

## Article

# Limited proteomic response in the marine snail *Melarhappe neritoides* after long-term emersion

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## Abstract

Rocky intertidal organisms are commonly exposed to environmental gradients, promoting adaptations to these conditions. Emersion time varies along the intertidal range and in the supralittoral zone is frequently larger than a single tidal cycle, even lasting for weeks. The planktonic-dispersing gastropod *Melarhappe neritoides* is a common species of the high shore, adapted to reduce water loss in order to survive during long-term emersion. In this study, we investigated the molecular response, at the proteome level, of *M. neritoides* collected in high-shore tide pools to a series of emersion periods, from 8 to 24 days, in laboratory conditions. We compared this response to individuals maintained submerged during this period, because this was their original habitat. We also included a reversion treatment in the study, in which emersed individuals were returned to the submerged conditions. Although we detected an increase in overall protein concentration with longer emersion periods, contrary to general expectation, the two dimensional electrophoresis (2DE)-based proteomic analysis did not show significant differences between the treatments at the level of individual protein spots, even after an emersion period of 24 days. Our results suggest that the metabolism remains unaltered independent of the treatment carried out or the changes are very subtle and therefore difficult to detect with our experimental design. We conclude that *M. neritoides* could be equally adapted to emersion and submersion without drastic physiological changes.

**Key words:** adaptation, 2DE proteomics, environmental gradient, intertidal zone, metabolic rate depression.

Intertidal species from rocky shores are exposed to striking environmental gradients, some of these changes are predictable because of the periodicity associated with the tides while others are more variable (Raffaelli and Hawkins 1996; Hochachka and Lutz 2001; Somero 2004; Alpert 2005). These organisms often develop different adaptations to those conditions that represent an important metabolic challenge for them (Sokolova and Pörtner 2001; Somero 2002, 2004). Littorinid snails are a particularly interesting group because they include taxa adapted to several environmental conditions and the physiological basis of some of these adaptations has been studied from different angles (Adey and Hayek 2005, reviewed in Rolán-Alvarez et al. 2015).

Adaptation to emersion is an important feature of many intertidal species, including littorinids, as they are exposed to this condition twice a day during low tide, and sometimes for days or weeks if they inhabit the supralittoral zone (McMahon 1988). At the molecular level an organism needs to balance its ATP consumption and production rates during emersion (Storey and Storey 2004). A mechanism involved in this response in a wide range of organisms, including marine invertebrates, is a strong metabolic rate depression (MRD), controlled by reversible protein phosphorylation and changes in ribosomal aggregation state (Larade and Storey 2002; Storey and Storey 2004). Therefore, regulation through protein

phosphorylation can affect the activity of a wide range of metabolic enzymes involved in ATP production (Larade and Storey 2002; Storey and Storey 2004). Despite this, there have not been many attempts to characterize the molecular response to emersion by two-dimensional electrophoresis (2DE)-based proteome analysis (e.g., Fields et al. 2014), a methodology that allows resolving proteins with differences in post-translational modifications (Rabilloud and Lelong 2011). For example, differences in protein phosphorylation can be detected by this technique because it causes a change in the isoelectric point, shifting the relative position of the protein spot in the 2DE gels. On the other hand, it has been generally recognized that intertidal organisms, especially those that inhabit the supralittoral zone, are able to survive long periods of emersion by activating different anaerobic pathways (Sokolova and Pörtner 2001; Larade and Storey 2009; Storey et al. 2013), while an aerobic metabolism is prevalent in the lower shore. Those changes should also be reflected at the proteome level (Diz et al. 2012), there are many 2DE-based proteomic studies that have recurrently reported the identification of a wide range of enzymes involved in different metabolic pathways (e.g., Wulff et al. 2008; Diz et al. 2012a, 2012b; Rocher et al. 2015).

*Melarhaphe neritoides* (L.) is a small littorinid gastropod (~3–5 mm), highly abundant and widely distributed, from the NE Atlantic shores to the Mediterranean (Fretter and Graham 1980). It is a dioecious species and has a planktonic larval phase of 4–8 weeks (Johannesson 1992; Cronin et al. 2000). The long distance dispersal by the larvae has been associated with the absence of genetic structure at a geographical scale using allozymes (Johannesson 1992), although this has not been properly evaluated with other molecular markers. *Melarhaphe neritoides* is considered a high-shore species although it is found across the entire rocky intertidal range (McGrath 1997). The settlement of the larvae occurs in the lower shore, then the juveniles graze on the microalgae growing on the rock and when they reach a certain size the snails start migrating upshore (Fretter and Manley 1977). It also has been reported that the settlement can occur, at lower frequency, in tide pools from the upper shore and these recruits will be part of the high-shore subpopulations (Fretter and Graham 1980; McGrath 1997). At this intertidal level, *M. neritoides* is commonly sheltered in crevices to avoid desiccation (Fretter and Manley 1977; Raffaelli and Hughes 1978; Britton 1995) and can be exposed to emersion for weeks (Britton 1992); it is proven to survive this stress for at least 5 months (Lysaght 1941). Lower shore and high-shore subpopulations from the same locality have been found to be morphologically different (shell size and shape) although they lacked genetic differentiation based on AFLPs (amplified fragment length polymorphisms) (Cuña et al. 2011). In contrast, these morphologically different subpopulations (morphs) showed significant differences at the proteome level (22%) when exposed to a common laboratory environment, suggesting a certain level of phenotypic plasticity in this species (García et al. 2013). Therefore, this species represents an interesting system to study the proteomic changes induced by emersion which could be involved in a physiologically adaptative strategy to this environmental condition.

In this study, we exposed *Melarhaphe neritoides* individuals dwelling in tide pools from the upper intertidal zone to long-term emersion in the laboratory and then analyzed the proteome changes observed in comparison with individuals that were submerged in seawater for the same amount of time. If the emersion condition represents a stressful environment for *M. neritoides*, promoting an adaptive response, then it is very likely that this change would be

observed at the proteome level and detected by 2DE-based proteomic analysis. Here we evaluate this response at the proteome level and discuss its ecological and evolutionary implications.

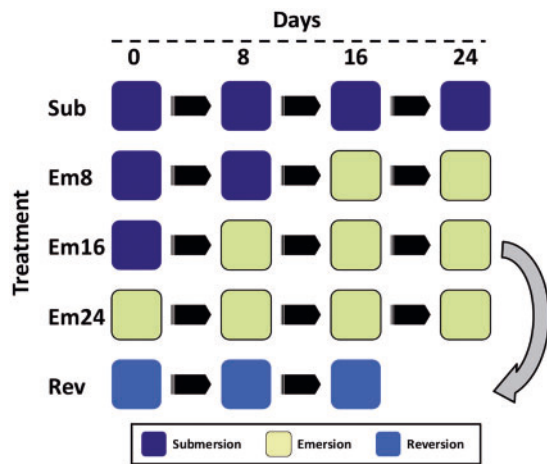
## Material and Methods

### Emersion treatments

Samples of *Melarhaphe neritoides* were collected in April 2013 at the exposed rocky shore of Silleiro in Galicia (42°6'45"N, 8°53'58"W; NW Spain). Approximately 500 adult individuals of the high-shore morph (3–5 mm shell height) were randomly collected within tide pools from a 3-m<sup>2</sup> area at the limit of the barnacle *Chthamalus stellatus* fringe (upper intertidal zone). The snails were kept in seawater and transported to the marine laboratory facilities at ECIMAT (Estación de Ciencias Mariñas de Toralla, Vigo, Spain). In the laboratory, groups of 30 random similarly sized individuals were placed in bags created with 12 × 12 cm squares of 1-mm nylon mesh. Then, each bag was introduced in a glass aquarium (18 × 10 × 6 cm) with an open sea water system (14.2 ± 0.8 °C) and kept submerged continuously for 30 days of acclimatization to laboratory conditions prior to the experimental treatments. The photoperiod was set to a 14/10-h light/dark cycle and the snails were able to graze on the algae growing on the nylon mesh during this time. Note that specimens of *M. neritoides* have been maintained alive (and showing shell growth) in similar conditions for 9 (Cuña et al. 2011) and 15 months (García et al. 2013), respectively.

In order to study the response at the proteome level induced by emersion we exposed the individuals to this environment for different periods (8, 16, 24 days), all after the 30-day acclimatization period in seawater. We chose these lengths in days for the treatments because we know that this species had a quick recovery, actively crawling, after 11 days of exposure to emersion (Britton 1995) and also could survive for months in these conditions (Lysaght 1941). In this experiment, we performed 5 different treatments and 3 biological replicates per treatment (Figure 1) as follows: 1) "Submersion" (Sub) treatment, the snails were kept submerged in seawater for 24 days, 2) "Emersion 8 days" (Em8) treatment, the snails were kept submerged for 16 days and subjected to emersion for 8 days, 3) "Emersion 16 days" (Em16) treatment, submerged for 8 days and 16 days of emersion, 4) "Emersion 24 days" (Em24) treatment, subjected to emersion for 24 days, 5) "Reversion" (Rev) treatment, the remaining snails from treatment Em16 were submerged again for 16 days, this included only 2 biological replicates. After each treatment all the snails were snap frozen in liquid nitrogen and kept at –80 °C until further proteomic analyses were performed. The individuals were not sexed (a not easy task in this species), and we assume that variation in the proportion of sexes could be an issue. However, a reasonably high number of individuals per pooled sample were used in this study, making unlikely that the differential sex distribution of individuals among replicates could mask any substantial effect of factors under study. Two technical replicates were also analyzed.

To subject the samples to emersion stress, the aquaria were emptied and the snails were dried with paper towels and then kept in the aquarium inside the original mesh bags and on top of a dry filter paper. The treatments were randomly assigned to the aquaria used during the experiment to avoid a possible effect of the location of the aquaria in our laboratory. All the samples were maintained under room temperature conditions (18–22 °C).



**Figure 1.** Schematic representation of the experimental design used in this study (see also section “Materials and Methods”). Two main experiments were carried out: 1) Emersion experiment (comparison between samples that were out of seawater during 8, 16, or 24 days and control samples, submerged in seawater during 24 days,) and 2) Reversion experiment (reversion treatment; 16 days of emersion + 16 days submerged in seawater). Three pooled samples (biological replicates; 20 individual each pool) were made for each treatment, excepting for the reversion experiment where only two pooled samples were made. The pooling process followed the same procedure in all cases. Note that in order to undertake the reversion experiment, samples were taken from the Em16 treatment group at day 24 followed by another 16 days submerged in seawater.

## 2DE proteomic analysis

Proteins were extracted from pools of 20 individuals that were made for each biological replicate and treatment. Pooling individual samples to make biological replicates helps to decrease the within-group variation, due to the higher natural interindividual variation, getting a higher statistical power to detect between-group changes (Diz et al. 2009). It also helps to avoid an underestimation of the number of proteins showing expression differences due to limited statistical power to detect gene expression differences with a low-mid effect size (Diz and Rolán-Alvarez 2014). A pooling strategy was successfully applied in previous littorinid studies to detect proteome variation (Martínez-Fernández et al. 2008; Martínez-Fernández et al. 2010; Diz et al. 2012b; García et al. 2013). We studied the behavior of snails in a subsample of the individuals ( $N=10$ ) for each treatment at the end of the experiment, by returning the snails to seawater and checking the percentage of those that for 1 h did not react (on average  $11.2\% \pm 6.51$ ). The different treatments, however, did not differ in frequency of reaction ( $G=4.83$ ;  $df=7$ ;  $P=0.6801$ ). These observations were used as a proxy for mortality rates or close to death. Shelled individuals were sonicated (Branson Digital Sonicator 250) in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol, and 1% carrier ampholytes [Bio Lyte 3-10 buffer, BioRad) using 10 blasts of 25% amplitude and 5 s each, with 10 s breaks, to maximize tissue disruption. Samples were centrifuged for 15 min at  $21,000 \times g$  ( $10^\circ\text{C}$ ), the pellet was discarded and the protein supernatant was kept at  $-80^\circ\text{C}$ . Protein concentration was measured using a modification of the Bradford method (Ramagli and Rodriguez 1985). Then,  $75 \mu\text{g}$  of total extracted protein was analyzed by 2DE gels in batches of 6 gels (biological replicates) per run, following a randomizing block design to control high run-to-run (technical) variation (see Diz and Skibinski 2007). The first

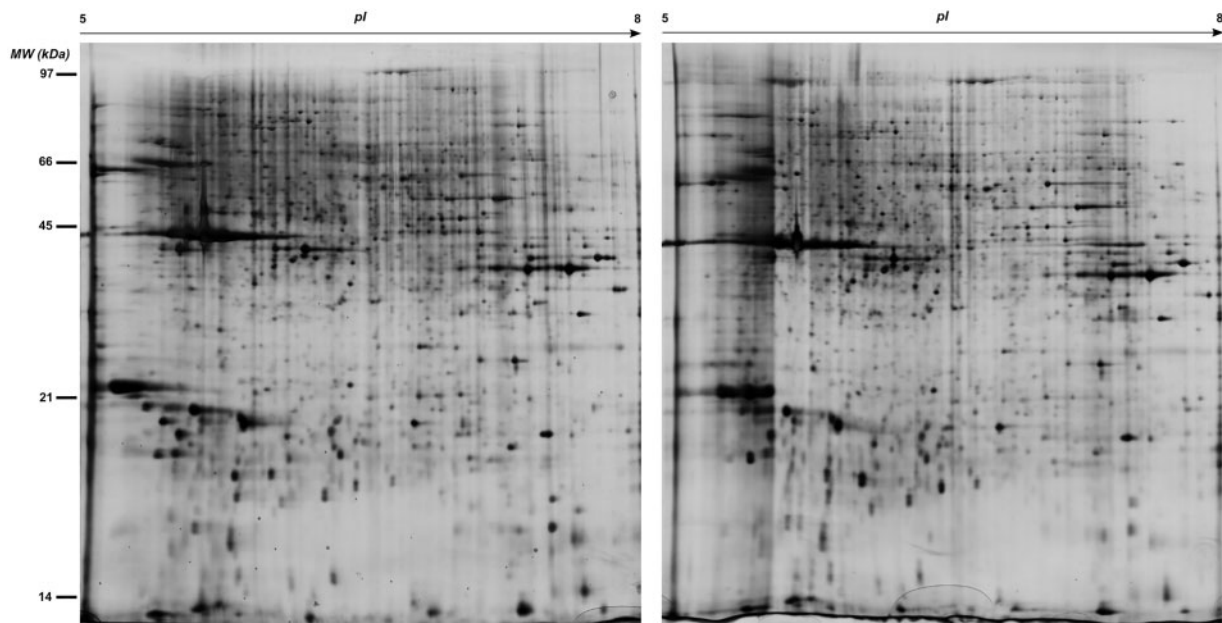
dimension of protein separation was carried out through IEF (iso-electric focusing) on immobilized pH gradient strips (pH 5–8/17 cm, BioRad) using a Protean IF System (BioRad) following manufacturer instructions to complete 60,000 volt-h per strip. An active strip-rehydration step, followed by 2 consecutive steps of strip equilibration ( $2 \times 15$  min) with dithiothreitol (10 mg/mL) and iodoacetamide (25 mg/mL) respectively, was included.

The second dimension of gel electrophoresis was carried out with labcast 12.5% polyacrylamide gels ( $22 \times 27 \times 0.1 \text{ cm}^3$ ; GE Healthcare) on an Ettan DALTsix electrophoresis unit (GE Healthcare) at  $20^\circ\text{C}$  (with an F12-MC refrigerated/heating circulator system; JULABO laborotechnik), and at a constant current of 15 W/gel for  $\sim 5$  h. Protein spots were visualized following a modified version of the silver staining method. Then, silver-stained 2DE gels were scanned with GS-800<sup>TM</sup> calibrated densitometer (BioRad) and analyzed with Progenesis SameSpots software version 4.1 (TotalLab Ltd) as described in García et al. (2013). The software calculates the absolute protein-spot volumes, which were normalized for each gel and then log-transformed in order to better fit normality and homoskedasticity (see Diz and Skibinski 2007). For each protein spot the log-transformed volume (dependent variable) was analyzed by one-way ANOVA for both emersion (fixed; Sub, Em8, Em16, and Em24) and reversion (fixed; Rev) treatments using a cutoff for significance of  $P < 0.05$ . We also applied a linear regression for the variable days of emersion against protein spot volume (dependent variable). In addition, we studied the total protein concentration of each sample as a new dependent variable to study one of the predictions of the MDR hypothesis (see introduction); that those individuals subjected to emersion should induce a general reduction of protein metabolism (*sensu* Storey and Storey 2004). SGoF ver.7.2 (Carvajal-Rodríguez et al. 2009) was used to perform SGoF (Sequential Goodness of Fit) and FDR (false discovery rate) multiple test corrections on the whole set of  $P$ -values obtained from the previous analyses. In order to gain statistical power, probabilities obtained from the different tests (emersion and reversion) above were combined following the Fisher test (see Sokal and Rohlf 1995, pp. 794–797). All statistical analyses were carried out using IBM SPSS Statistics v.22.0 software.

## Results

Bidimensional gels were initially checked for quality (Figure 2) and then technical variation was calculated in order to guarantee the support of further statistical inferences. One of the three biological replicates of the Em16 treatment was discarded because the 2D gel did not pass the quality filter, the gel presented high proportion of streaking areas and poorly defined spots, and therefore it was not included in the analyses. The coefficient of variation for normalized protein concentration across technical replicates ( $CV=22.3 \pm 4.0$ ) was comparable to that observed in similar studies (Diz et al. 2012b; García et al. 2013). Moreover, the correlation coefficient between technical replicates across normalized volumes was very high ( $r=0.950 \pm 0.026$ ).

We analyzed a total of 590 protein spots, the summary of the statistical analyses of the proteomic response to emersion and reversion treatments is presented in Table 1. Several protein spots differed significantly between the emersion treatments and the control (submersion), 11 spots with ANOVA and 28 spots with univariate regression (after analyzing each of the 590 spots separately). Between the reversion treatment and its corresponding emersion (Em16) treatment, 27 spots differed significantly (ANOVA). However, none



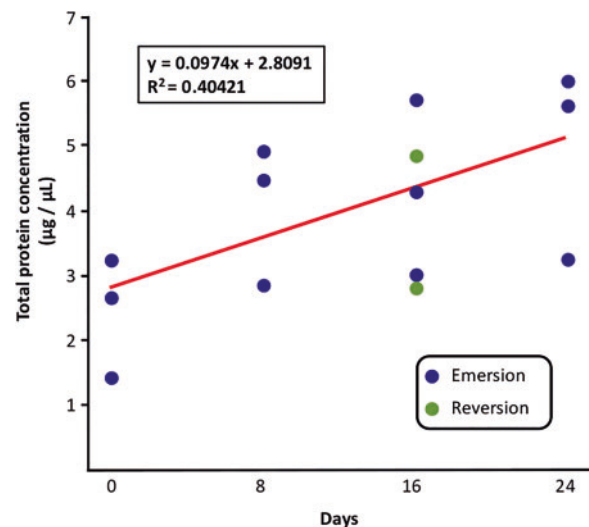
**Figure 2.** Two-dimensional electrophoresis gels showing the proteome of *M. neritoides*. A representative 2DE gel from a pooled sample (20 individuals per pool) for the submerged 24 days (Sub24) and emerged 8 days (Em8) treatments are showed on the left- and right-hand side, respectively. MW: molecular weight; pI: isoelectric point. Statistical information for analyses of these protein spots is given in Table 1.

**Table 1.** Summary of the results from the statistical tests using 590 protein spots from the 2DE analysis of *M. neritoides*

Test	Method	Gels	$P \leq 0.05$	FDR	SGoF
Sub versus Em	ANOVA	11	11	0	0
	Regression	11	28	0	0
Em16 versus Rev	ANOVA	4	27	0	0
Combined	ANOVA	15	19	0	0
	Regression	15	15	0	0

2DE analysis of *M. neritoides* was used for comparing submersion and emersion treatments (sub vs. em) and comparing emersion at 16 days with reversion treatments (Em16 vs. rev). In addition, we combined probabilities from both tests to identify possible spots that showed a significant result using ANOVA and regression analysis (combined). Gels represent the number of 2DE gels analyzed, equivalent to the number of pooled samples (20 snails per pool).  $P \leq 0.05$  represents the number of protein spots that showed significant differences in the expression values without multiple test correction. Multiple test correction was carried out using FDR and SGoF, none of the protein spots was significant after the corrections.

of these spots remained significant after multiple test correction (SGoF and FDR). Therefore, we cannot rule out the possibility that they could represent false positives and this degree of protein differentiation could be caused by random effects. We then combined the probabilities that were previously obtained in these two comparisons (emersion and reversion), but again none of the tests remained significant after multiple test adjustment (Table 1). However, contrary to the general expectation of the MRD hypothesis, when total protein concentration for the different treatments was analyzed we detected a significant positive relationship between total protein concentration and emersion time, excluding the reversion (Rev) treatment from the analysis ( $F_{\text{regression}} = 6.8$ ;  $df_1 = 1$ ;  $df_2 = 10$ ;  $P = 0.0263$ ; see Figure 3).



**Figure 3.** Total protein concentration ( $\mu\text{g}/\mu\text{L}$ ) for each pool of individuals measured using a modification of Bradford method. Each point represents a biological replicate for a specific treatment; the result from the linear regression analysis is shown. Sub: submersion; Em8, Em16, and Em24 (8, 16, and 24 days of emersion, respectively); Rev (16 days of emersion and a recovering period of 16 days submerged in seawater).

## Discussion

The intertidal rocky shore is a highly heterogeneous ecosystem and exposure to emersion is one of the environmental variables that changes across the intertidal range, being more extreme at the supralittoral fringe. Eulittoral organisms are exposed to emersion for hours whereas supralittoral organisms could be exposed for days or weeks, depending on the spring tides, waves from storms, and spray.

Adaptation to these conditions is important because it determines the zonation distribution of the different species, although temperature, competition, and predation are other important variables. A general metabolic switch from aerobic to anaerobic metabolism has been claimed to occur in several marine organisms affected by anoxia, among other environmental stressors (Storey and Storey 2004), and this is common in the supralittoral zone. These changes typically include a MRD effect, the up and down regulation of several key gene products and the accumulation of different side-products from catabolism and glycolysis (Larade and Storey 2009; Storey et al. 2013). The marine snail *M. neritoides* is characteristic of the supralittoral fringe, it is found at the highest limit of the littoral zone, where other littorinids are not found (Chappuis et al. 2014), therefore, it is exposed to long periods of emersion and high temperatures.

In our aim to determine, at the proteome level, the effects of differential exposure to emersion in *M. neritoides* (from continuous submersion to 24 days of emersion) we conducted a manipulative experiment in laboratory conditions. The 2DE proteomic analysis did not find any significant protein spot differences between the "Submersion" and the "Emersion" or "Reversion" treatments. The environmental differences between these treatments are clear, for example, 1 group of individuals were kept in seawater for 54 days (including 30 days of acclimatization) and another were submerged for 30 days and then exposed to the air (emersion) for 24 days, but these treatments did not show statistical differences at the proteome level, which is very striking. Here we discuss different hypotheses that could explain this unexpected result. The first cause to take into account is related to the methodology and the experimental design. We know from a previous study in *M. neritoides* (García et al. 2013) that 2DE proteomic analysis is able to detect significant differences (22% of the spots analyzed) between two divergent morphs inhabiting upper and lower shore intertidal levels when using the same pooling approach, number of sample replicates, and analyzing a similar number of protein spots (532, García et al., 2013; 590, this study), our technical error was also similar to their error values. Moreover, the 2DE gel images from this study (see Figure 2) show a general protein spot pattern similar to that obtained in the previous *M. neritoides* 2DE-based study (García et al. 2013). Furthermore, we did not find an accumulation of protein spots at the low molecular weight region of the 2DE gels, thus we assume that protein degradation during sample preservation or protein extraction procedure was negligible. Therefore, we should have enough statistical power in our analyses and the 2DE methodology should be adequate for our initial goal of detecting significant protein changes, assuming high effect sizes also in the current experiment. On the other hand, the experimental design could have a series of limitations, for example, we did not include different sampling sites or subpopulations along the intertidal range like García et al. (2013). We only studied individuals found in high-shore tide pools because these would be exposed to long-term submersion (tide pools) and emersion (high-shore crevices) throughout their lives, which are the conditions that we tested in this experiment. We assume that studying other subpopulations is also important, mostly because certain individuals would never be exposed to long-term emersion and therefore learning about their proteomic response to this condition would be interesting, but this was not under the scope of the current study. We also decided to study a single locality because the intertidal zone is so heterogeneous that it would be difficult to replicate exactly the same environment in 2 or more localities and this would add noise to our proteomic analysis. For example, García et al. (2013) found

16% of protein spots significantly differentiated between *M. neritoides* morphs in the same locality of this study, while only 6% varied in a second locality. Since we did not detect significant differences between the treatments in this study, more sampled localities added to this study would probably not improve our design. It is evident that this study would be improved with more biological replicates to account for the possible technical or biological variability or with more treatments (e.g., shorter or longer emersion periods, shorter submersion periods). However, this fact does not explain why using the current experimental design with 3 biological replicates and pooled samples, similar to that followed in García et al. (2013), a design that was proven to have enough statistical power to detect significant differences between subpopulations of this species, was not able to detect any proteome differences in this new experiment that tested the effect of such notably different environmental conditions (emersion and submersion in seawater).

Previous studies in other littorinids found up to 10% of transcriptomic (MRD) reduction and up to 50% of protein synthesis reduction under anoxia (Sokolova and Pörtner 2001; Larade and Storey 2002, 2009; Storey and Storey 2004; Storey et al. 2013). However, intertidal upper shore subpopulations of *Littorina saxatilis* presented a rather limited increment of the anaerobic metabolism (1%–2%), a moderate MRD effect, and an enhanced ability for water conservation and storage of fermentable substrates (Sokolova and Pörtner 2001; Sokolova and Pörtner 2003). In our study, the lack of significant protein spot differences between any of the treatments carried out suggests the lack of important metabolic changes (e.g., affecting one or several pathways), regulation through widespread protein phosphorylation or an MRD effect. This may perhaps suggest that *M. neritoides* does not suffer from anoxia during long-term emersion like in the current experiment. It is common that *M. neritoides* can be emersed for the period between 2 successive spring tides (several days), sometimes even longer (weeks). *Melarhaphé neritoides* is able to reduce evaporative water loss during long-term emersion conditions (Britton 1995). This characteristic would allow the individuals to retain water during this period of emersion, also taking into account that high temperatures were not applied to the snails because that would be a new variable in the experiment and could represent a different stress condition. Therefore, a small amount of water could be enough to maintain the proteome unaltered although it is reasonable to think that even with that amount of water the condition is very different from the submerged individuals. The present results suggest that *M. neritoides* is capable of resisting emersion for up to 24 days without significant changes at the proteome level, in comparison to submersion. If this is the case, it means that emersion should not represent a stress for this species and therefore the metabolism is the same under both environmental conditions. Alternatively, as we mentioned before, the changes at the proteome level could be so subtle that they were not statistically detected in our experiment.

On the other hand, we detected a significant positive trend in the total amount of proteins existing in the homogenates of individuals subjected to progressively longer emersion times, while the "Reversion" treatment showed a slight, but not statistically significant, decrease in concentration (Figure 3), opposite to the MRD hypothesis expectation. One possibility to explain the differences in protein concentration is the reduction of tissue hydration between treatments and the consequent increase in protein concentration. Even though *M. neritoides* is able to largely reduce the amount of evaporative water loss, some water loss does still inevitably occur during emersion. It is common to find in other littorinids an evaporative

water loss proportional to the emersion time (e.g., Britton 1995). This hypothesis would not necessarily imply changes in metabolism. Alternatively, it could be due to a state of hypometabolism during emersion where protein degradation rates (proteolysis) are substantially lowered compared to submersion conditions to compensate the lower protein synthesis rate (Storey and Storey 2004). In other words, both degradation and synthesis may decrease during emersion, but if degradation rates decrease to a greater extent, then protein concentration will increase, explaining the observed result.

In summary, we did not find evidence of proteomic changes in *M. neritoides* between submersion and emersion conditions. There was an overall increase of protein concentration in the tissue homogenates of samples from the emersion treatment which may perhaps suggest a strategy of acclimatization to emersion, the ability to resist certain level of dehydration. Two parameters of the design that could be changed in future experiments would be to analyze samples coming directly from the shore (without acclimatization) and also to test shorter periods of submersion and emersion. It is possible that if these treatments were too long they could have activated a similar response against this stress condition, showing nonsignificant differences between the treatments. Although this hypothesis seems unlikely because the treatments were very different. *Melarhappe neritoides* is a marine species which has evolved several adaptations to survive under long and extreme periods of desiccation, perhaps using different physiological adaptations to deal with extreme changes in their environmental conditions (submerged and emerged) without substantial changes in the proteome.

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## Author contributions

APD, ER-A, and JG designed the study. JG and MR-A collected the samples and carried out the experimental treatments. APD, MA-R, and MRR carried out the 2DE proteomic analysis. APD and ER-A carried out the statistical analysis. APD, ER-A, and JG wrote the manuscript.

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## References

Adey WH, Hayek LC, 2005. The biogeographic structure of the western North Atlantic rocky intertidal. *Cryptogamie Algol* 26:35–66.  
 Alpert P, 2005. The limits and frontiers of desiccation-tolerant life. *Integr Comp Biol* 45:685–695.  
 Britton JC, 1992. Evaporative water loss, behavior during emersion, and upper thermal tolerance limits in seven species of eulittoral fringe Littorinidae (Mollusca: Gastropoda) from Jamaica. In: Grahame J, Mill PJ, Reid DG eds. *Proceedings of the 3rd International Symposium in Littorinid Biology*. London: The Malacological Society of London, 69–83.

Britton JC, 1995. The relationship between position on shore and shell ornamentation in two size-dependent morphotypes of *Littorina striata*, with an estimate of evaporative water loss in these morphotypes and in *Melarhappe neritoides*. *Hydrobiologia* 309:129–142.  
 Carvajal-Rodríguez A, de Uña-Alvarez J, Rolán-Alvarez E, 2009. A new multitest correction (SGoF) that increases its statistical power when increasing the number of tests. *BMC Bioinformatics* 10:1–14.  
 Chappuis E, Terradas M, Cefali ME, Mariani S, Ballesteros E, 2014. Vertical zonation is the main distribution pattern of littoral assemblages on rocky shores at a regional scale. *Estuar Coast Shelf Sci* 147:113–122.  
 Cronin MA, Myers AA, O'riordan RM, 2000. The reproductive cycle of the intertidal gastropod *Melarhappe neritoides* on the West and South coasts of Ireland. *Biol Environ: Proc R Ir Acad* 100:97–106.  
 Cuña V, Saura M, Quesada H, Rolán-Alvarez E, 2011. Extensive microgeographical shell polymorphism in a planktotrophic marine intertidal snail. *Mar Ecol Prog Ser* 427:133–143.  
 Diz AP, Dudley E, Skibinski DOF, 2012. Identification and characterization of highly expressed proteins in sperm cells of the marine mussel *Mytilus edulis*. *Proteomics* 12:1949–1956.  
 Diz AP, Martínez-Fernández M, Rolán-Alvarez E, 2012a. Proteomics in evolutionary ecology: linking the genotype with the phenotype. *Mol Ecol* 21:1060–1080.  
 Diz AP, Páez de la Cadena M, Rolán-Alvarez E, 2012b. Proteomic evidence of a paedomorphic evolutionary process within a marine snail species: a strategy for adapting to extreme ecological conditions? *J Evol Biol* 25:2569–2581.  
 Diz AP, Rolán-Alvarez E, 2014. High proteome variation between ecotypes of *Littorina saxatilis* cannot be explained by tissue heterogeneity or a common-garden x ecotype effect. *J Exp Mar Biol Ecol* 451:69–73.  
 Diz AP, Skibinski DOF, 2007. Evolution of 2-DE protein patterns in a mussel hybrid zone. *Proteomics* 7:2111–2120.  
 Diz AP, Truebano M, Skibinski DOF, 2009. The consequences of sample pooling in proteomics: An empirical study. *Electrophoresis* 30:2967–2975.  
 Fields PA, Eurich C, Gao WL, Cela B, 2014. Changes in protein expression in the salt marsh mussel *Geukensia demissa*: evidence for a shift from anaerobic to aerobic metabolism during prolonged aerial exposure. *J Exp Biol* 217:1601–1612.  
 Fretter V, Graham A, 1980. The prosobranch molluscs of Britain and Denmark. Part 5-Marine Littorinacea. *J Mollus Stud* 7:243–284.  
 Fretter V, Manley R, 1977. The settlement and early benthic life of *Littorina neritoides* (L.) at Wembury, S. Devon. *J Mollus Stud* 43:255–262.  
 García SD, Diz AP, Sá-Pinto A, Rolán-Alvarez E, 2013. Proteomic and morphological divergence in micro-allopatric morphotypes of *Melarhappe neritoides* in the absence of genetic differentiation. *Mar Ecol Prog Ser* 475:145–153.  
 Hochachka PW, Lutz PL, 2001. Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp Biochem Physiol B: Biochem Mol Biol* 130:435–459.  
 Johannesson K, 1992. Genetic variability and large scale differentiation in two species of littorinid gastropods with planktotrophic development *Littorina littorea* (L.) and *Melarhappe (Littorina) neritoides* (L.) (Prosobranchia: Littorinacea), with notes on a mass occurrence of *M. neritoides* in Sweden. *Biol J Linn Soc* 47:285–299.  
 Larade K, Storey KB, 2009. Living without oxygen: anoxia-responsive gene expression and regulation. *Curr Genomics* 10: 76–85.  
 Larade K, Storey KB, 2002. Reversible suppression of protein synthesis in concert with polysome disaggregation during anoxia exposure in *Littorina littorea*. *Mol Cell Biochem* 232:121–127.  
 Lysaght A, 1941. The biology and trematode parasites of the gastropod *Littorina neritoides* (L.) on the Plymouth Breakwater. *J Mar Biol Assoc UK* 25:41–67.  
 Martínez-Fernández M, Páez de la Cadena M, Rolán-Alvarez E, 2010. The role of phenotypic plasticity on the proteome differences between two sympatric marine snail ecotypes adapted to distinct micro-habitats. *BMC Evol Biol* 10:65.  
 Martínez-Fernández M, Rodríguez-Piñeiro AM, Oliveira E, Páez de la Cadena M et al., 2008. Proteomic comparison between two marine snail ecotypes

- reveals details about the biochemistry of adaptation. *J Proteome Res* 7:4926–4934.
- McGrath D, 1997. Population structure and recruitment of the intertidal gastropod *Melarhaphé neritoides* (L.) on an exposed rocky shore on the South coast of Ireland. *Biol Environ: Proc R Ir Acad* 97B:75–79.
- McMahon RF, 1988. Respiratory response to periodic emergence in intertidal molluscs. *Am Zool* 28:97–114.
- Rabilloud T, Lelong C, 2011. Two-dimensional gel electrophoresis in proteomics: a tutorial. *J Proteomics* 74:1829–1841.
- Raffaelli D, Hawkins S, 1996. *Intertidal Ecology*. London: Chapman & Hall.
- Raffaelli DG, Hughes RN, 1978. The effects of crevice size and availability on populations of *Littorina rudis* and *Littorina neritoides*. *J Anim Ecol* 47:71–83.
- Ramagli LS, Rodriguez LV, 1985. Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6:559–563.
- Rocher B, Bultelle F, Chan P, Foll F, Letendre J et al., 2015. 2-DE Mapping of the blue mussel gill proteome: the usual suspects revisited. *Proteomes* 3:3–41.
- Rolán-Alvarez E, Austin CJ, Boulding EG, 2015. The contribution of the genus *Littorina* to the field of evolutionary ecology. *Oceanogr Mar Biol Annu Rev* 53:157–214.
- Sokal RR, Rohlf FJ, 1995. *Biometry: The Principles and Practice of Statistics in Biological Research*. New York: W.H. Freeman.
- Sokolova IM, Pörtner H-O, 2003. Metabolic plasticity and critical temperatures for aerobic scope in a eurythermal marine invertebrate (*Littorina saxatilis*, Gastropoda: Littorinidae) from different latitudes. *J Exp Biol* 206:195–207.
- Sokolova IM, Pörtner H-O, 2001. Physiological adaptations to high intertidal life involve improved water conservation abilities and metabolic rate depression in *Littorina saxatilis*. *Mar Ecol Prog Ser* 224:171–186.
- Somero GN, 2004. Adaptation of enzymes to temperature: searching for basic "strategies". *Comp Biochem Physiol B: Biochem Mol Biol* 139:321–333.
- Somero GN, 2002. Thermal physiology and vertical zonation of intertidal animals: optima, limits, and costs of living. *Integr Comp Biol* 42:780–789.
- Storey KB, Lant B, Anozie OO, Storey JM, 2013. Metabolic mechanisms for anoxia tolerance and freezing survival in the intertidal gastropod, *Littorina littorea*. *Comp Biochem Physiol A: Mol Integr Physiol* 165:448–459.
- Storey KB, Storey JM, 2004. Metabolic rate depression in animals: transcriptional and translational controls. *Biol Rev* 79:207–233.
- Wulff T, Jessen F, Roepstorff P, Hoffmann EK, 2008. Long-term anoxia in rainbow trout investigated by 2-DE and MS/MS. *Proteomics* 8:1009–1018.