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Comparative proteomics and codon substitution analysis reveal mechanisms of differential resistance to hypoxia in congeneric snails

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ABSTRACT

Although high-throughput proteomics has been widely applied to study mechanisms of environmental adaptation, the conclusions from studies that are based on one species can be confounded by phylogeny. We compare the freshwater snail *Pomacea canaliculata* (a notorious invasive species) and its congener *Pomacea diffusa* (a non-invasive species) to understand the molecular mechanisms of their differential resistance to hypoxia. A 72-h acute exposure experiment showed that *P. canaliculata* is more tolerant to hypoxia than *P. diffusa*. The two species were then exposed to three levels of dissolved oxygen (6.7, 2.0 and 1.0 mg L⁻¹) for 8 h, and their gill proteins were analyzed using iTRAQ-coupled LC-MS/MS. The two species showed striking differences in protein expression profiles, with the more hypoxia tolerant *P. canaliculata* having more up-regulated proteins in signal transduction and down-regulated proteins in glycolysis and the tricarboxylic acid cycle. Evolutionary analysis revealed five orthologous genes encoding differentially expressed proteins having clear signal of positive selection, indicating selection has acted on some of the hypoxia responsive genes. Our case study has highlighted the potential of integrated proteomics and comparative evolutionary analysis for understanding the genetic basis of adaptation to global environmental change in non-model species.

1. Introduction

Rapid globalization in recent decades has greatly facilitated species introduction around the world [1,2]. Successfully established introduced species, so-called invasive species, have threatened the invaded ecosystems by reducing biodiversity, altering community structure and compromising ecosystem services [3,4]. While it is difficult to predict whether a species will become invasive following its introduction, several niche- and trait-based characteristics are known to be associated with establishment success [5,6]. Climate suitability, resource availability, presence of potential competitors, community diversity and frequency and scale of disturbances are some of the niche-related characteristics [7-9]; whereas growth rate, life span, fecundity, dispersal ability, dietary spectrum, and tolerance of environmental stressors are some of the trait-based characteristics [10]. Indeed, a review by Kolar & Lodge (2001) [11] showed that 86% of the invasive species could be predicted by a subset of life-history traits including fast growth, high fecundity, high dispersal ability, wide food spectrum, phenotypic plasticity and tolerance of extreme abiotic conditions.

There has been substantial interest in studying how invasive species respond to extreme environmental conditions because the results can help not only predict their range of expansion and manage their impact, but also may reveal the adaptive mechanisms underlying their invasiveness [2,9]. Comparative studies using closely related strains or species, when combined with transcriptomic and proteomic techniques, have provided scientists with unprecedented opportunities to study the molecular mechanisms regulating animal responses to environmental stressors including hypoxia [12]. Such studies have advantages over single-species studies in that they can distinguish effects of adaptive variation from those of phylogeny [13-16]. High-throughput transcriptomic and proteomic methods are especially suitable for such studies because it is expected that environmental stressors will alter the expression of many genes and proteins involved in various critical biological processes such as stabilization of protein structure, repairing damaged DNA, and regulating protein turnover, energy production and redox homeostasis [17-19]. In order to determine whether congeneric apple snails differ in their tolerance to hypoxia, and whether such tolerance is accompanied by physiological and proteomic responses, we compared the two species of apple snails, the invasive *Pomacea canaliculata* and its non-invasive congener species *Pomacea diffusa* (Fig. S1, Supporting Information). These apple snails belong to Ampullariidae, a family of Gastropoda which are considered as emerging models in evolutionary studies due to their biogeography, speciation and physiological adaptation [20]. *Pomacea canaliculata*, a native of freshwater wetlands of South America, has invaded East and Southeast Asia, the southern United States, and Hawaii in the 1980s [21]. In its invaded regions, this species has become a pest, causing tremendous loss to rice and taro farming [22], and affecting wetland biodiversity and function by herbivory [23,24]. Tolerance of environmental stressors, especially desiccation, heat, cold, and hypoxia, has been considered as an important trait determining the success of invasion in *P. canaliculata* [25-29]. In southern China where the climate is divided into a warm

and wet summer and a cold and dry winter [30], this species is often found in shallow-water bodies that experience high temperature in summer and hypoxia in winter. Our field surveys found that *P. canaliculata* is often present in stagnant shallow water bodies, especially ponds and abandoned farmlands with dissolved oxygen $< 1 \text{ mg L}^{-1}$ [30]. It is apparently even more tolerant (Fig. S2, Supporting Information) to hypoxia than the Nile tilapia *Oreochromis niloticus*, a hypoxia tolerant fish that has invaded southern China as well. In contrast, *Pomacea diffusa*, another species of native snail in the freshwater wetlands of South America, is not considered an invasive species. This species is a common ornamental and tank cleaning species in the aquarium trade around the world, and the only species in the family Ampullariidae allowed to be transported across states without a permit issued by the United States Department of Agriculture [31]. There have been scattered reports of *P. diffusa* in Asia [5], New Zealand [32], and southern United States [31], but this species has not been found to build up large populations or become an agricultural pest. Although the two species of *Pomacea* are both of South American origin, the native range of *P. canaliculata* is a small area of southern South America from northeastern Argentina to southern Uruguay [21], whereas that of *P. diffusa* is central Bolivia to the western Amazon basin of Brazil (Kenneth A. Hayes, personal communication). Therefore, *P. canaliculata* evolved in an area with greater climate extremes than *P. diffusa* and may have higher resistance to hypoxia.

In the present study, we conducted two experiments. The first experiment was designed to compare the resistance of the two *Pomacea* species to hypoxia. The second experiment was designed to identify proteins that are responsive to hypoxia stress in both species and those that are differentially expressed between the two species. Since adaptive evolution might have contributed to the differential protein expression [33], we also analyzed the base substitution pattern of genes encoding the differentially expressed proteins. Because the statistical power of such base substitution analysis is weak when only two species are compared [34], we adopted a phylogenetic comparative approach [35] to determine genes that have contributed to the adaptive divergence in resistance to hypoxia in apple snails by including our newly sequenced transcriptome data from *Pomacea maculata* [36] in the analysis. Our hypothesis was that there could be sequence divergence between the two congeneric species and the nucleotide/amino acid mutations that could affect protein functions, leading to differential gene expression. A similar approach of base substitution analysis has been applied to understand the involvement of positive selection in protein sequence divergence among closely related species [37]. Previous studies have also provided evidence of adaptive evolution underlying gene expression in response to stresses at the transcriptome level [38,39]. Although other genetic mechanisms such as gene duplication, *cis/trans*-regulation and epigenetic modifications can also be involved in hypoxia resistance, our study, with limited transcriptomic data for the non-model organisms, can provide a snap shot of the involvement of positive selection in differential protein expression. The differential expressed proteins which have also undergone positive selection can serve as

candidates for further studies, for instance gene silence or mutation to verify the function.

2. Material and methods

2.1 Snail maintenance and hypoxia exposure system

A stock culture of *P. canaliculata* was established using adults collected from a vegetable farming area in Yuen Long (22°15'N, 114°10'E), Hong Kong and a stock culture of *P. diffusa* was established using adults purchased from an aquarium shop in Hong Kong. The stock cultures were established several years ago [27,29,36], and they had been maintained in identical conditions in the same air-conditioned room for more than six generations before the study. The two species were separately reared in 250-L aquaria with a submerged heater to maintain the water temperature at 25 ± 1 °C. The aquaria were each supplied with a submerged pump and canister filter to circulate water, and remove food waste and snail feces. Oxygen in the water was supplemented by continuous aeration with an air pump. Snails were fed with carrot, lettuce and fish feed and leftovers were removed daily using a hand net. The culture room was illuminated by fluorescent light with a photoperiod of 14 h light: 10 h dark to facilitate the development of biofilm. Snails were transferred from stock cultures to experimental aquaria for acclimation for at least one month before use.

During the experiments, both species of snails were kept in aquaria filled with 3.5 L water. The normoxia control was established by pumping compressed air into the water continuously to maintain the ambient dissolved oxygen (DO) level of 6.7 mg L⁻¹ (range 6.6 to 6.8 mg L⁻¹). The normoxia condition represented the condition the snails in our stock culture experienced. Two hypoxic conditions were chosen to represent different levels of hypoxia. As indicated by previous studies, 2.0 mg O₂ L⁻¹ represented a mild hypoxic exposure at which many species would survive for at least several days; while 1.0 mg O₂ L⁻¹ represented an extreme hypoxia condition at which few species would survive for more than several days [40,41]. The hypoxia treatments were created by mixing compressed nitrogen gas and air before introducing the mixed gas into the exposure water [42]. The discharge rates of the two gases were adjusted to reach the following DO levels: 2.0 mg L⁻¹ (range 1.8 to 2.2 mg L⁻¹) and 1.0 mg L⁻¹ (range 0.8 to 1.2 mg L⁻¹). The DO levels in the experimental chambers were monitored continuously using a Stable Optical Oxygen System (TauTheta Instruments LLC, Colorado, USA) coupled with an optical sensor inserted into the exposure water. Since apple snails can ventilate their lung by crawling on aquarium wall near water surface and extending their respiratory siphon into air [43], a plastic grid (pore size 1.5 cm) was fixed horizontally 10 cm below the water surface, a distance longer than their respiratory siphon, to make sure the snails were truly exposed to the different oxygen treatments. The experiments were conducted at 25 ± 1 °C.

2.2 Experiment 1. Differential mortality under hypoxia

For each species, 36 snails (2.3 to 2.5 cm shell length) were starved for one day before use to avoid the confounding effect of feces decomposition on oxygen content. Snails were introduced into experimental aquaria and exposed to the different normoxia and hypoxia treatments for 72 hours, during which snail survival was checked every few hours. Dead snails, defined as those that did not move and had no response to physical stimuli, were removed to avoid fouling due to tissue decomposition. We confirmed the death of these snails by transferring them to normoxia condition and none of them recovered. The cumulative mortality in each treatment was plotted against time to show the treatment- and time-dependent mortality pattern [42].

2.3 Experiment 2. Responses of the proteome to acute hypoxia exposure

2.3.1 Exposure, protein extraction and labeling

Snails of *P. canaliculata* (shell length 25.9 to 32.8 mm, $n = 9$, three biological replicates) and *P. diffusa* (shell length 28.6–37.6 mm, $n = 9$, three biological replicates) were exposed to the normoxia (6.7 mg O₂ L⁻¹) or hypoxia (2.0 and 1.0 mg O₂ L⁻¹) treatments for 8 hours, after which they were dissected, and their gills were placed in 8 M urea and stored in -80°C until use. We did not use a longer exposure time to avoid sampling snails that might enter a moribund state before they mobilized the hypoxia responsive mechanisms, especially for the more sensitive species *P. diffusa* exposed to 1.0 mg O₂ L⁻¹. The gills were used in proteomic analysis because they are the organs used by apple snails for respiration when they are under water, and therefore should be responsive to hypoxia conditions. We did not sample the lung because it is supposed to be used in aerial respiration by apple snails when they crawl out of water. During protein extraction, gill samples were placed on ice and homogenized using a plastic pestle, sonicated using a Branson Digital Sonifier to break cell membranes, and centrifuged at 15,000 g for 15 min under 4°C. Supernatant containing proteins were collected and purified with a 2D-cleanup kit (Bio-Rad, Hercules, California, USA). The purified protein pellets were reconstituted with 8 M urea and quantified with a RC-DC kit (Bio-Rad). Afterwards, an aliquot containing 200 µg protein was used for Isobaric Tags for Relative and Absolute Quantitation (iTRAQ) labelling. Proteins were reduced using 5 mM triscarboxyethyl phosphine hydrochloride at 60°C for 1 h and then alkylated using 10 mM methylethanethiosulfonate at room temperature for 20 min. Protein solutions were diluted 8-fold using 50 mM triethylammonium bicarbonate, then digested with sequencing-grade trypsin (Promega, Madison, WI) with an enzyme-to-protein ratio of 1:50 (w/w) for 16 h at 37°C. After digestion, samples were desalted with Sep-Pak C18 cartridges (Waters, Milford, MA) and dried in an Eppendorf vacuum concentrator. Peptides derived from the 6.7, 2.0 and 1.0 mg O₂ L⁻¹ treatments were labeled with iTRAQ reagents 114, 116 and 117 (AB Sciex, Framingham, MA), respectively. Labeled peptides which came from one biological replicate (including 6.7, 2.0 and 1.0 mg O₂ L⁻¹ treatments) were pooled together and dried in an Eppendorf

concentrator. This sample pooling reduced variability in protein concentrations introduced during sample fractionation and LC-MS/MS analysis that is normally associated with label free proteomics.

2.3.2 Fractionation and LC-MS/MS analysis

The dried samples were reconstituted in Buffer A (10 mM KH_2PO_4 and 20% acetonitrile (ACN), pH 3.0), and then fractionated with a PolySULFOETHYL strong cation-exchange column (200 x 4.6 mm, 5 μm particle size, 200-Å pore size, PolyLC, Columbia, Maryland) on a Waters 2695 high performance liquid chromatography system. A 50-min gradient was used to fractionate samples at a flow rate of 1 ml/min: 100% Buffer A for 5 min, 0-30% Buffer B (10 mM KH_2PO_4 , 0.5 M KCl and 20% ACN, pH 3.0) with a linear changing gradient for the following 28 min, 30-100% (linear changing gradient) Buffer B for 5 min, 100% Buffer B for 5 min, and 100% Buffer A for the final 7 min. Fourteen fractions were collected, dried and desalted.

Each fraction was reconstituted with Solution A (0.1% formic acid and 2% ACN) and analyzed twice with an LTQ-Orbitrap Elite coupled with an Easy-nLC 1000 (Thermo Fisher, Bremen, Germany). Peptides were separated with a C18 capillary column (50 μm x 15 cm, packed with Acclaim PepMap RSLC C18, 2 μm , 100 Å, nanoViper, Thermo Scientific) by using a 90 min gradient: 100% Solution A (0.1% formic acid in Milli-Q water) for the initial 5 min, followed by a 55 min linear changing gradient of 0-30% Solution B (0.1% formic acid in ACN), 30-98% Solution B (linear changing gradient) for 10 min, and maintained at 98% Solution B for 10 min, then re-equilibrated at 100% Solution A for the final 10 min. Mass spectrometry scans (350-1,600 m/z ranges) were acquired in the positive ion mode with a resolution of 60,000. The five most abundant multiple-charged ions with a combined abundance threshold > 500.0 counts were selected for fragmentation under both high-energy collision-induced dissociation (HCD) and collision-induced dissociation (CID) conditions in order to maximize the number of identified proteins. Part of the HCD spectra were used for iTRAQ quantification in C-trap and the rest of the HCD spectra were combined with CID spectra were used for identifying peptides in LTQ. The isolation width was set as 2.0 m/z for both HCD and CID which had activated dynamic exclusion to remove duplicate precursors. The parameters of HCD fragmentation were 10 ms of activation time, 45% of normalized collision energy, and a full scan with FTMS at a resolution of 15,000 in a centroid mode. The parameters of CID fragmentation were 10 ms of activation time, activation Q of 0.25, and 35% of normalized collision energy.

2.3.3 Protein identification

Raw mass spectrometry data were converted into .mgf files using Thermo Proteome Discoverer 1.3.0.339 (Thermo Finnigan, CA). Each file was filtered by a python script [29] to remove unpaired scans and separated into the HCD and CID data. For HCD and CID data which belong to the same precursor, reporter groups (i.e. 114-

117.5 Da) in CID scans were replaced by those of normalized HCD scans. The HCD data file and modified CID file were separately searched against a *P. canaliculata* [44] and a *P. diffusa* [29] database using Mascot version 2.3.2 (Matrix Sciences, London, UK). The search parameters were set as no more than two missed cleavages, ± 5 ppm for precursor ions, ± 0.6 Da for fragments, fixed modification for methylthio of cysteine, and variable modification for oxidation (methionine) and deamidation (asparagine and glutamine). Search criteria for HCD files were similar to those for CID files with the exception of 20 mmu fragment ions tolerance. Data with ion scores less than 28 (corresponding to 95% confidence level) were removed. The remaining data were exported as a .csv file. Peptides which contained < 7 amino acids were further removed as short peptides might have match in decoy database. In the database search, false discovery rate was set as 1% at the protein level. Peptides which matched more than one protein were deleted as these peptides might have matched to conserved regions in protein groups and could cause bias in quantitation. In addition, peptides which were unlabeled, matched to decoy database and had erratic ratio between iTRAQ reporters (e.g., $116/114 < 0$ or > 100) were removed. Median normalization was performed in each replicate. Proteins detected in all three biological replicates and contained at least 4 summed peptides were used for quantification based on the summed intensity of matched peptides.

2.3.4 Determination of differentially expressed proteins

Orthologs in the two species were identified by searching the translated transcriptome of the two species (40996 and 39527 sequences for *P. diffusa* and *P. canaliculata*, respectively) using the reciprocal best hit method (RBH) with BLASTp (NCBI-blast 2.2.26) under an *E*-value threshold of 1.0×10^{-5} [29,44]. For each pair of ortholog, a two-way analysis of variance (ANOVA) was performed with hypoxia and species used as dependent variables. Because some of the proteins without an ortholog in the other species might also respond to hypoxia, a separate one-way ANOVA was conducted for each species (with hypoxia as the dependent variable), followed by the Tukey tests to determine the hypoxia treatment effects. For both two-way and one-way ANOVA, we chose a *P* value threshold of 0.02 [14,45]. Analyses were performed using an R script (R Development Core Team, 2009) [15,46]. We explored the use of different criteria to account for the multiple hypothesis testing problem in order to define DEPs, an approach that has been suggested in the literature [47]. Results after applying Bonferroni [48] and Benjamini-Hochberg (BH) [49] correction methods show a low number of DEPs (Table S1). However these two methods could be too stringent for our proteomic study, which is mainly exploratory, so we arbitrarily decided to avoid the use of any particular correction method but using the *P* value threshold of 0.02. The use of this *P*-value cut-off, instead of 0.05, reduced the number of DEPs substantially (~50%).

2.3.5 Functional classification

Genes in the transcriptome of both species were searched against databases including NCBI nr, KEGG, SwissProt and COG using BLASTx with a threshold of E-value of 1.00×10^{-5} . Gene Ontologies (GOs) annotations (i.e. Biological Process, Molecular Function, and Cellular Component) of each gene were obtained from Blast2GO [29]. The differentially expressed proteins (DEPs) were classified based on their Gene Ontology (GO). With further manual correction, the DEPs were assigned into several Biological Process GO terms. Proteins without GO annotation or known functions were grouped as 'Others'. The protein expression data of DEPs were $\log_2$ transformed before conducting clustering analysis using Cluster 3.0 software [27]. Their expression patterns were compared using Spearman Rank correlation similarity matrix and the centroid linkage method, and visualized using Java Treeview [27].

2.3.6 Evolutionary analysis of orthologous protein-coding genes

Previous pairwise comparison calculated the Ka and Ks ratios between two sequences by averaging over all amino acids in a gene. As most amino acids in a protein are under functional and structural constraints, thus the Ka/Ks ratio is rarely larger than 1 [50]. Here we adopted a phylogenetic comparative approach [35] which are based on codons to determine the genes that may have contributed to the adaptive divergence in resistance to hypoxia in apple snails by including the transcriptome data from one additional species in the same genus (*P. maculata*) [36]. Using the RBH method, the differentially expressed orthologs (DEOs) between *P. canaliculata* and *P. diffusa* were searched against the translated transcriptomes of *P. maculata*, which were sequenced by Illumina Hiseq 2000 and assembled by Trinity (release 20130225). The orthologs between *P. canaliculata* and the other species were used for the following multiple-species sequence analysis.

Orthologous sequences among the three species were aligned using MUSCLE implemented in ParaAT 1.0 [51]. Gaps in the aligned codons were removed. A likelihood ratio test (LRT) was then applied to compare ω (Ka/Ks) ratios under different pairs of models using the codeml tool implemented in PAMLx [52]. Briefly, the neutral model hypothesized that no codons with a ω value >1 (ℓ_0), and the alternative model with sites having a ω value >1 (ℓ_1). The number of twice the difference of log-likelihood values between alternative and null models ($2\Delta\ell$) was calculated and compared with the Chi-square distribution with a degree of freedom of 2. Two pairs of site models were used. The first pair included the null model M1a (two classes of sites with rates $0 < \omega_0 < 1$ and $\omega_1 = 1$) and the positive selection model M2a (three classes of sites with rates $0 < \omega_0 < 1$, $\omega_1 = 1$, and $\omega_2 > 1$). The second pair compared the M7 model which expects a beta distribution for ω ($0 < \omega < 1$) and the M8 model which has one more class of sites with $\omega > 1$. In both the M2a and M8 models, a Bayes Empirical Bayes (BEB) approach was applied to calculate the posterior probability of each amino acid site belonging to the site class of diversifying selection.

3. Results

3.1 Differential tolerance to hypoxia

The 72-h experiment showed that *P. canaliculata* is more tolerant to hypoxia than *P. diffusa* (Fig. 1). For *P. canaliculata*, all individuals in the 6.7 and 2.0 mg L⁻¹ treatments survived through the experiment period. In the 1.0 mg L⁻¹ treatment, dead snails (41.6%) were first observed at 46 h, and all snails were dead by 54 h. For *P. diffusa*, all individuals exposed to 6.7 mg L⁻¹ survived through the exposure period. At 2.0 and 1.0 mg L⁻¹, dead snails were first found at 18 h; by 65 h all individuals in both treatments were found dead.

We encountered some difficulty in obtaining sufficient snails of similar sizes to conduct an experiment with replicate tanks. Nevertheless, we have performed several experiments with a similar design, and each time with different numbers of snails that were available to us. The results were consistent with the data shown in Fig. 1. We believe our conclusions about the differential sensitivity of the two species of snails to hypoxia are robust, as these results are consistent with our field observation that *P. canaliculata* can be found in ponds or ditches with very low dissolved oxygen levels in the water, whereas *P. diffusa* has not been reported from such low oxygen environment.

3.2 Proteome analysis

In *P. canaliculata*, a total of 2759 proteins were identified, including 2105, 2049 and 2060 in three biological replicates, respectively. There were 1430 shared proteins among the three replicates. In *P. diffusa*, a total of 2550 proteins were identified, including 1951, 1989 and 1973 from the three biological replicates, respectively. There were 1378 shared proteins among the three replicates.

Reciprocal best hit identified 13,308 orthologous proteins between the two species. A two-way ANOVA of these orthologs revealed a total of 78 proteins with significant expression changes due to hypoxia (36 proteins), species (51 proteins) or their interaction (18 proteins) (Fig. 2). The DEPs were classified into 14 GO terms: amino acid metabolism (4 proteins), cell fate (4 proteins), cytoskeletal organization (8 proteins), energy metabolism (7 proteins), immune response (2 proteins), localization (4 proteins), oxidation-reduction (6 proteins), protein folding (3 proteins), protein modification (7 proteins), proteolysis (7 proteins), transcriptional and translational processes (12 proteins), signal transduction (5 proteins), and others (9 proteins). The following discussion of the two-way ANOVA results focus on the species effects, because the oxygen effects will be discussed in the one-way ANOVA results. The 51 proteins that showed species-specific responses could be roughly classified into two categories and within each category, the two species showed overall opposite expression trends. These proteins belonged to several functional groups: transcription and translation (7 proteins), energy metabolism (6 proteins), proteolysis (6 proteins), oxidation-reduction (5 proteins), protein modification (5 proteins), cytoskeletal organization (4 proteins), cell fate (3 proteins), localization (3 proteins), protein

folding (2 proteins), signal transduction (2 proteins), amino acid metabolism (2 proteins), immune response (1 protein) and others (5 proteins). There were 18 proteins showing interactions between species and hypoxia treatments. They belonged to cell fate (1 protein), immune response (1 protein), signal transduction (2 proteins), energy metabolism (3 proteins), protein folding (2 proteins), proteolysis (2 proteins), transcription and translation (3 proteins), protein modification (2 proteins) and an undefined functional group (2 proteins).

One-way ANOVA conducted in each species revealed a hypoxia level-dependent expression pattern. In *P. canaliculata*, when compared with the 6.7 mg L⁻¹ control, the 2.0 and 1.0 mg L⁻¹ treatments resulted in 48 and 28 DEPs, respectively (Fig. 3A). DEPs of two hypoxic treatments covered almost all the important functional categories, but their expression trends or levels are quite different with the 2.0 mg L⁻¹ treatment showing more alerting or repairing responses and the 1.0 mg L⁻¹ treatment showing irreversible damage. For example, the DEPs in the 2.0 mg L⁻¹ treatment included up-regulation of immune responses (e.g., molluscan defense molecule precursor protein) and molecular chaperones (e.g., chaperonin containing T-complex polypeptide subunit zeta) to protect the organism from exogenous pathogens and repair denatured proteins. In contrast, in the more hypoxic 1 mg L⁻¹ treatment, the damage caused by hypoxia is more severe as indicated by the significant up-regulation of programmed cell death 6-interacting protein which is involved in apoptosis. Filamin-C isoform 4, a cytoskeletal organization related protein, was significantly down-regulated indicating suppression of cell growth and proliferation in order to reallocate energy to cell survival. In *P. diffusa*, when compared with the 6.7 mg L⁻¹ control, the 2.0 and 1.0 mg L⁻¹ treatments resulted in 49 and 25 DEPs, respectively (Fig. 3B). The 2.0 mg L⁻¹ treatment caused higher up-regulation of immune proteins (e.g., G-type lysozyme) and stronger induction of oxidation-reduction related proteins (e.g., ectoine hydroxylase) than the 1.0 mg L⁻¹ treatment. Compared to repairing response of 2.0 mg L⁻¹ treatment, the more severe hypoxic 1.0 mg L⁻¹ treatment caused overall suppression of several non-essential cellular pathways. For example, there was significant down-regulation of some cytoskeletal organization related proteins such as dynein, light chain, roadblock-type 2-like protein suggesting a reduced activity of cell proliferation in order to save energy for other vital cellular processes and extend surviving time.

3.3 Estimation of Ka/Ks

Among the 78 DEOs between *P. canaliculata* and *P. diffusa*, 62 had orthologs in all three *Pomacea* species. The log-likelihood test values, average Ka/Ks (ω) and positively selected amino acid sites for the 62 orthologs are shown in Table S2. Comparison of M1a vs. M2a and M7 vs. M8 indicated that there was significant difference between the two pairs of models ($\omega > 1$, $P < 0.05$) in five genes (Table 1). These included two uncharacterized proteins (CAPTEDRAFT_173803 and RE16407p); a protein which functions in amino acid metabolism (CAPTEDRAFT_158431); a protein in the electron transport chain which functions in

cellular oxidation-reduction hemostasis (cytochrome *c* oxidase subunit VIb); and a protein which is known to be involved in cytoskeletal organization (K03H1.5). Analysis of their sequences using the BEB method revealed several positively selected amino acid sites, with a posterior probability $\geq 90\%$ (Table 1). Among the five genes, only RE16407p has a ω larger than 1 indicating overall positive selection on the whole gene sequence. For the other four genes, although the ω value was less than 1, applying the BEB method revealed several positively selected amino acid sites. For instance, in cytochrome *c* oxidase subunit VIb, the 34th amino acid has a posterior probability $\geq 90\%$ of having undergone positive selection in this gene that plays a key role in the respiratory electron transport chain of mitochondria.

4. Discussion

Since oxygen is critical for energy production in metazoans, understanding how hypoxia affects energy metabolism and cellular homeostasis has been a focus of research over the last decades [53,54]. It is well known that, animals may decrease metabolism in the oxidative phosphorylation pathway in response to hypoxia, thus reducing energy production through lowering protein synthesis and ion pump activities, and/or activating the anaerobic pathway [55,56]. Several groups of evolutionarily conserved genes, particularly those regulating DNA and protein structural stability, cell cycle and oxidation-reduction, are also involved in hypoxia responses [57-59].

Nevertheless, different organisms have different thresholds of resistance to hypoxia, and it is imperative to distinguish whether such difference is caused by adaptive variation or phylogeny. Comparing the responses of the two apple snail species to hypoxia has revealed the complex protein expressional networks that are involved. Sequence divergence at DNA level has given us some candidates which may participate into the genetic bases of differential protein expression. A striking result of the two-way ANOVA was that there were only a few DEPs showing identical expressional trends between the two species, and many of the DEPs exhibited opposite responses to hypoxic exposure in the two species, suggesting genetically closely related species can differ substantially in their responses to the stress. The differentially expressed orthologs (DEOs) can be classified into several functional groups, whose expression patterns will be discussed below.

4.1 Hypoxia sensing and signal transduction

In order to actively avoid hypoxia, many aquatic species can sense the oxygen level quickly, but their oxygen sensors and downstream signal transduction pathways have not been identified [58]. Among the candidate oxygen sensors are respiration chain proteins mitochondrial cytochrome *a3* and cytochrome *c* oxidase, whose upregulation can enhance reactive oxygen species (ROS) production, which stabilizes hypoxia induction factors [60,61]. Support for this mitochondrial model of hypoxia signaling pathway in the present study comes from the discovery of a cytochrome *c* oxidase

subunit VIb, which was down-regulated in both *P. canaliculata* and *P. diffusa* exposed to hypoxia. In addition, the DEPs in the two apple snails include a variety of signaling and transduction related proteins, among them are a guanine nucleotide-binding protein, a molecular switch that regulates a variety of cellular processes such as sensual perception, transportation and differentiation [62]; as well as several proteins (i.e., calcium-dependent protein kinase isoform 2, phosphoglycerate kinase 1 and tyrosine-protein kinase HTK16) that are known to participate in post-translational modifications such as phosphorylation and demethylation, implying epigenetic control of gene expression, protein synthesis and intracellular signaling [63].

4.2 Protein synthesis

Since protein synthesis is one of the main cellular energy-consuming processes under standard metabolic rate [64], reducing the energy demand of protein synthesis to a minimum is a critical survival strategy, which has been reported in fish under hypoxic conditions [57,65]. In the present study, a variety of translational and transcriptional chaperones participating in protein biosynthesis (e.g., elongation factor 1 alpha, ribosomal protein L22 and ribosomal protein S5) showed significant changes in both species. For instance, histone-binding protein Caf1 and ribosomal protein L22 were down-regulated in *P. canaliculata*, but up-regulated in *P. diffusa* under the two hypoxic treatments (Fig. 4). When energy supply is limited, reallocation of cellular energy between essential and non-essential ATP-demanding processes becomes important, and energy spared by transcriptional and translational arrest can be allocated to other critical biological processes such as ionic homeostasis [66]. The opposite expression patterns of translational factors between the two species in our study indicates that *P. canaliculata* can quickly respond to acute hypoxia stress by activating translational arrest, but *P. diffusa* lacks this ability which could have implications for energy reallocation and protein synthesis.

4.3 Energy metabolism

In the present study, hypoxia exposure has led to the differential expression of many enzymes in several energy metabolic and amino acid metabolic processes between the two species. For instance, malate dehydrogenase precursor which is involved in the tricarboxylic acid (TCA) cycle was down-regulated in *P. canaliculata* but up-regulated in *P. diffusa* (Fig. 4). Fructose-biphosphate aldolase, an enzyme catalyzing fructose 1,6-bisphosphate into glyceraldehyde 3-phosphate reversely in glycolysis, was down-regulated in *P. canaliculata* but up-regulated in *P. diffusa* in the two hypoxic treatments. In the glycolytic pathway, glucose is catalyzed into pyruvate, and pyruvate is catalyzed into lactate by lactate dehydrogenase under hypoxic condition, or participate into the TCA cycle under normal condition. In *P. canaliculata*, lactate dehydrogenase was significantly down-regulated, which suggests the suppression of anaerobic metabolism in this hypoxia tolerant species; but there was no evidence for activation or suppression of anaerobic metabolism in *P. diffusa* under the 8-h

exposure to hypoxia. Because even though anaerobic metabolism could generate ATP, this pathway of energy production depletes fermentable glycogen store quickly due to its low efficiency, and leads to excessive accumulation of deleterious end products such as protons, ethanol and lactate that would eventually compromise the snail's ability to tolerate hypoxia [66,67]. We can speculate with a suppression of anaerobic respiration in *P. canaliculata* under the acute hypoxia condition in our study. However it does not mean that this snail does not use this well-characterized pathway of hypoxia responses under longer hypoxia exposure [67].

4.4 Effects of different oxygen concentrations

One-way ANOVA revealed 17 overlapped differentially expressed proteins (DEPs) between two hypoxic treatments in *P. canaliculata*, and 15 overlapped DEPs between the two hypoxic treatments in *P. diffusa*. The shared DEPs belong to several conserved functional groups, including "energy metabolism", in the two species, indicating changes in metabolism is a common hypoxia response in the apple snails.

In accordance with previous studies [40], we used 2.0 mg O₂ L⁻¹ as the hypoxic threshold. In both species, this hypoxia treatment induced the highest number of DEPs which included most of the common functional categories. Nevertheless, the DEPs of the two species showed different expressional trends. For example, several proteins functioning in protein modification (e.g., tyrosine-protein kinase HTK16 and CAPTEDRAFT_173271) were differentially expressed in *P. diffusa* but not in *P. canaliculata*. Since protein modification (e.g., phosphorylation) is involved in signaling and downstream gene expression, the significant changes in *P. diffusa* indicated disruption of cellular homeostasis which required repair and recovery. In addition, metabolic suppression was more obvious in *P. canaliculata* under 2 mg O₂ L⁻¹ as indicated by the reduced expression levels in most of the metabolism related enzymes. The reduced metabolic level in the invasive snail may contribute to conserve the cellular fermentable fuels and help the organism to survive hours or days.

We adopted 1.0 mg O₂ L⁻¹ as an extreme hypoxia condition, as reported in previous studies [41]. Under this hypoxia treatment, proteins in many functional groups were differentially expressed, but the DEPs differed in function or expressional trend between the two apple snails. For instance, several cytoskeletal organization related proteins (e.g., filamin-C isoform 4 and CAPTEDRAFT_221766) were down-regulated in *P. canaliculata*, but not in *P. diffusa*. Since proteins involved in cytoskeletal structure and locomotion are highly abundant, reducing their expression in *P. canaliculata* might be an important energy-saving strategy. In *P. canaliculata*, there was also translational arrest, indicated by the down-regulation of eukaryotic translation initiation factor 3 subunit K. Protein synthesis is a major energy-consuming process, therefore, translational arrest could reduce protein synthesis and help *P. canaliculata* conserve the limited energy under severe hypoxia.

4.5 Evolutionary analysis of orthologs

As the mutation of most amino acids in a protein is under functional and structural constraints, the Ka/Ks ratio, calculated for the whole protein coding gene sequence, is rarely larger than 1 [50]. By adopting codon-based site models which allow the Ka/Ks ratio to vary among codons [50,52], we found that five of the 62 orthologous DEOs contained positively selected amino acid sites. Due to the incomplete gene annotation, four of them have either unknown function (CAPTEDRAFT_173803 and RE16407p) or not-well characterized function (CAPTEDRAFT_158431 and uncharacterized protein K03H1.5). These uncharacterized proteins should be good candidates for functional analysis to determine their exact role in hypoxia responses. Cytochrome *c* oxidase subunit VIb, however, is a DEO with known functions. As a subunit of ETC complex IV, this protein is involved in the electron transport chain (ETC) by transferring electrons from reduced cytochrome *c* to oxygen. This result indicates that the mitochondrial enzyme involved in energy production has been a target of positive selection in apple snails, and this selection can have implications in their divergence in stress resistance. This result is consistent with previous reports of several cytochrome *c* oxidase subunits having undergone positive selection in other animals adapted to hypoxic environments [68,69]. In order to further examine the locations and consequence of mutated sites on proteins with Ka/Ks > 0.5, we built three-dimensional structures using PHYRE2 [70]. However, the 3D structures were in low confidence. Nevertheless, the list of differentially expressed proteins with accelerated substitution rates can serve as candidates for more detailed structural and functional studies.

4.6 Hypothesis on cellular responses to acute hypoxia

Our proteomic data indicate that molecular responses involved in the acute hypoxia responses are complex (Fig. 4). Apple snails may detect low oxygen level using extracellular (e.g., hemocyanin protein) or intracellular (e.g., cytochrome *c* oxidase) oxygen sensors, then activate signal transduction to regulate downstream energy demanding processes such as translation or energy generating pathways including glycolysis, the TCA cycle, lipid metabolism, and anaerobic metabolism. In addition, since low oxygen levels may lead to the accumulation of cellular reactive oxygen species (ROS) and protein denaturation, molecular chaperones and proteins involved in proteolysis are also regulated. An interesting observation from this study is that proteins in several important cellular pathways (e.g., TCA cycle, electron transport chain and glycolysis) exhibited differential responses between the congeneric snails exposed to hypoxia. In particular, there was an obvious metabolic suppression in the invasive snail *P. canaliculata* under acute exposure to hypoxia, as indicated by the down-regulation of several key enzymes in the ETC, glycolysis, TCA cycle and translational processes, consistent with the view of Boutilier and St-Pierre (2000) [66] that metabolic suppression could conserve cellular fermentable fuels and extend the survival time of organisms to hours or days. In conclusion, the expression profiles of proteins involved in energy metabolism, cytoskeleton and transcriptional and

translational processes revealed some common responses to hypoxia stress in both snails but also a clear higher tolerance to hypoxia in the invasive *P. canaliculata*, which is in accordance with its ability to live in stagnant hypoxia waters [30].

5. Conclusions

The integrated physiological, proteomic, transcriptomic and base substitution analyses employed in this study has given us a better understanding of the genetic basis of differential resistance to hypoxia between the two congeneric snails. The list of differentially expressed proteins with accelerated substitution rates can serve as candidates for more detailed functional studies. Our streamlined comparative approach of congeneric species can be applied to understand some of the molecular mechanisms of adaptation to global environmental change in non-model species. Nevertheless, it should be pointed out that, other molecular mechanisms, such as gene duplication and epigenetic modifications, can also be involved in hypoxia resistance.

Conflict of interest

The authors declare no competing financial interest.

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References

- [1] D.L. Strayer, Alien species in fresh waters: ecological effects, interactions with other stressors, and prospects for the future. *Freshwater Biol.* 55 (2010) 152–174.
- [2] E.V. Moran, F. Hartig, D.M. Bell, Intraspecific trait variation across scales: implications for understanding global change responses. *Glob. Chang. Biol.* 22 (2016) 137–150.
- [3] D. Pimentel, R. Zuniga, D. Morrison, Update on the environmental and economic costs associated with alien-invasive species in the United States. *Ecol. Econ.* 52 (2005) 273–288.
- [4] D. Simberloff, J.L. Martin, P. Genovesi, V. Maris, D.A. Wardle, J. Aronson et al., Impacts of biological invasions: what's what and the way forward. *Trends Ecol. Evol.* 28 (2013) 58–66.
- [5] K.R. Hayes, S.C. Barry, Are there any consistent predictors of invasion success? *Biol. Invasions* 10 (2008) 483–506.
- [6] E. McKnight, E. Garcia-Berthou, P. Srean, M. Rius, Global meta-analysis of native and nonindigenous trophic traits in aquatic ecosystems. *Glob. Chang. Biol.* 23 (2017) 1861–1870.
- [7] M.A. Davis, J.P. Grime, K. Thompson, Fluctuating resources in plant communities: a general theory of invasibility. *J. Ecol.* 88 (2000) 528–534.
- [8] D.M. Blumenthal, Interactions between resource availability and enemy release in plant invasion. *Ecol. Lett.* 9 (2006) 887–895.
- [9] C. Bellard, W. Thuiller, B. Leroy, P. Genovesi, M. Bakkenes, F. Courchamp, Will climate change promote future invasions? *Glob. Chang. Biol.* 19 (2013) 3740–3748.
- [10] M.H. Williamson, A. Fitter, The characters of successful invaders. *Biol. Conserv.* 78 (1996) 163–170.
- [11] C.S. Kolar, D.M. Lodge, Progress in invasion biology: predicting invaders. *Trends Ecol. Evol.* 16 (2001) 199–204.
- [12] G.N. Somero, Comparative physiology: a "crystal ball" for predicting consequences of global change. *Am. J. Physiol., Reg. Integr. Comp. Physiol.* 301 (2011) R1–R14.
- [13] Y.M. Dong, G.N. Somero, Temperature adaptation of cytosolic malate dehydrogenases of limpets (genus *Lottia*): differences in stability and function due to minor changes in sequence correlate with biogeographic and vertical distributions. *J. Exp. Biol.* 212 (2009) 169–177.
- [14] L. Tomanek, M.J. Zuzow, The proteomic response of the mussel congeners *Mytilus galloprovincialis* and *M. trossulus* to acute heat stress: implications for thermal tolerance limits and metabolic costs of thermal stress. *J. Exp. Biol.* 213 (2010) 3559–3574.
- [15] B.L. Lockwood, G.N. Somero, Transcriptomic responses to salinity stress in invasive and native blue mussels (genus *Mytilus*). *Mol. Ecol.* 20 (2011) 517–29.
- [16] M.D. García-Molina, V. Muccilli, R. Saletti, S. Foti, S. Masci, F. Barro, Comparative proteomic analysis of two transgenic low-gliadin wheat lines and non-transgenic wheat control. *J. Proteomics* (2017) doi:

- 10.1016/j.jprot.2017.06.010.
- [17] C. Xu, E. Li, Y. Liu, X. Wang, J.G. Qin, L. Chen, Comparative proteome analysis of the hepatopancreas from the Pacific white shrimp *Litopenaeus vannamei* under long-term low salinity stress. *J. Proteomics* 162 (2017) 1–10.
- [18] K.B. Storey, J.M. Storey, Metabolic rate depression in animals: transcriptional and translational controls. *Biol. Rev.* 79 (2004) 207 – 233.
- [19] J. Trapp, A. McAfee, L.J. Foster, Genomics, transcriptomics and proteomics: enabling insights into social evolution and disease challenges for managed and wild bees. *Mol. Ecol.* 26 (2017) 718–739.
- [20] K.A. Hayes, R.H. Cowie, A. Jorgensen, R. Schultheiss, C. Albrecht, S.C. Thiengo, Molluscan models in evolutionary biology: apple snails (Gastropoda: Ampullariidae) as a system for addressing fundamental questions. *Am. Malacol. Bull.* 27 (2009) 47–58.
- [21] K.A. Hayes, R.L. Burks, A. Castro-Vazquez, P.C. Darby, H. Heras, P.R. Martin, et al., Insights from an integrated view of the biology of apple snails (Caenogastropoda: Ampullariidae). *Malacologia* 58 (2015) 245–302.
- [22] R.H. Cowie, Apple Snails (Ampullariidae) as Agricultural Pests: Their Biology, Impacts and Management. In *Molluscs as Crop Pests*, Barker, G. M., Eds., CABI Publishing: Wallingford, U.K., 2002, pp 145–192.
- [23] N.O.L. Carlsson, C. Bronmark, L.A. Hansson, Invading herbivory: The golden apple snail alters ecosystem functioning in Asian wetlands. *Ecology* 85 (2004) 1575–1580.
- [24] L. Fang, P.K. Wong, L. Lin, C.Y. Lan, J.W. Qiu, Impact of invasive apple snails in Hong Kong on wetland macrophytes, nutrients, phytoplankton and filamentous algae. *Freshwater Biol.* 55 (2010) 1191–1204.
- [25] Y. Yusa, T. Wada, S. Takahashi, Effects of dormant duration, body size, self-burial and water condition on the long-term survival of the apple snail, *Pomacea canaliculata* (Gastropoda : Ampullariidae). *Appl. Entomol. Zool.* 41 (2006) 627–632.
- [26] M. Giraud-Billoud, I.A. Vega, M.E.R. Tosi, M.A. Abud, M.L. Calderon, A. Castro-Vazquez, Antioxidant and molecular chaperone defences during estivation and arousal in the South American apple snail *Pomacea canaliculata*. *J. Exp. Biol.* 216 (2013) 614–622.
- [27] J. Sun, H. Mu, H.M. Zhang, K.H. Chandramouli, P.Y. Qian, C.K.C Wong, J.W. Qiu, Understanding the regulation of estivation in a freshwater snail through iTRAQ-based comparative proteomics. *J. Proteome Res.* 12 (2013) 5271–5280.
- [28] K. Yoshida, K. Matsukura, N.J. Cazzaniga, T. Wada, Tolerance to low temperature and desiccation in two invasive apple snails, *Pomacea canaliculata* and *P. maculata* (Caenogastropoda: Ampullariidae), collected in their original distribution area (Northern and Central Argentina). *J. Molluscan Stud.* 80 (2014) 62–66.
- [29] H. Mu, J. Sun, L. Fang, T.G. Luan, G.A. Williams, S.G. Cheung, C.K.C. Wong, J.W. Qiu, Genetic basis of differential heat resistance between two species of congeneric freshwater snails: insights from quantitative proteomics and base

- substitution rate analysis. *J. Proteome Res.* 14 (2015) 4296–4308.
- [30] K.L. Kwong, P.K. Wong, S.S.S. Lau, J.W. Qiu, Determination of the distribution of apple snails in Hong Kong two decades after their initial invasion. *Malacologia* 50 (2008) 293–302.
- [31] T.A. Rawlings, K.A. Hayes, R.H. Cowie, T.M. Collins, The identity, distribution, and impacts of non-native apple snails in the continental United States. *BMC Evol. Biol.* 7 (2007).
- [32] K.J. Collier, N.J. Demetras, I.C. Duggan, T.M. Johnston, Wild record of an apple snail in the Waikato River, Hamilton, New Zealand, and their incidence in freshwater aquaria. *New Zealand Nat. Sci.* 36 (2011) 1–9.
- [33] I.G. Romero, I. Ruvinsky, Y. Gilad, Comparative studies of gene expression and the evolution of gene regulation. *Nat. Rev. Genet.* 13 (2012) 505–16.
- [34] N.L. Clark, W.J. Swanson, Pervasive adaptive evolution in primate seminal proteins. *PLoS Genet.* 1 (2005) e35.
- [35] A. Whitehead, D.L. Crawford, Neutral and adaptive variation in gene expression. *PNAS* 103 (2006) 5425–5430.
- [36] H. Mu, J. Sun, H. Heras, K.H. Chu, J.W. Qiu, An integrated proteomic and transcriptomic analysis of perivitelline fluid proteins in a freshwater gastropod laying aerial eggs. *J. Proteomics* 155 (2017) 22–30.
- [37] G.D. Findlay, W.J. Swanson, Proteomics enhances evolutionary and functional analysis of reproductive proteins. *Bioessays* 32 (2010) 26–36.
- [38] M.D. Vigeland, M. Spannagl, T. Asp, C. Paina, H. Rudi, O. Rognli, S. Fjellheim, S.R. Sandve, Evidence for adaptive evolution of low-temperature stress response genes in a Pooideae grass ancestor. *New Phytologist* 199 (2013) 1060–1068.
- [39] X. Zhao, H. Yu, L. Kong, S. Liu, Q. Li, Comparative transcriptome analysis of two oysters, *Crassostrea gigas* and *Crassostrea hongkongensis* provides insights into adaptation to hypo-osmotic conditions. *PLoS one* 9 (2014) e111915.
- [40] R. Vaquer-Sunyer, C.M. Duarte, Thresholds of hypoxia for marine biodiversity. *PNAS* 105 (2008) 15452–15457.
- [41] R.J. Diaz, Overview of hypoxia around the world. *J. Environ. Qual.* 30 (2001) 275–281.
- [42] S.G. Cheung, H.Y. Chan, C.C. Liu, P.K.S. Shin, Effect of prolonged hypoxia on food consumption, respiration, growth and reproduction in marine scavenging gastropod *Nassarius festivus*. *Mar. Pollut. Bull.* 57 (2008) 280–286.
- [43] M.E. Seuffert, P.R. Martin, Dependence on aerial respiration and its influence on microdistribution in the invasive freshwater snail *Pomacea canaliculata* (Caenogastropoda, Ampullariidae). *Biol. Invasions* 12 (2010) 1695–1708.
- [44] J. Sun, M. Wang, H. Wang, H. Zhang, X. Zhang, V. Thiyagarajan, P.Y. Qian, J.W. Qiu, *De novo* assembly of the transcriptome of an invasive snail and its multiple ecological applications. *Mol. Ecol. Resour.* 12 (2012) 1133–1144.
- [45] P.A. Fields, M.J. Zuzow, L. Tomanek, Proteomic responses of blue mussel (*Mytilus*) congeners to temperature acclimation. *J. Exp. Biol.* 215 (2012) 1106–1116.
- [46] B.L. Lockwood, J.G. Sanders, G.N. Somero, Transcriptomic responses to heat

- stress in invasive and native blue mussels (genus *Mytilus*): molecular correlates of invasive success. *J. Exp. Biol.* 213 (2010) 3548–58.
- [47] A.P. Diz, A. Carvajal-Rodríguez, D.O.F. Skibinski, Multiple hypothesis testing in proteomics: a strategy for experimental work. *Mol. Cell. Proteomics* 10 (2011), M110. 004374.
- [48] R.J. Simes, An improved Bonferroni procedure for multiple tests of significance. *Biometrika* 73 (1986), 751–754.
- [49] Y. Benjamini, Y. Hochberg, Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Series B* (1995), 289–300.
- [50] Z.H. Yang, R. Nielsen, Codon-substitution models for detecting molecular adaptation at individual sites along specific lineages. *Mol. Biol. Evol.* 19 (2002) 908–917.
- [51] Z. Zhang, J.F. Xiao, J.Y. Wu, H.Y. Zhang, G.M. Liu, X.M. Wang, L. Dai, ParaAT: A parallel tool for constructing multiple protein-coding DNA alignments. *Biochem. Biophys. Res. Commun.* 419 (2012) 779–781.
- [52] B. Xu, Z.H. Yang, pamlX: a graphical user interface for PAML. *Mol. Biol. Evol.* 30 (2013) 2723–2724.
- [53] P.W. Hochachka, L.T. Buck, C.J. Doll, S.C. Land, Unifying theory of hypoxia tolerance: Molecular metabolic defense and rescue mechanisms for surviving oxygen lack. *PNAS* 93 (1996) 9493–9498.
- [54] J.J. Childress, B.A. Seibel, Life at stable low oxygen levels: Adaptations of animals to oceanic oxygen minimum layers. *J. Exp. Biol.* 201 (1998) 1223–1232.
- [55] P.W. Hochachka, P.L. Lutz, Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp. Biochem. Phys. B* 130 (2001) 435–459.
- [56] P.W. Hochachka, G.N. Somero, *Biochemical Adaptation-Mechanism and Process in Physiological Evolution*, Oxford University Press: New York 2002.
- [57] A.Y. Gracey, J.V. Troll, G.N. Somero, Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *PNAS* 98 (2001) 1993–1998.
- [58] R.S.S. Wu, Hypoxia: from molecular responses to ecosystem responses. *Mar. Pollut. Bull.* 45 (2002) 35–45.
- [59] L. Tomanek, Environmental proteomics: changes in the proteome of marine organisms in response to environmental stress, pollutants, infection, symbiosis, and development. *Annu. Rev. Mar. Sci.* 3 (2011) 373–399.
- [60] N.S. Chandel, G.R.S. Budinger, S.H. Choe, P.T. Schumacker, Cellular respiration during hypoxia-role of cytochrome oxidase as the oxygen sensor in hepatocytes. *J. Biol. Chem.* 272 (1997) 18808–18816.
- [61] P.R. Castello, P.S. David, T. McClure, Z. Crook, R.O. Poyton, Mitochondrial cytochrome oxidase produces nitric oxide under hypoxic conditions: implications for oxygen sensing and hypoxic signaling in eukaryotes. *Cell Metab.* 3 (2006) 277–287.
- [62] I.R. Vetter, A. Wittinghofer, Signal transduction-the guanine nucleotide-binding switch in three dimensions. *Science* 294 (2001) 1299–1304.
- [63] K. Engholm-Keller, M.R. Larsen, Technologies and challenges in large-scale phosphoproteomics. *Proteomics* 13 (2013) 910–931.

- [64] D.F.S. Rolfe, G.C. Brown, Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* 77 (1997) 731–758.
- [65] C. Ton, D. Stamatiou, C.C. Liew, Gene expression profile of zebrafish exposed to hypoxia during development. *Physiol. Genomics* 13 (2003) 97–106.
- [66] R.G. Boutilier, J. St-Pierre, Surviving hypoxia without really dying. *Comp. Biochem. Phys. A* 126 (2000) 481–490.
- [67] K.B. Storey, J.M. Storey, Tribute to PL Lutz: putting life onpause'—molecular regulation of hypometabolism. *J. Exp. Biol.* 210 (2007), 1700–1714.
- [68] Y. Luo, W. Gao, Y. Gao, S. Tang, Q. Huang, X. Tan, J. Chen, T. Huang, Mitochondrial genome analysis of *Ochotona curzoniae* and implication of cytochrome *c* oxidase in hypoxic adaptation. *Mitochondrion* 8 (2008) 352–7.
- [69] G.R. Scott, P.M. Schulte, S. Egginton, A.L.M. Scott, J.G. Richards, W.K. Milsom, Molecular evolution of cytochrome *c* oxidase underlies high-altitude adaptation in the bar-headed goose. *Mol. Biol. Evol.* 28 (2011) 351–363.
- [70] L.A. Kelley, S. Mezulis, C.M. Yates, M.N. Wass, M.J.E. Sternberg, The Phyre2 web portal for protein modeling, prediction and analysis, *Nat. Protoc.* 10 (2015) 845–858.

Figure Legends

Figure 1. Mortality rate of *P. canaliculata* (A) and *P. diffusa* (B) under control (6.7 mg L⁻¹) and two hypoxia conditions (2.0 and 1.0 mg L⁻¹) in a 72-h experiment ($n = 12$ in each treatment for both species). Note that all snails in the control survived through the experiment, but the data cannot be seen from (A) as they were covered by those from the 2.0 mg L⁻¹ treatment.

Figure 2. Expression patterns of 78 orthologous proteins that had significant effects of oxygen concentration, species or their interaction. The column on the left with white cells represents the control, whose protein expression level was normalized to 1 against other treatments. Significant *P*-values are highlighted in bold red. Pc = *P. canaliculata*, Pd = *Pomacea diffusa*, 2.0 and 1.0 represents the 2.0 and 1.0 mg L⁻¹ hypoxia treatment, respectively. The level of fold change (red = up-regulation, blue = down-regulation) is indicated by a color key on the upper left.

Figure 3. Expression patterns of differentially expressed proteins between control (6.7 mg L⁻¹) and two hypoxia treatments (2.0 and 1.0 mg L⁻¹) in *P. canaliculata* (A) and *P. diffusa* (B). The column on the left with white cells represents the control, whose protein expression level was normalized to 1 against other treatments. Significant *P*-values are highlighted in bold red. The level of fold change (red = up-regulation, blue = down-regulation) is indicated by a color key on the upper left. On the right of the heat map are protein annotation, functional classification, and *P*-value of *post hoc* test.

Figure 4. Possible pathways involved in differential hypoxia responsive protein expression in *P. canaliculata* (Pc) and *P. diffusa* (Pd). Protein names in red color represent differentially expressed proteins (including one-way and two-way ANOVA results) detected in our study, and those in blue are detected proteins not showing differential expression. The green symbols “↑” and “↓” represent up- or down-regulation. Abbreviations: G6P: Glucose-6-phosphate; F6P: Fructose-6-phosphate; F1,6PP: Fructose-1,6-bisphosphate; G3P: glyceraldehyde-3-phosphate; DHAP: Dihydroxyacetone phosphate; 3PG: 3-phosphoglycerate; 1,3BPG: 1,3-Bisphosphoglycerate; 2PG: 2-Phosphoglycerate; PEP: Phosphoenolpyruvate; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Table 1. Log-likelihood values and base substitution parameter estimates for 5 orthologous protein-coding genes estimated using two pairs of site models.

Description	Model type	lnL	$2\Delta\ell^a$	Ka/Ks ^b	Parameters ^c	BEB residues ^d
CAPTEDRAFT_17 3803	M1a	-1308.844		0.242	$P_0 = 0.758, P_1 = 0.242; \omega_0 = 0, \omega_1 = 1.000$	---
	M2a	-1304.997	7.694	0.566	$P_0 = 0.914, P_1 = 0, P_2 = 0.086; \omega_0 = 0, \omega_1 = 1.000, \omega_2 = 6.558$	235Q(*), 280T(**)
	M7	-1309.044		0.200	$p = 0.005, q = 0.020$	---
	M8	-1305.000	8.093	0.566	$P_0 = 0.914 (P_1 = 0.086); p = 0.005, q = 2.563, \omega = 6.558$	235Q(**), 280T(**)
RE16407p	M1a	-547.628		0.421	$P_0 = 0.580, P_1 = 0.420; \omega_0 = 0, \omega_1 = 1.000$	---
	M2a	-544.707	5.842	1.050	$P_0 = 0.842, P_1 = 0, P_2 = 0.158; \omega_0 = 0, \omega_1 = 1.000, \omega_2 = 6.648$	---
	M7	-548.007		0.300	$p = 0.005, q = 0.011$	---
	M8	-544.707	6.600	1.050	$P_0 = 0.842 (P_1 = 0.158); p = 0.005, q = 1.933, \omega = 6.648$	98T(*), 108V(*)
CAPTEDRAFT_15 8431	M1a	-1581.134		0.188	$P_0 = 812, P_1 = 0.188; \omega_0 = 0, \omega_1 = 1.000$	---
	M2a	-1572.552	17.164	0.594	$P_0 = 0.955, P_1 = 0, P_2 = 0.045; \omega_0 = 0.082, \omega_1 = 1.000, \omega_2 = 11.499$	1M(**), 2S(**), 4A(**), 14E(*), 41T(*)
	M7	-1581.170		0.200	$p = 0.005, q = 0.020$	---
	M8	-1572.553	17.229	0.594	$P_0 = 0.955 (P_1 = 0.045); p = 8.953, q = 99.0, \omega = 11.509$	1M(**), 2S(**), 4A(**), 10R(*), 12L(*), 14E(**), 17L(*), 41T(**)
Cytochrome c oxidase subunit VIb	M1a	-351.061		0.143	$P_0 = 0.857, P_1 = 0.143; \omega_0 = 0, \omega_1 = 1.000$	---
	M2a	-347.325	7.471	0.721	$P_0 = 0.920, P_1 = 0, P_2 = 0.080; \omega_0 = 0, \omega_1 = 1.000, \omega_2 = 8.997$	---
	M7	-351.310		0.200	$p = 0.005, q = 0.021$	---
	M8	-347.325	7.970	0.721	$P_0 = 0.920 (P_1 = 0.080); p = 0.005, q = 99.0, \omega = 8.997$	34T(*), 37E(*)
Uncharacterized protein K03H1.5	M1a	-5921.905		0.459	$P_0 = 0.541, P_1 = 0.459; \omega_0 = 0, \omega_1 = 1.000$	---
	M2a	-5916.229	11.353	0.662	$P_0 = 0.530, P_1 = 0.460, P_2 = 0.010; \omega_0 = 0, \omega_1 = 1.000, \omega_2 = 19.940$	11F(**), 12K(*), 410E(*)
	M7	-5922.363		0.500	$p = 0.005, q = 0.005$	---
	M8	-5916.287	12.152	0.651	$P_0 = 0.988 (P_1 = 0.012); p = 0.025, q = 0.030, \omega = 16.268$	11F(**), 12K(**), 410E(*)

^a Numbers in bold indicate significantly difference between the compared models (M1a

vs. M2a and M7 vs. M8).

^b This is the average Ka/Ks ratio across all the sites.

^c p and q are shape parameters in the beta distribution. ω_0 is the ratio of Ka/Ks sites with a proportion of P_0 . ω_1 is the Ka/Ks of completely neutral sites with a proportion of P_1 . ω_2 is the Ka/Ks of positive selection sites with a proportion of P_2 .

^d Probability of amino acid site under positive selection predicted by Bayes Empirical Bayes (BEB) analysis. *, $\Pr(\omega > 1) > 90\%$; **, $\Pr(\omega > 1) > 95\%$; ***, $\Pr(\omega > 1) > 99\%$.

Significance: Rapid globalization in recent decades has greatly facilitated species introduction around the world. Successfully established introduced species, so-called invasive species, have threatened the invaded ecosystems. There has been substantial interest in studying how invasive species respond to extreme environmental conditions because the results can help not only predict their range of expansion and manage their impact, but also may reveal the adaptive mechanisms underlying their invasiveness. Our study has adopted a comparative approach to study the differential physiological and proteomic responses of two congeneric snails to various hypoxic conditions, as well as codon substitution analysis at transcriptomic level to detect signals of positive selection in hypoxia-responsive genes. The integrated physiological, proteomic and transcriptomic approach can be applied in other non-model species to understand the molecular mechanisms of adaptation to global environmental change.

Graphical abstract

Highlights

- Two species of *Pomacea* snails have differential susceptibility to hypoxia
- Proteomics was used to study the genetic bases of the differential resistance to hypoxia
- Significant changes occurred in signal transduction, glycolysis and TCA cycle
- Five genes encoding differentially expressed proteins are positively selected