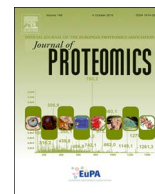




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Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

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

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ARTICLE INFO

Keywords:

Stress response
Invasive species
Pomacea
Proteomics
Epigenetics

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.

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.

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 [11] showed that 86% of the invasive species

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<https://doi.org/10.1016/j.jprot.2017.11.002>

Received 23 June 2017; Received in revised form 21 October 2017; Accepted 1 November 2017
1874-3919/ © 2017 Published by Elsevier B.V.

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 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

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