischka *et al.*, 2011; Lischka & Riebesell, 2012) and shell integrity (Comeau *et al.*, 2012b; Lischka *et al.*, 2011; Manno *et al.*, 2012; Orr *et al.*, 2005) with decreasing seawater pH. However, the possible effects of ocean acidification on physiological processes in pteropods has received much less attention (Gazeau *et al.*, 2013 for a review). One study showed that respiration rates of four subtropical and tropical pteropods species that naturally migrate into oxygen minimum zones were not affected by elevated carbon dioxide while the one species studied that do not migrate shown reduced oxygen consumption (Maas *et al.*, 2012). Results on polar species were more complex and influenced by other external parameters such as temperature (Comeau *et al.*, 2010b) or phytoplankton abundance (Seibel *et al.*, 2012). A recent study reported that low salinity in consort with lower pH negatively affects the swimming activity of the pteropod *Limacina retroversa* (Manno *et al.*, 2012). Such finding is consistent with a growing body of evidence

that elevated CO_2 and low pH causes behavioral disturbances in fish, molluscs and other marine organisms (Briffa *et al.*, 2012; Clements & Hunt, 2015; Watson *et al.*, 2014). Two recent transcriptomic studies have examined the responses of pteropods to elevated CO_2 (Koh *et al.*, 2015; Maas *et al.*, 2015). However sample sizes and high inter-individual variability severely limited the numbers of differentially expressed genes that could be detected (Maas *et al.*, 2015) or no attempts were made to link gene expression data with physiological parameters (Koh *et al.*, 2015).

In the present study, both physiological and molecular approaches were used to investigate the response of the Mediterranean pteropod, *Heliconoides inflatus,* to pH values projected to occur by 2100 under a moderate emissions trajectory (RCP6.0). *Heliconoides inflatus* (d'Orbigny, 1836) is a shelled species formerly known as *Limacina inflata*. It is one of the most common warm-water cosmopolitan pteropods and is widely distributed in the tropical and subtropical regions of all oceans. It is an epiplanktonic species living primarily in the upper 300 - 600 m of the water column depending on the season (Bé & Gilmer, 1977). Both adults and juveniles undertake diurnal vertical migration, moving to surface waters at night. In adult stages, nocturnal migration occurs year round but is more pronounced during periods when they are deeper living (Feb-Mar). Nocturnal migration of juveniles is seasonally dependent (Rampal, 1975).

Individuals were subjected to pH_T 8.1 (control pH, on the total scale) or pH_T 7.9 for 3 days. In addition to changes in gene expression caused by the treatment, rates of calcification and respiration were measured in the two experimental conditions. Changes in mRNA levels were assessed by RNA sequencing (Illumina technology), note that proteins levels were not measured in the present study. Experimental and control gene expression levels were compared by mapping the individual reads onto a *de novo* reference transcriptome assembly

(see *Supporting Information*). A global approach based on Gene Ontology enrichment analysis was first implemented in order to identify which molecular pathways were being repressed or activated in response to low pH. This step was followed by a targeted approach focused on genes involved in calcification and respiration to facilitate comparison between the physiological and molecular results.

MATERIAL AND METHODS

Collection of *Heliconoides inflatus*

Heliconoides inflatus individuals (Fig. 1) were collected in the Bay of Villefranche-sur-mer (43˚40' N, 7˚18' E) between 16th and 29th February 2012. Sampling was undertaken using very slow oblique tows of 2-4 min in duration at up to 100 m depth with a 57 cm diameter WP2 plankton net (200 µm mesh size). Plankton samples were immediately transported to the Laboratoire d'Océanographie de Villefranche. *Heliconoides inflatus* individuals were inspected under binocular microscope to assess their condition and reproductive status (as *H. inflatus* broods its young, care taken to ensure that brooding females were not included in the experiments). Using a wide aperture pipette, individuals were transferred one by one to filtered seawater medium (pore size 0.2 µm).

pH manipulation

Individuals were exposed for 3 d to pH_T 8.1 (control) or pH_T 7.9 (treatment). The desired pH conditions were maintained using a pH-stat system (IKS Karlsbard) that bubbled filtered seawater (pore size 0.2 µm) with either ambient air (control) or CO_2 (treatment) until the expected pH was reached; the pH electrode was calibrated on the total scale using Tris/HCl and 2-aminopyridine/HCl buffer solutions with a salinity of 38.0 (Dickson *et al.*, 2007). Three separate experiments were performed to assess (1) respiration rate, (2) ^{45}Ca uptake, and

(3) gene expression. In each experiment, 25 individuals of *H. inflatus* were transferred to 2 L sterilized borosilicate glass bottles, together with 1 ml of *Isochrysis galbana* culture to feed the animals. Three replicates were set up for each pH condition for (1) and (3), and four replicates for (2). Bottles were then closed and sealed with parafilm for 72 h of incubation, unless stated otherwise. Total alkalinity (A_T), pH_T, and dissolved inorganic carbon (C_T) were measured at the start and end of the incubations. A_T samples were filtered on GF/F and measured potentiometrically using a Tritando 80, Metrohm titrator and a Metrohm, electrode plus glass electrode. 60 ml samples was also taken at the start and finish of incubations and poisoned with $HgCl_2$ for determination of C_T. Samples were measured using an AIRICA (Marianda, Kiel) with Licor analyser. All other parameters of the carbonate chemistry were calculated using the R package *seacarb* (Lavigne & Gattuso, 2013; R Development Core Team, 2010), constants for K1 and K2 were taken from Lueker *et al.* (2000), Kf from Perez & Fraga (1987) and Ks from Dickson (1990) (Table 1).

Experiments on ^{45}Ca uptake were conducted at the Marine Environment Laboratory of the International Atomic Energy Agency (IAEA) in Monaco, while incubations for gene expression and respiration rates measurements were conducted at Laboratoire d'Océanographie de Villefranche.

Experiment 1: respiration rates

Six, 60 ml biological oxygen demand (BOD) bottles with oxygen sensitive microspots (PreSens, Planar, 5 mm in diameter) glued to the inner wall were prefilled with experimental seawater (prepared as described above), 3 control pH and 3 low pH. Animals were incubated for 48 h in seawater adjusted to the experimental treatment levels; after 48 h, 60 actively swimming animals were selected from each treatment and transferred to BOD bottles containing the same seawater (20 individuals in each bottle). Two additional BOD bottles

were prepared with oxygen sensitive microspots and filled with only the treatment seawater to act as blanks. All eight bottles were connected to an oxygen sensor (OXY-4 mini, PreSens), placed into a temperature-controlled bath at 14˚C and oxygen concentration was measured semi-continuously (every 15 s) over the following 24 h. At the end of the incubation, animals were removed from the BOD bottles, placed into a petri dish and fixed using 90% ethanol. Individuals were photographed under a binocular microscope, and the maximum shell diameter measured using the imaging software AxioVision (version 6.1.7601); for consistency with the calcification results, wet weights were calculated from the size weight relationship. The average shell diameter of animals used in the respiration experiments was 497.77 ± 12.93 µm and the average wet weight was 11.27 ± 1.09 µg, organisms ranged in wet weight from 0.63 to 67.65 µg (see Table S8).

Oxygen consumption rate was calculated by regressing oxygen concentration through time. The initial 2 h of incubation were excluded to avoid any stress effect caused by the transfer from 2 L glass bottles to the BOD bottles. Respiration rates were calculated for the entire pool of individuals in one replicate after correcting for oxygen consumption rates in the blank incubations and expressed as µmol O_2 h^{-1} (µg wet weight)$^{-1}$.

Experiment 2: ^{45}Ca uptake

Pteropods were photographed under a binocular microscope, and the maximum shell diameter measured using the imaging software AxioVision, version 6.1.7601a; they were then cultured (n= 25 per bottle) in conditions similar to the one described above and the 2 L culture bottles spiked with a $^{45}CaCl_2$ (Radioisotope Centre Polatum, Poland; $T_{1/2}$ = 163 d) solution for a final concentration of *ca.*130 Bq ml^{-1}. At the end of the 72-h incubation, all the individuals from each of the four replicates were filtered (200 µm mesh), pooled and placed

into a scintillation vial. The shells were dissolved with 0.5 N HCl and the soft tissues removed using a stereomicroscope and fine forceps. The soft tissue was rinsed into the vial with Milli-Q water to ensure that all the liquid containing the dissolved shell was collected. The solution was then neutralized with 1 N NaOH and allowed to evaporate on a heated plate before addition of 10 ml of scintillation fluid (Ultima GoldTM XR, Perkin Elmer). ^{45}Ca activity was counted using a Packard scintillation counter (Tri-Carb, Packarb 1600 TR or Perkin Elmer 2900 TR), and corrected for quenching by employing external standard (0.4 $Bq.ml^{-1}$) quench correction curves. Counting time was adjusted between 10 min and 24 h to obtain relative errors below 5 %. The incorporation of ^{45}Ca in pteropods shell was expressed according to the following equation (see Martin *et al.*, 2011):

$$Q_{Ca} = [(A_{shell} / A_{sw}) \times C_{sw}] \times 10^3$$

where Q_{Ca} is the amount of Ca incorporated per shell (nmol g^{-1}), A_{shell} is the total ^{45}Ca activity in each shell (in Bq), A_{sw} is the ^{45}Ca activity (in Bq g^{-1}) in seawater during the time of exposure, and C_{sw} is the total Ca concentration in Mediterranean seawater (0.0114 mmol g^{-1}). ^{45}Ca uptake was expressed as nmol.μg^{-1} wet weight. The pteropod weight was calculated from a previously determined size-weight relationship (see paragraph below).

Experiment 3: Gene expression

At the end of the 3-d incubations, the *H. inflatus* individuals from each of the three replicates were collected from the experimental jars by filtering the water over a 200 µm mesh. Specimens were rinsed with 0.45 µm filtered seawater, immediately snap-frozen in liquid nitrogen and stored at -80 °C until further treatment. Twenty-five individuals were pooled for each of the three replicates.

Size-weight relationship:

A size-weight relationship was used to estimate the individual weight for ^{45}Ca uptake and respiration. The relationship between *H. inflatus* shell size and overall weight was based on 118 individuals representative of the full size range of individuals collected in the Bay of Villefranche-sur-mer in February 2012. Individuals were photographed under a binocular microscope, and the maximum shell diameter measured using the imaging software AxioVision, version 6.1.7601. Individuals were dried with tissue paper prior to weighting with a Mettler Toledo microbalance (precision = 0.1 µg) and the following relationship was derived:

$$W = -a + b \times D^2$$

where W is the wet weight (including the shell), a and b are constants and D is the shell diameter (Fig. S1).

Statistical analyses

For both respiration rates and ^{45}Ca uptake, mean differences between the two treatments were tested using a paired *t*-test, due to a temporal difference between replicates. Respiration experiments were all performed at the same time so a standard *t*-test was used. Statistical analyses were performed using R.

RNA extraction and transcriptome sequencing

Total RNA was extracted using RNeasy Plus Micro Kit (Qiagen) according to the manufacturer instructions. RNA quantity and quality were assessed using a NanoDrop ND-1000 spectrophotometer and denaturing gel electrophoresis using standard methods (Sambrook & Russel, 2001). Before being shipped on dry-ice to the Macrogen sequencing facilities in Seoul, South Korea, each RNA sample was precipitated in ethanol and sodium

acetate (2x and 0.1x sample volume, respectively), and stored at -80 °C. Libraries were prepared with Illumina TruSeq Stranded RNA-seq kit by Macrogen (South Korea). The libraries were sequenced using the Illumina HiSeq2000 platform, producing an average of 63 million sequence reads (100 bp paired-end) per sample.

De novo assembly and data analysis

The reads from all the *H. inflatus* samples were trimmed from low quality regions and sequencing adaptors using libngs (https://github.com/sylvainforet/libngs) with a minimum quality of 20 and a minimum size of 75 bp. The trimmed reads were then assembled using Trinity (Grabherr *et al.*, 2011). The sequences of the resulting assembly were clustered using cdhit-est (Fu *et al.*, 2012) with a similarity threshold of 90% and a word size of 8 bp. The multi-modal GC profile of this initial assembly is suggestive of contamination (Fig. S2a), probably originating from the *Isochrysis galbana* culture provided to the animals. In order to remove these potential contaminants, the transcripts were blasted against a database containing the NCBI refseq proteins augmented with sequences from the molluscs *Biomphalaria glabrata* (https://www.vectorbase.org/organisms/biomphalaria-glabrata) and *Lymnea stagnalis* (Sadamoto *et al.*, 2012). Sequences were then classified as putative *H. inflatus* sequences if they satisfied the following criteria: e-value less than $1e^{-10}$, a minimum bit-score of at least 100, and a delta bit-score of at least 100. Here delta bit-score refers to the difference between the best mollusc hit and the best non-mollusc hit. After the above filtering, the distribution of GC content appeared unimodal (Fig. S2b). Due to the stringent filtering procedure, the sequences in this reduced set are very likely to be bona-fide pteropod sequences. Characteristics of the transcriptome assemblies before and after filtering are presented as Table S1. Protein-coding sequences were predicted using Transdecoder (http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3875132/) and annotated using Blast2GO

(Conesa *et al.*, 2005). The reads were mapped back to the assembled transcripts using Bowtie2 (Langmead & Salzberg, 2012). The number of fragments mapping to each transcript was computed using RSEM. Only the longest transcript for each assembled trinity locus was used. Differential gene expression was inferred using EdgeR (Robinson *et al.*, 2010) using a FDR threshold of 5%.

GO enrichment analysis was carried out with the Blast2GO software (Conesa *et al.*, 2005) using a Fisher's exact test with a FDR threshold of 5% to reduce false-positive predictions of enriched GO terms. For each gene category of interest, BlastP and HMMER domain searches (e-value cut-off = $1e^{-}5$) were performed on the sets of differentially expressed genes. An additional blast onto the NCBI *nr* database confirmed the identification of each sequence. In addition for the category "calcification", the set of differentially expressed genes was searched against a database for metazoan biomineralization proteins (https://peerj.com/preprints/1983/).

RESULTS

Respiration and calcification

There was no difference in respiration rate between individuals kept under control and low pH (Fig. 2b and Table S9; *t*-test, t = -0.05, df = 4, p-value = 0.97). However, individuals incubated in the low pH treatment exhibited a 37% decrease in gross calcification (^{45}Ca uptake) compared to control treatment (Fig. 2a; paired *t*-test, t=6.1, df=3, p-value=0.009).

Transcriptomic analyses

Transcript levels differed significantly between the two experimental conditions; relative to the control (pH_T 8.1), 1.8% (400 transcripts) and 0.8% (173 transcripts) of *H. inflatus* transcripts were up- and down-regulated, respectively, at the lower pH condition (adjusted *P*

<0.05). Relatively small changes were observed amongst genes that were up-regulated: in approximately 74% of cases, expression changed between 2 and 10-fold in low pH conditions, whereas changes of ≥100-fold were observed for fewer than 3% of the up-regulated transcripts. In contrast, a much larger proportion of the down-regulated genes showed higher fold changes, 47% of down-regulated transcripts showing more than 100 fold-changes (Fig. 3).

Ion transport, protein synthesis and mitochondrion activity

Gene Ontology (GO) analyses were conducted to infer the overall impacts of the experimental manipulation. Amongst the up-regulated genes, only a single GO category, *Ion transport* (GO:0006811), was significantly enriched (Table 2). This category comprises 26 genes including acid sensing ion channels, potassium, sodium, calcium and proton transporters, phospholipids and amino acid transporters, as well as neuronal transporters (see Table S2).

In contrast, many GO categories were enriched amongst the down-regulated genes (Table 2), a large proportion of these being related to protein synthesis and mitochondrial activity. For example, 75 clusters associated with protein synthesis were significantly down-regulated in the low pH condition (Table 3 and Table S3), with some transcripts levels being essentially undetectable (e.g.: a GTP-binding translation elongation factor, comp474736_c0_seq3). This category comprises 24 clusters encoding subunits of the 40S ribosomal protein, 26 encoding subunits of the 60S ribosomal protein as well as several other genes encoding proteins involved in regulation of transcription, protein translation initiation and elongation (Table 3 and Table S3), suggesting that the entire protein synthesis machinery was affected. Amongst mitochondrion-related genes, 22 components of the electron transport chain (including proteins from 3 of the 4 mitochondrial inner membrane complexes as well as several ATP synthase subunits, see Table 3 and Table S4) were affected, which suggests that oxidative

metabolism is suppressed in the low pH condition. The magnitude of changes in expression was extremely high in this category; with four cytochrome *c* oxidase subunits completely turned-off in the low pH treatment compared to control.

Acid-base regulation

Amongst the differentially expressed genes, 8 were potentially involved in acid-base regulation (Table 3 and Table S5). Two mRNA encoding for Na^+-K^+-ATPases, responsible for establishing and maintaining the electrochemical gradients of Na^+ and K^+ ions across the plasma membrane, were up-regulated in the low pH condition (average of 4.9-fold). Two mRNA encoding for vacuolar-type H^+-ATPases, generating proton gradients across membranes of numerous cell types, were also up-regulated more than 5-fold. One mRNA encoding for a carbonic anhydrase (comp456540_c0_seq1) was up-regulated 39 times in the low pH condition compared to control. Carbonic anhydrases are ubiquitous enzymes that catalyze the interconversion of HCO_3^- and CO_2 and are involved in a range of physiological processes that include pH homeostasis (see for review Pastorekova *et al.*, 2004; Supuran, 2008). Three transcripts encoding members of the SLC15A4 family (Solute carrier family 15 member 4) were also up-regulated. SLC15A4 are proton oligopeptide co-transporters that are required for lysosomal pH regulation and V-type H^+-ATPase integrity (Kobayashi *et al.*, 2014).

Calcification

The set of differentially expressed genes was searched against a database for database for metazoan biomineralization proteins (https://peerj.com/preprints/1983/) and 26 candidates were identified. All were up-regulated in response to elevated CO_2 (Table 3 and Table S6). They include 9 transcripts encoding a putative metalloproteinase, an alkaline phosphatase, two chitin synthases, a transcript encoding a collagen protein and four transcripts encoding cartilage matrix proteins. Two transcripts encoding mucin-like proteins were also highly up-

regulated (average of 17.5-fold). We found two perlucin-like transcripts together with a C-type lectin transcript to be up-regulated, the latter being 600-times up-regulated in the low pH condition compared to control. C-type lectins have been proposed to be involved in avian eggshell calcification (Mann & Siedler, 2004) and perlucin previously found to be involved in Mollusca biomineralization (Mann *et al.*, 2000). Interestingly, one transcript showing similarities with a dentin sialophosphoprotein was 9-fold up-regulated under elevated CO_2. Sialophosphoprotein has been shown to be involved in tooth and bone formation (Prasad *et al.*, 2010). Finally, the two transcripts encoding subunits of the vacuolar-type proton ATPase and the transcript encoding a carbonic anhydrase that were up-regulated in this experiment and mentioned in the "*Acid-base regulation*" section could also play a role in pteropod calcification.

Nervous system

Twenty-two percent of the up-regulated transcripts in the low pH condition (88 of the 400 up-regulated clusters) were genes putatively involved in the functioning of the nervous system. Those 88 transcripts include several neural cell-adhesion molecules, proteins involved in the maintenance and formation of the nervous system, various neuropeptides and neuropeptide receptors, key players in synaptic vesicle and recovery at the synapses, as well as ion channels potentially involved in synapse communication (Table 3 and Table S7).

This set of genes comprised several ligand-gated ion channels and their associated proteins from three types of synapses: GABAergic, cholinergic and glutamaergic. This includes the $GABA_A$ receptor ($GABA_AR$), which is of particular interest in relation to recent physiological studies showing that elevated CO_2 alters the behavior of fish (Heuer & Grosell, 2014; Nilsson *et al.*, 2012) and molluscs (Watson *et al.*, 2014). In the present study, a transcript encoding a subunit of the $GABA_AR$ was up-regulated 16-fold in low pH compared to control conditions. A transcript encoding a glycine receptor subunit was also up-regulated

(GlyR, 7.7-fold up-regulated), an inhibitory ligand-gated ion channel that is known to co-localize with $GABA_AR$ on some hippocampal neurons (Table 3 and Table S7) (Lévi *et al.*, 2004). A surprising number of transcripts involved in cholinergic synapses were also up-regulated. Fourteen transcripts encoding subunits of acetylcholine receptors (nicotinic and muscarinic), together with one voltage-gated potassium channel and three transcripts encoding acetylcholinesterase were up-regulated (Table 3 and Table S7). Acetylcholine receptors occur both on neurons (in ganglia and brain) and on muscles. The diffusion of Na^+ and K^+ across the receptor causes depolarization that opens voltage-gated sodium/potassium channels and allows firing of the action potential and potentially muscular contraction. Acetylcholinesterases hydrolyze the neurotransmitter acetylcholine and are, therefore, essential for the termination of synaptic transmission in cholinergic synapses.

Finally, the mRNA of a glutamate receptor responsible for the glutamate-mediated postsynaptic excitation of neural cells, and the mRNA of a glutamate transporter, were also up-regulated at lower pH (4.8 and 4.7 times respectively).

Only two transcripts with potential roles in the nervous system were down-regulated in response to elevated CO_2: a transcript encoding a voltage-dependent calcium channel subunit and a transcript encoding a putative tenascin-R-like protein (see Table 3 and Table S7). It should be noted that 10 transcript encoding putative tenascin-R-like protein were differentially regulated; one being down-regulated 42-fold and the other ones being up-regulated on average 44-fold under low pH. Tenascin-R proteins (TN-R) are extracellular matrix proteins exclusive to the central nervous system in vertebrates (Anlar & Gunel-Ozcan, 2012). They have versatile roles and can act as adhesive or anti-adhesive molecules towards various neural and non-neural cells but also inhibitors or enhancers of neurite outgrowth (Pesheva & Probstmeier, 2000).

Lastly, the mRNA of an amiloride-sensitive cation channel 4 (ASIC 4 or ACCN4) was up-regulated about 7-fold under low pH. These channels have been implicated in synaptic transmission, pain perception as well as mechano-reception in mammals, and were found in zebrafish neurons (Chen *et al.*, 2007).

DISCUSSION

Physiological and molecular tools were used to investigate the response of the Mediterranean pteropod *Heliconoides inflatus* to seawater pH likely to be reached by 2100 under a moderate emissions trajectory (RCP6.0). *Heliconoides inflatus* individuals were subjected to pH_T 8.1 (control) or pH_T 7.9 for 3 d and changes in gene expression, calcification and respiration were measured. Gross calcification strongly decreased in low pH conditions while genes potentially involved in calcification were up-regulated, suggesting that pteropods attempt to maintain calcification rates when faced with unfavorable conditions. Interestingly, a large number of genes related to nervous system structure and function were also up-regulated in the low pH treatment, including a $GABA_A$ receptor subunit. This is particularly interesting given that $GABA_A$ receptor disturbances have been documented in several marine organisms after exposures to elevated CO_2 (Heuer & Grosell, 2014; Nilsson *et al.*, 2012; Watson *et al.*, 2014).

It is important to recognize that our experiment was an acute exposure of pteropods to low pH conditions for only three days, and physiological and molecular effects could be different under longer exposure, as previously shown in corals (Moya *et al.*, 2015). While our study may seem short in comparison to similar studies on other organisms, the difficulties in maintaining these planktonic molluscs under laboratory conditions (Howes *et al.*, 2014) places our study amongst the longest experiments for non-polar pteropods species.

Suppression of metabolism and protein synthesis

During the course of the present experiment, oxygen consumption did not differ between pH_T 7.9 and pH_T 8.1. Previous studies also found no effect of lower pH on the respiration of *Limacina helicina* (Comeau *et al.*, 2010b), *Clio pyramidata, Hyalocylis striata, Cavolinia longirostris, Creseis virgula* (Maas *et al.*, 2015; Maas *et al.*, 2012) and *Creseis acicula* (Comeau *et al.*, 2012b) while it decreased in *L. helicina forma antarctica* (Seibel *et al.*, 2012) and *Diacria quadridentata* (Maas *et al.*, 2012), suggesting that the natural seawater chemistry may influence their resilience to ocean acidification. However in the present study, exposure of *H. inflatus* to pH_T 7.9 for 3 d led to a decreased expression of metabolism-related genes, indicating that metabolism was suppressed under acidified conditions. The apparent contradiction between the transcriptomic and physiological measurements of the present study may be due to the long turnover times typical of respiratory complex proteins. The latter is not unexpected as all four respiratory complexes contain subunits encoded in the mitochondrial genome. Half-lives of human mitochondrial proteins range from 6 to 16 days (Eden *et al.*, 2011). Studies on the effects of elevated CO_2 on pteropods (Maas *et al.*, 2015), sea urchins (O'Donnell *et al.*, 2010; Todgham & Hofmann, 2009) and coral larvae (Moya *et al.*, 2012) have congruently documented the suppression of metabolic gene expression under elevated CO_2 as observed in the present study. It is widely accepted that metabolic depression is an adaptive strategy for survival in short-term energy limitation in aquatic organisms (Seibel & Walsh, 2003) and is accomplished, at least in part, by shutting down processes such as protein synthesis (Guppy & Withers, 1999), and in particular mitochondrial protein synthesis (Kwast & Hand, 1996). In our experiment, the entire protein synthesis machinery, including transcripts of initiation and elongation factors as well as transcripts of ribosomal proteins, was subject to down-regulation at low pH. Depression of metabolic gene expression

upon exposure to acute stress potentially allows the reallocation of transcriptional resources (and energy) to more immediate demands such as pH homeostasis.

Acid-base regulation

pH homeostasis is crucial for a large range of systemic and cellular functions, including calcification and neural function, but the pathways involved, as well as the efficiency of compensation mechanisms of acid-base imbalance differ between taxa and are often species-specific (Clements & Hunt, 2015; Melzner *et al.*, 2009). Although acid-base regulation is often achieved in molluscs by controlling levels of bicarbonate ions (Pörtner, 2008), in the present case, the expression of bicarbonate transporter genes did not differ between low pH and control treatments. In contrast, eight genes involved in acid-base regulation were found to be up-regulated, including carbonic anhydrases and V-type H^+-ATPases. This observation suggests that pH homeostasis in *H. inflatus* could be achieved by means other than active bicarbonate transport. For example, carbonic anhydrases interconvert CO_2 into HCO_3^- while the protons that this generates are removed by V-type H^+-ATPases, contributing to the accumulation of bicarbonate ions and pH homeostasis at lower pH. Consistent with the strong decrease in calcification rates observed in this study at low pH, prior research on bivalves suggests that bicarbonate buffering can be partly achieved by the dissolution of the $CaCO_3$ exoskeletons (Lindinger *et al.*, 1984; Michaelidis *et al.*, 2005). However, this strategy is of limited usefulness in the case of pteropods, considering the thin shell of these planktonic molluscs.

Irrespective of the nature of the strategy used by pteropods to achieve acid-base balance, the extent to which compensation is achieved is unknown, and this has important implications for other physiological processes. In fish, for example, the compensatory response during acid-base regulation is claimed to be responsible for the observed disturbances in neural function

and behavior (Heuer & Grosell, 2014). An additional consideration is that several proteins involved in acid-base regulation are also involved in other physiological processes, such as calcification and neural functioning (e.g. carbonic anhydrases, V-type H^+-ATPases). At this stage, however, it is unclear in which processes the differentially expressed isoforms identified in this study are involved. Understanding the mechanisms underlying basic processes such as acid-base regulation or calcification is a prerequisite for predicting the effects of future ocean conditions on pteropod populations.

Calcification decreases despite increased expression of calcification genes

The major (37%) decrease in gross calcification observed after exposure of *H. inflatus* to pH_T 7.9 is consistent with data for the Mediterranean pteropod species *Creseis acicula* (Comeau *et al.*, 2012b reported a 30% decrease). The decline in calcification could be the result of an active reduction in this energetically expensive process at low pH in order to permit reallocation of energy to other processes. However, this hypothesis is difficult to reconcile with the observation that a number of genes involved in calcification were up-regulated at lower pH. Although the detail of where these genes function in the calcification process is unknown, these data imply that the observed decrease in gross calcification reflects the inability of the pteropod to sustain calcification rates under acidic conditions, rather than active down-regulation of the calcification process.

Amongst the calcification-related genes up-regulated in our study are two perlucin transcripts and a C-type lectin which were also differentially expressed in similar experiments on the pteropods *Clio pyramidata* (Maas *et al.*, 2015) and *Limacina helicina* (Koh *et al.*, 2015). While comparisons between these studies are complicated by major differences in experimental design, it is noteworthy that following a 10 h exposure of *C. pyramidata* to a pH level similar to the one used in the present experiment (pH_T 7.8), a perlucin homolog was

one of the few differentially expressed genes, with a 154-fold increase in expression (Maas *et al.*, 2015). In contrast, Koh *et al.* (2015) documented an opposite trend for a C-type lectin in *L. helicina* under more extreme (pH 7.5 and 6.5) conditions than those used here. One factor complicating comparison of data reported here with published analyses is that many of the genes involved in calcification are members of large multi-gene families, hence the assignment of orthology is difficult. Large numbers of perlucin and C-type lectins are known to be present in *C. pyramidata* (Maas *et al.*, 2015), and in the present study, approximately 300 contigs containing C-type lectin domains (Pfam PF00059) were retrieved from the *H. inflatus* transcriptome (data not shown). C-type lectin domain proteins have a diverse range of roles, including cell-cell adhesion, immune response and apoptosis (Drickamer, 1999). It is therefore likely that different isoforms have different roles in the response of pteropods to elevated CO_2.

The contrast between physiological and molecular results suggests that pteropods unsuccessfully attempt to maintain calcification rates under unfavorable conditions. If shelled pteropods could calcify faster than their shell dissolves, as has been observed for some aragonite-based corals (Rodolfo-Metalpa *et al.*, 2011), then they could survive under near future ocean conditions (Ries, 2012). However, when considered in conjunction with the extensive shell dissolution that has previously been documented for Ω_a levels ~ 1 (Bednaršek *et al.*, 2012b), the data presented here implies a poor prognosis for shelled pteropod populations.

Low pH affects the nervous system

In the present study, 20% of the genes up-regulated under low pH are likely to function in the nervous system. This is particularly interesting in light of recent studies showing that ocean acidification influences the behavior of both vertebrates and invertebrates in multiple ways

(see Table 1 and 2 of Clements & Hunt, 2015 for a review). Some of the previous studies implicate the GABA-A receptor ($GABA_AR$, up-regulated 16-fold in the present study) in behavioral changes by demonstrating that gabazine, a specific $GABA_AR$ antagonist, almost completely restores the behavioral performance of fishes (Chivers *et al.*, 2014; Hamilton *et al.*, 2014; Lai *et al.*, 2015; Nilsson *et al.*, 2012) and gastropod molluscs (e.g.Watson *et al.*, 2014). The $GABA_A$ receptor is a ligand-gated ion channel activated by GABA, the most common inhibitory neurotransmitter in vertebrates and some invertebrates (Jessen et al 1979). $GABA_A$ has specific conductance for Cl^- and HCO_3^-, two ions likely to be impacted by ocean acidification (Nilsson *et al.*, 2012). Under normal conditions, when $GABA_A$ opens, letting Cl^- and HCO_3^- ions flow into the cell, depolarization of the membrane is prevented, reducing neural activity. $GABA_AR$ are pH sensitive proteins; minor changes in external pH are sufficient to affect their function, affecting neuronal excitability (Wilkins *et al.*, 2005). When extracellular pH decreases, most animals (depending on their acid-base regulation strategy) excrete Cl^- and accumulate HCO_3^- in order to prevent cellular acidosis (Heuer & Grosell, 2014), which renders $GABA_AR$ signaling excitatory (Lambert & Grover, 1995), thereby affecting behavior and causing dramatic shifts in sensory preferences (Nilsson *et al.*, 2012). Whether this hypothesis applies to pteropods remains to be determined, and this will depend on elucidating the acid-base balance strategies of the taxon. If pteropods accumulate HCO_3^- (through carbonic anhydrase rather than bicarbonate transporters) in order to avoid cellular acidosis, a mechanism similar to that of fishes is likely to be present. If the acid-base balance is not bicarbonate dependent, $GABA_AR$ should not be specifically affected. However, irrespective of the acid-base strategy used, if compensation is incomplete then more pH sensitive (neural and other) proteins will be affected at low pH. In the present study, the up-regulation of many other genes involved in neuron function, including several other voltage-dependent neural proteins belonging to glutamatergic and cholinergic synapses,

supports the latter hypothesis and suggests that the effect of low pH might be less specific than proposed in fishes, and could potentially affect other cellular functions. Alternatively, if $GABA_A$ receptor function is altered, that could have wide ranging effects leading to responses in other transmitter systems, which in turn could explain the widespread changes seen in neural gene expression.

A limitation of our study is the lack of behavioral observations that could provide context for the interpretation of the observed changes in neural gene expression. To our knowledge, only one study has investigated the effects of low pH (in combination with altered salinity) on the behavior of pteropods (Manno *et al.*, 2012). It found that the combined stressors negatively affected upward swimming. In part, the lack of behavioral studies on shelled pteropods is due to the difficulty of maintaining them under laboratory conditions (Howes *et al.*, 2014). Clearly, more work is needed to uncover the role of $GABA_A$ receptors in the behavior of invertebrates under elevated CO_2, as well as a better understanding of the link between acid-base regulation and neural function in molluscs.

Planktonic molluscs such as pteropods are likely to normally experience large changes in the carbonate chemistry due to diel vertical migrations in the water column (Maas *et al.*, 2012). This life history strategy suggests that pteropods should have molecular mechanisms to cope with sudden changes in pH. This diel migration also involves substantial changes in temperature, a parameter that may act synergistically with CO_2, and potentially amplify or attenuate the pteropod response to high CO_2 alone (Clements & Hunt, 2015). Scrutinizing the combined effect of CO_2 and temperature on *H. inflatus*, as done by Comeau *et al.* on the arctic species *L. helicina*, will be the next important step towards better understanding the impacts of global changes in climate and ocean chemistry on these key planktonic organisms.

In the present study, strong decrease in calcification was observed at Ω_a levels around 2. Comeau *et al.* (2010b) have shown that the Arctic pteropod *Limacina helicina* is unable to

precipitate calcium carbonate at aragonite saturation state close to 1 and that gross calcification declines well above this value according to a logarithmic relationship. The projected increase in temperature does not ameliorate the situation. Both net and gross calcification also decline well before an aragonite saturation state of 1 in the Mediterranean pteropod *Creseis acicula* (Comeau *et al.*, 2012b). At ecologically relevant changes in saturation state in the Mediterranean or tropical/sub-tropical open ocean, saturation states are not expected to go below 1. If these organisms have significant changes in calcification at the reported levels, the scientific community needs to pay particular attention to non-polar pteropods in these regions.

This study advances our understanding of the responses of pteropods to ocean acidification and illustrates the utility of combining physiological and molecular approaches. When considered in the context of previous work on fish (Chivers *et al.*, 2014; Hamilton *et al.*, 2014; Lai *et al.*, 2015; Nilsson *et al.*, 2012) and other molluscs (Watson *et al.*, 2014), the major impacts of ocean acidification on expression of genes likely to function in the pteropod nervous system lead us to recommend that future studies of the impacts of ocean acidification on animal biology should include behavioral observations together with physiological and molecular measurements.

Comprehensive studies of this kind will clearly be needed in order to accurately predict the impacts of ocean acidification on individual species and the food webs in which they are involved.

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Figure Legends

Figure 1. Individual of *Heliconoides inflatus.*

Figure 2. (a) Calcium uptake and **(b)** respiration rates measured at control (pH_T 8.1) and low (pH_T 7.9) pH levels. Asterisk indicates significant difference between control and low pH (t = 6.0, df = 3, p-value = 0.01).

Figure 3. Distribution of the log2 (fold-change) of the differentially expressed genes in response to the low pH treatment (adjusted P <0.05).

Supporting information captions

Figure S1. Shell diameter-weight relationship of *Heliconoides inflatus* individuals used in the present study. Individuals were photographed under a binocular microscope, and the maximum shell diameter measured using the imaging software AxioVision, version 6.1.7601. Individuals were dried with tissue paper prior to weighting with a Mettler Toledo microbalance (precision = 0.1 µg).

Figure S2. Distribution of GC content **(a)** before and **(b)** after filtering the contaminating data.

Table S1. Characteristics of the transcriptome assemblies before and after filtering the contaminating data.

Table S2. Genes responsive to the low pH treatment belonging to the GO category *Ion transport* (GO:0006811).

Table S3. Protein synthesis genes responsive to low pH treatment.

Table S4. Mitochondrion genes responsive to low pH treatment.

Table S5. Genes potentially involved in acid-base regulation that were affected by the low pH treatment.

Table S6. Genes potentially involved in pteropod calcification that were affected by the low pH treatment.

Table S7. Genes potentially involved in pteropod nervous system that were affected by the low pH treatment.

Table S8. Shell diameters (µm) and weights (µg) of all individuals used in the respiration experiments. Number in brackets next to pH correspond to the replicate.

Table S9. Results of the respiration experiments showing the µmol O_2 consumed per hour per µg of wet weight. Number in brackets next to pH correspond to the replicate.

Data accessibility: The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and are accessible through GEO Series accession number GSE77934 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE77934).

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Table 1. Carbonate chemistry parameters in the control and low pH treatments. pH_T and A_T were measured while all other parameters were estimated using the R package *seacarb*. C_T, dissolved inorganic carbon; A_T, total alkalinity, Ω_a, saturation state of aragonite. Three separate experiments were performed to assess (1) respiration rate, (2) ^{45}Ca uptake, and (3) gene expression. Experiments (1) and (3) were performed at Laboratoire d'Océanographie de Villefranche and experiment (2) was performed at IAEA.

Treatment	**pH_T**	**pCO_2 (µatm)**	**HCO_3^- (µmol kg^{-1})**	**CO_3^{2-} (µmol kg^{-1})**	**C_T (µmol kg^{-1})**	**A_T (µmol kg^{-1})**	**Ω_a**
Control pH (1) and (3)	8.1	410	2091	188	2294 ± 10	2552 ± 4	2.8
Low pH (2)	7. 9	617	2208	139	2370 ± 11	2550 ± 9	2.1
Control pH (1) and (3)	8.1	382	2059	196	2269 ± 17	2540 ± 5	2.9
Low pH (2)	7.9	720	2243	123	2393 ± 21	2561 ± 21	1.9

Table 2. Gene Ontology enrichment table. GO enrichment analysis was carried out with the Blast2GO software using a Fisher's exact test with a False Discovery Rate (FDR) threshold of 5% to reduce false-positive predictions of enriched GO terms. Down, categories enriched in the set of down-regulated genes in the low pH treatment; Up, category enriched in the set of up-regulated genes in the low pH treatment; MF, Molecular Function; CC, Cellular Component; BP Biological Process. The last column indicates the number of differentially expressed genes in each category.

Up- or down-regulated	Category	GO-ID	Term	FDR	# genes
DOWN	MF	GO:0003735	Structural constituent of ribosome	8E-34	46
		GO:0019843	rRNA binding	2E-06	8
		GO:0003729	mRNA binding	9E-05	9
		GO:0016491	Oxidoreductase activity	4E-02	21
		GO:0017111	Nucleoside-triphosphatase activity	4E-02	20
	CC	GO:0005739	Mitochondrion	5E-08	30
		GO:0015935	Small ribosomal subunit	9E-07	7
		GO:0005576	Extracellular region	2E-03	29
		GO:0005875	Microtubule associated complex	3E-03	4
		GO:0005730	Nucleolus	4E-03	13
		GO:0022625	Cytosolic large ribosomal subunit	4E-02	3
	BP	GO:0006091	Generation of precursor metabolites and energy	3E-07	16
		GO:0034655	Nucleobase-containing compound catabolic process	4E-07	16
		GO:0044403	Symbiosis encompassing mutualism through parasitism	9E-07	16
		GO:0044765	Single-organism transport	4E-05	35
		GO:0006605	Protein targeting	1E-04	13
		GO:0042254	Ribosome biogenesis	6E-04	10
		GO:0061024	Membrane organization	2E-03	14
		GO:0008584	Male gonad development	2E-02	3
UP	BP	GO:0006811	Ion transport	2E-02	26

Table 3. Summary of the genes responsive to low pH treatment. See supporting information for more detail about specific processes. Down, down-regulated genes in the low pH treatment; Up, up-regulated genes in the low pH treatment.

Process	Category/gene family	Number of genes	Expression
Protein synthesis	40S ribosomal proteins	24	down
	60S ribosomal proteins	26	down
	Other clusters involved in protein synthesis	19	down
Mitochondrion	Complex I	4	down
	Complex II	0	down
	Complex III	3	down
	Complex IV	10	down
	Complex V	6	down
Acid-base regulation	V-type H^+-ATPase	2	up
	Na^+-K^+-ATPase	2	up
	Carbonic anhydrase	1	up
	Solute carrier family 15 member 4	3	up
Calcification	Metalloproteinases	9	up
	Alkaline phosphatase	1	up
	Chitin synthase	2	up
	Cartilage matrix proteins	4	up
	Mucins	2	up
	C-type lectins	3	up
	Dentin sialophosphoprotein	1	up
	Collagen	1	up
	V-type H^+-ATPase	2	up
	Carbonic anhydrase	1	up
Nervous system	GABAergic synapses	2	up
	Cholinergic synapses	18	up
	Glutamaergic synapses	2	up
	Tenascin-R like	10	9 up, 1 down
	Acid-sensing ion channel	1	up
	Neuronal cell adhesion	15	up
	Neuropeptide receptors	3	up
	Neuronal differentiation and survival	6	up
	Postsynaptic scaffolding protein	4	up
	Vesicular and membrane trafficking	9	up
	Synaptic plasticity	1	up
	Receptor	1	up
	Neuro-transmiter transporters	4	up
	Calcium homeostasis	8	up
	Transporter	1	up
	Electrical coupling	1	up
	Unknown role	2	up