

Article

Proteomic analysis of F₁ hybrids and intermediate variants in a *Littorina saxatilis* hybrid zone

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Abstract

Proteomic analysis was carried out on the Crab (upper-shore) and Wave (lower-shore) ecotypes of *Littorina saxatilis* from a hybrid zone at Silleiro Cape, Spain. Proteome profiles of individual snails were obtained. Protein expression in F₁ hybrid snails bred in the laboratory and snails with intermediate shell phenotypes collected from the mid-shore were compared with Crab and Wave ecotypes using analytical approaches used to study dominance. Multivariate analysis over many protein spots showed that the F₁ snails are distinct from both ecotypes but closer to the Wave ecotype. The intermediate snails are highly variable, some closer to the Crab and others to the Wave ecotype. Considered on a protein by protein basis, some proteins are significantly closer in expression to the Crab and others to the Wave ecotype for both F₁ and intermediate snails. Furthermore, a significant majority of proteins were closer in expression to the Wave ecotype for the F₁, consistent with the multivariate analysis. No such significant majority toward either the Crab or Wave ecotype was observed for the intermediate snails. The closer similarity of F₁ and Wave ecotype expression patterns could be the result of similar selective pressures in the similar mid-shore and low-shore environments. For a significantly larger number of proteins, intermediate snails were closer in expression to the ecotype having the lower expression, for both Crab and Wave ecotypes. This is somewhat unexpected as lower expression might be expected to be an indication of impairment of function and lower fitness. Proteomic analysis could be important for the identification of candidate proteins useful for gaining improved understanding of adaptation and barriers to gene flow in hybrid zones.

Key words: F₁ hybrids, gene expression, marine invertebrates, molecular phenotype, reproductive isolation, speciation.

The study of hybridization and hybrid zones is important for understanding the causes of speciation (Barton and Hewitt 1985; Harrison 1990, 1993; Gompert et al. 2017). Different hybrid zones between ecologically divergent ecotypes of the marine intertidal gastropod *Littorina saxatilis* have been well studied across three regions

from North East Atlantic coasts (Sweden, Britain and Spain) (reviewed in Galindo and Grahame 2014; Rolán-Alvarez et al. 2015).

Here we study the *L. saxatilis* hybrid zone from Galicia (North West Spain), where a ridged and banded ecotype is thought to have evolved to resist crab predation in the upper-shore (Crab ecotype)

due to its large and robust shell with a small aperture, and a smooth and unbanded ecotype has evolved to resist wave action in the lower-shore (Wave ecotype) due to its small and thin shell with a large aperture (Johannesson et al. 1993; Rolán-Alvarez et al. 1997; Butlin et al. 2014). They meet and hybridize at the mid-shore (reviewed in Rolán-Alvarez 2007) and have been described as an example of *in situ* divergence due to ecologically based divergent selection existing at these different microhabitats (crab predation and wave action) in the face of gene flow (Rolán-Alvarez et al. 2004; Quesada et al. 2007; Galindo et al. 2009). They show partial reproductive barriers due to divergent natural selection and positive size-assortative mate choice (Johannesson et al. 2010; Rolán-Alvarez et al. 2015; Boulding et al. 2017) and can be crossed successfully in the laboratory to produce F₁ hybrids (Saura et al. 2011). In the field, at the mid-shore, it is possible to observe a small proportion of mating pairs between ecotypes and also low frequencies of intermediate snails with mixed shell characters (ridged-unbanded, smooth-banded; referred to as intermediates hereafter) (reviewed in Rolán-Alvarez 2007). These intermediates show viabilities, fertilities, and sexual selection on phenotypic traits, which are the average of those of the pure ecotypes (Rolán-Alvarez et al. 1997; Johannesson et al. 2000; Cruz et al. 2001). Use of genome-wide Amplified Fragment Length Polymorphism (AFLP) markers in this hybrid zone in Galicia showed that these phenotypic intermediates are not necessarily the offspring (F₁) of a heterospecific cross between the pure ecotypes, but certain snails showed introgression between the parental ecotypes. This pattern was highly dependent on environmental characteristics of the locality studied (Galindo et al. 2013). Moreover, selection on shell morphology of these intermediates was variable even at the scale of micro-habitat within locality (Galindo et al. 2014). A more recent study using Single Nucleotide Polymorphisms (SNPs) derived from double digest Restriction-site Associated DNA (ddRAD) markers detected a very low proportion of introgressed individuals (1.8%) within the studied intermediates (Kess et al. 2018). The genetic constitution and ancestry of these intermediate snails between the Crab and Wave ecotypes is thus not fully understood in the Galician hybrid zone of *L. saxatilis*. In this study, we use proteomics to study expression in these intermediate snails compared with the two ecotypes as a complementary approach to previous genetic analyses (Galindo et al. 2013; Kess et al. 2018).

Proteomic analysis has been performed so far only on the parental ecotypes (Crab and Wave) (Martínez-Fernández et al. 2008, 2010; Diz et al. 2012a; Diz and Rolán-Alvarez 2014). Those studies provide evidence that up to 30% of proteins show significant differences in expression between ecotypes which persist from embryonic through to adult stages. This study is novel in analyzing the proteome of F₁ snails bred in the laboratory for comparison with the proteome of the intermediate snails collected on the shore and in determining the proteome of individual snails, the earlier studies having used protein samples pooled from more than one individual. We also test the hypothesis of sex difference in protein expression and its interaction with ecotype.

The study of hybrid trait values in relation to parental values in divergently selected traits is key to understanding speciation mechanisms (Thompson et al. 2020). An analytical approach to this is that used to quantify dominance and is applied here to the intermediate snails as well as the laboratory-bred F₁ snails. In a review of nearly 200 studies of F₁ hybrids and their parent populations, specific trends in relation to dominance in divergently selected traits have been noted in diverse species (Thompson et al. 2020). Hybrids usually resemble one parent more closely than the other, the direction

of resemblance differs between traits, and dominance of higher and lower trait values is equally likely. Here we consider these three trends as hypotheses to test in the *L. saxatilis* Galician hybrid zone treating each of the many proteins in a proteomics dataset as a separate trait. We discuss the proteomic nature of the intermediate snails and the relevance of these results to our understanding of speciation and adaptation in *Littorina*.

Materials and Methods

Sampling and laboratory acclimation

Samples were collected in July 2010 across the *L. saxatilis* hybrid zone at Silleiro Cape (42°06'16.7"N, 8°53'56.3"W; NW Spain). Snails were randomly collected from each of the typical parental microhabitats, the barnacle belt in the upper-shore (Crab ecotype: large, banded, and ridged shell—also referred to as RB ecotype in previous studies using populations from NW Spain) and from the mussel belt in the lower-shore (Wave ecotype: small, unbanded, and smooth shell—referred also as SU ecotype in previous studies using populations from NW Spain). Phenotypically, intermediate snails (called intermediates hereafter) were collected in the mid-shore, a patchy environment (barnacles and mussels). Their phenotypes include smooth shells with dark bands, ridged and uncolored shells (unbanded), and ridged with two or more incomplete bands (following Johannesson et al. 1993; Cruz et al. 2001).

After the sampling, all the snails were brought alive to the marine laboratory (Toralla Marine Science Station - ECIMAT, University of Vigo) in order to keep them in controlled laboratory conditions. The snails were randomly assigned to different aquaria where individual phenotypes remained clearly identifiable. Further details on the culture system are given in Saura et al. (2011). After 1 week, the individuals were flash frozen in liquid nitrogen and kept at −80 °C until total protein extraction.

Rearing F₁ hybrids in the laboratory

We also studied three F₁ hybrids that were reared in the marine laboratory (Toralla Marine Science Station - ECIMAT, University of Vigo) for 6 months. *Littorina saxatilis* is a dioic species with internal fertilization, the female carries the developing embryos inside the shell (ovoviparity) until they are born as crawlaways (Reid 1996). These F₁ individuals are from three different broods resulting from several attempted laboratory crosses between a Crab ecotype female and a Wave ecotype male following Saura et al. (2011), both ecotypes also being collected at Silleiro Cape as were the intermediate individuals. The crosses were carried out using virgin juvenile females in order to avoid the possibility that the females had previously mated in the field, as *L. saxatilis* presents multiple paternity (Panova et al. 2010). The three F₁ hybrids were flash frozen in liquid nitrogen and kept at −80 °C until total protein extraction.

It is possible that any protein expression differences between the individuals collected from the shore and the laboratory-reared F₁ individuals could be attributed in part to environmental differences. The former were maintained under laboratory conditions for 1 week after collection, whereas the latter were reared for several months under laboratory conditions. However, this is an unlikely explanation for the results we report because we have previously shown that patterns of protein expression in these *L. saxatilis* ecotypes are only marginally affected by the environment where the snails live (Martínez-Fernández et al. 2010).

Proteomic analysis

The proteomic analysis of individual snails was carried out by 2-D electrophoresis (2-DE) following the procedure described in [García et al. \(2013\)](#) with slight modifications. Prior to total protein extraction, the shells were removed from the snails and the sex was determined. Whole snails without shells were submerged individually into 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate [CHAPS], 1% dithiothreitol [DTT], and 1% immobilized pH gradient [IPG]), the samples were sonicated (10 × 5 s pulses with 10 s breaks) using a Branson Digital Sonifier 250 (Branson, Brookfield, CT) and centrifuged for 20 min at 21,000 g (4 °C), then the supernatant containing the proteins was stored at −80 °C.

A total of 24 intermediate, 6 Crab ecotype, 6 Wave ecotype, and 3 laboratory-reared F₁ snails were analyzed individually by 2-DE. Equal numbers of males and females were analyzed within each category of the snails collected from Silleiro Cape. The same 452 protein spots were quantified in all of the individual snails. Representative images showing the distinct differences between the Crab, Wave, and intermediate snail shells are shown in [Figure 1](#). Protein concentration was measured using a modification of the Bradford method ([Ramagli and Rodríguez 1985](#)). Then, ~100 µg of total protein were separated through isoelectric focusing (first-dimension separation) on IPG strips (ReadyStrip™, pH 5–8/17cm; Bio-Rad, Hercules, CA) using a Protean IEF System (Bio-Rad) following manufacturer's instructions. The second dimension was carried out by electrophoresis (constant current of 12 W/gel for 5 h) on 12.5% polyacrylamide gels using an Ettan DALTsix electrophoresis system (GE Healthcare, Chicago, IL) connected to a refrigerated circulator system (20 °C). Protein spots were visualized using a modified version of the silver staining method, then the gels were scanned using a GS-800™ Calibrated Imaging Densitometer (Bio-Rad). The analysis of the gel images was performed with the software Progenesis SameSpots version 3.3 (Nonlinear Dynamics Ltd. Newcastle, UK) which was used for semi-automatic alignment of the gels, protein spot detection and spot volume measurements. A final spot-filtering step was carried out in order to exclude technical artifacts or spots that were not well-defined. Absolute spot volumes for each gel were corrected by background subtraction and normalized using the default method that takes into account the differences in the total spot volume across 2-DE gels in Progenesis SameSpots version 3.3 for further statistical analyses. [Supplementary Figure S1](#) gives an example of a 2-DE gel on which is marked the position of 38 spots which were a focus of study as described in the Results section. Normalized spot volumes were transformed to a

logarithmic scale to fit normality and homoscedasticity assumptions of parametric tests (see [Diz et al. 2017](#) and references therein). Hierarchical clustering (using the UPGMA method) and heat map analyses were carried out using correlation values for distance calculations and the average linkage criterion for clustering on log-normalized protein spot volume data in ClustVis ([Metsalu and Vilo 2015](#)). Principal component analysis (PCA) was also carried out on the logarithm of normalized spot volume using ClustVis with unit variance scaling and singular value decomposition with imputation to calculate the PCs.

Dominance analyses

In this study, the logarithm of normalized protein spot volume was used to study expression differences between the ecotypes and F₁ and intermediate snails. Hereafter the abbreviation Hy (for hybrid) is used as a convenience to stand for both the F₁ individuals which are true hybrids and the intermediates recognizing that there is uncertainty about the genetic status of the latter. Similarly, we use the word dominance to describe the position of Hy for the intermediates as a convenience to avoid introducing new terminology. Thus the word dominance is used as a generic term when applied to both F₁ and intermediates. In addition, the words dominance, overdominance, and underdominance are used to describe the phenotypic value of Hy relative to that of the two parental ecotypes and the mid-parent value (the average of the Crab and Wave ecotypes; [Figure 2](#)). In the context of our descriptive use of the word (following [Thompson et al. 2020](#)), dominance occurs when the value of Hy is different from the mid-parent and closer to that of one ecotype than the other, but has a value between that of the two parental ecotypes. Overdominance occurs when Hy has a spot volume greater than both ecotypes, and underdominance occurs when Hy has spot volume less than both ecotypes. These descriptions of phenotypic dominance make no assumptions about the genetic basis of protein expression in terms of the number of underlying loci and the dominance relationships of the alleles at these loci. For a given spot, eight possible general patterns illustrating dominance, overdominance, and underdominance are possible if we take into account that the Wave value could be greater than Crab (Patterns 1–4) or the other way round (Patterns 5–8; [Figure 2](#)).

If spot volumes of Crab, Wave, and Hy are regarded as phenotypes for the purpose of estimating dominance by analogy with a quantitative genetics model ([Falconer and Mackay 1996](#); [Caballero 2020](#)), then values of $+a$ or $-a$ can be assigned to the two parental ecotypes and the value d to Hy. The values of a and d for the eight patterns of dominance are shown in [Figure 2](#). If the value of Hy coincides with the mid-parent, then $d = 0$, representing the lack of dominance.

Transformations of the scale of measurement of the trait, such as the conversion to log values of the protein spot volume in this study will affect the value of d . Then if Hy lies between both parental ecotypes, this could change the sign of d . However, this will rarely happen if Hy is situated far from the mid-parent and will not happen at all with overdominance or underdominance which is prevalent for significant spots here, as previously mentioned. Logarithmic transformation will also compress higher values of spot volume and this could lead to an overestimation of the number of spots showing dominance of higher expression. However, dominance of lower expression predominates in our results (see Results section), which thus could not be explained by the transformation.

Statistical analysis of dominance

Two methods were used to assess dominance statistically. In the first method, referred to here as the mid-parent method, one-way analysis

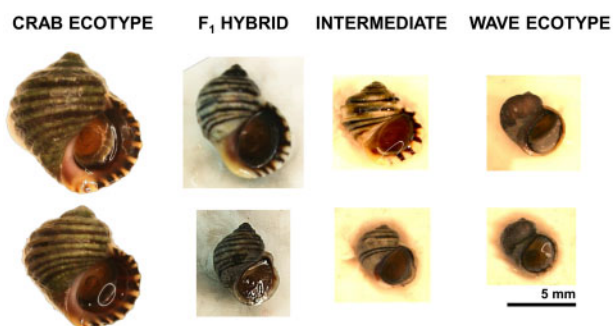


Figure 1. Shell images of representative individuals of *L. saxatilis* for the different categories used in the proteomic (2-DE) analysis. Crab ecotype (large, ridged, and banded), laboratory-reared F₁ hybrid, intermediate snails collected in the field, and Wave ecotype (small, smooth, and unbanded).

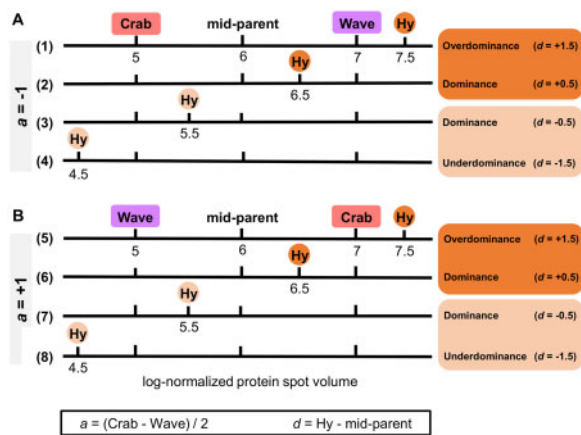


Figure 2. Scheme of the Patterns 1–8 of dominance, overdominance, and underdominance that could be obtained from the proteomic analysis performed in this study. The mean of log-normalized protein spot volume of the Hy individuals (F₁ or intermediates) is shown in relation to that of the parental ecotypes (Crab and Wave) and the mid-parent (the average of Crab and Wave). The scale 4.5–7.5 is an approximate representation of the values of log-normalized spot volume observed in the data. When Hy has a value between that of the two parental ecotypes there is dominance, if Hy exceeds both Crab and Wave there is overdominance and when Hy is less than both ecotypes there is underdominance. The values of *a* (additive genetic variance) and *d* (dominance variation) are indicated for each pattern. If *a* = −1, the spot volume of Wave is greater than the Crab (A, Patterns 1–4), and if *a* = +1, the Crab is greater than the Wave (B, Patterns 5–8). Patterns of dominance for higher expression (spot volume) are shown in dark orange (e.g., 6.5 and 7.5) and for lower expression in light orange (e.g., 4.5 and 5.5).

of variance (ANOVA) was used to compare log-normalized spot volume between Hy (F₁ or intermediates) and the mean of Crab and Wave ecotypes on a spot by spot basis (mid-parent value; see Figure 2). The ANOVA was carried out with F₁ hybrids and intermediates separately. A significant result provides evidence that *d* is not equal to zero, and therefore evidence of dominance as defined in the previous section, that is the F₁ or intermediates have spot volume which differs from the mid-parent. In each analysis, a *P*-value was obtained for every protein spot analyzed. Then two different multiple testing adjustment methods were used, the Sequential Goodness of Fit (SGoF) method using the SGoF software (Carvajal-Rodríguez et al. 2009) and the Stouffer method (Z-scores; referred to as SStouffer hereafter; see Whitlock 2005) applied sequentially and implemented in Excel. Using more than one multiple testing methods provides some flexibility in assessing the number of significant results depending on research priorities (Diz et al. 2011). A significance level of *P* < 0.05 was used with both multiple testing methods.

In the second method for assessing dominance statistically, referred to here as the “SNK method” because it incorporates the Student–Newman–Keuls (SNK) test, a 2-way ANOVA, again applied on a spot by spot basis, was first used to compare log-normalized spot volume between the parental ecotypes (Crab vs. Wave; fixed factor Ecotype) and sexes (Female vs. Male; fixed factor Sex). Hy individuals were excluded from this first step. This ANOVA was used to determine candidate proteins accounting for ecotype differentiation and which would also thus be good candidates in the search for dominance. Then, multiple test adjustments (SGoF and SStouffer) were carried out and the significant spots (*P* < 0.05) for the factor Ecotype, were considered as candidate proteins to assess dominance. In the SNK method, these candidate spots were used as the starting point to assess dominance. One-way

ANOVAs for each candidate spot, including both parental ecotypes and Hy (F₁ or intermediates in separate analyses), were carried out. Then, the significance of the three comparisons among Crab, Wave, and Hy (F₁ or intermediates) was determined by the *post-hoc* studentized range distribution SNK test. Two different results were considered as being compatible with a dominance effect. First, when the spot volume of Hy (F₁ or intermediates) is not significantly different to one of the 2 parental ecotypes, but both of these are significantly different from the other ecotype. For example, if Hy (F₁ or intermediates) and Wave are not significantly different from each other, but both are significantly greater than Crab, then Wave dominance is concluded (in line with Patterns 1 and 2 in Figure 2A and Patterns 7 and 8 in Figure 2B). The second criterion is when the spot volume of Hy is either significantly greater than both ecotypes (Patterns 1 and 5; overdominance), or significantly less than both ecotypes (Patterns 4 and 8; underdominance). For protein spots that do not meet any of the above criteria, it is concluded that there is no evidence for dominance. In presenting the results of both the mid-parent and SNK dominance assessment methods, the word dominance is used as a generic term to describe statistically significant dominance, overdominance, and underdominance as defined above.

The frequencies of protein spots showing significant Crab or Wave dominance after multiple testing were compared against a 1:1 expectation using a G-test (G-test calculator spreadsheets; see McDonald 2014). A test against a 1:1 expectation was also performed to compare the frequency of spots showing dominance for higher expression (higher spot volume; Patterns 1–2 and 5–6, Figure 2) and those showing dominance for lower expression (lower spot volume; Patterns 3–4 and 7–8, Figure 2).

Statistical analyses for the mid-parent and SNK methods were accomplished in SPSS version 24 (IBM Corp., Armonk, NY) software.

Results

In the mid-parent method, the number of protein spots showing significant deviation from the mid-parent after multiple testing adjustment (SGoF and SStouffer) is shown in Table 1. SStouffer generates a greater number of significant spots than SGoF. For both datasets (F₁ and intermediates) there is no evidence of a difference in frequency of Hy (i.e., F₁ or intermediate) being closer to Crab or Wave than the mid-parent (non-significant *P*-values, Table 1). However, for the intermediate snails, the ecotype with lower expression is closer to the mid-parent for a significant majority of spots (*P* < 0.001). An overall picture of the possible patterns of phenotype expression comparing Crab, Wave, and Hy for the mid-parent method is given in Figure 3A for the analysis of F₁ hybrids and in Figure 3B for intermediates. In each case, the genotypic values of *a* and *d* are plotted for each of the 452 protein spots. The spots that are significant for dominance, that is for *d* in the test of Hy against mid-parent, are highlighted in the plot and their frequencies correspond to those shown in Table 1. The majority of spots showing significant dominance are those which fall above (top quadrants) or below (bottom quadrants) the curves for *a* or −*a*, and thus show overdominance and underdominance, respectively. For the intermediates (Figure 3B), it is clear that the number of significant spots showing underdominance is greater than those showing overdominance, and this is reflected in the significant result of the G-test between high and low expression (Table 1).

In the SNK method for the factor Ecotype, 38 spots (8.4%) are significant after SGoF and 75 (16.6%) are significant after SStouffer (Table 1). For the factor Sex, 9 spots (2.0%) are significant after

Table 1. Summary of results of proteomic analysis with the number of protein spots showing dominance of Crab or Wave ecotype, and high or low expression for F₁ and intermediates for the mid-parent and SNK methods of dominance assessment, with SGoF and SStouffer multiple testing adjustment methods

			Expression dominance							
Analyses	Multiple test correction	N	No dominance	High		Low		G-test P-values		
				Crab	Wave	Crab	Wave	Crab:Wave	Higher: Lower	
Mid-parent	SGoF	F ₁	452	413	8	12	7	12	0.148	0.873
		Intermediates	452	348	12	13	42	37	0.695	0.000
	SStouffer	F ₁	452	352	20	35	26	19	0.423	0.317
		Intermediates	452	285	21	26	62	58	0.938	0.000
SNK	SGoF	F ₁	38	19	3	10	2	4	0.035	0.104
		Intermediates	38	21	1	4	6	6	0.466	0.085
	SStouffer	F ₁	75	50	3	11	3	8	0.008	0.548
		Intermediates	75	44	5	5	9	12	0.590	0.046

Note that the word dominance is used generically to describe the similarity to Crab or Wave ecotype for both F₁ and intermediate snails (see Materials and Methods section). The final 2 columns show P-values for G-test against 1:1 expectations (see text).

SGoF and 28 (6.2%) are significant after SStouffer. The corresponding values for the interaction (Ecotype × Sex) are 11 (2.4%) and 43 (9.5%) spots after SGoF and SStouffer, respectively. The significance of the factor Ecotype in the ANOVA analysis derives from the comparison Crab vs. Wave, thus separation of these ecotypes in clustering or PCA component plots is to be expected. The F₁ and intermediates are excluded from the ANOVA analysis, thus their positioning in cluster or PCA in relation to the ecotypes is not predictable *a priori*.

The results of the dominance analysis using the SNK method are displayed in Table 1. The number of spots showing significant dominance is lower than for the mid-parent method. This might be due in part to the initial screening for candidate loci using 2-way ANOVA in the SNK method. The spots that are significant with SNK tend to have high absolute values of the genotypic value *a*, the wide separation of the two ecotypes favoring a statistically significant difference between them. In the dataset using F₁ hybrids, the results of the G-test for Crab: Wave against the 1:1 expectation gives significant P-values indicating a preponderance of Wave dominance over Crab for both SGoF and SStouffer (Table 1). This significant result was not observed with the mid-parent method. In line with the results for intermediates with the mid-parent method, the G-tests provide evidence for a greater number of spots showing dominance of lower expression, with one quite low P-value for SGoF (*P* = 0.085) and a significant P-value for SStouffer.

In summary, Table 1 presents evidence for significant dominance with different protein spots showing dominance of Crab or Wave and lower or higher expression. Both the mid-parent and SNK methods suggest a significant preponderance of spots showing dominance of lower expression in intermediates, and the SNK method in addition provides evidence of a preponderance of spots showing dominance of Wave over Crab for the F₁ hybrids.

The heat map constructed from the 38 spots declared significant after SGoF shows that the Crab and Wave ecotypes are clearly differentiated into separate clusters as expected, and these in turn are differentiated from the cluster of the three F₁ individuals (Figure 4). The intermediates fall into three distinct clusters. The largest cluster links closely to the three F₁ individuals. Of the other two clusters, one links to Crab the other to Wave. A similar heat map was

obtained for the 75 candidate spots significant after SStouffer (Supplementary Figure S2). The corresponding PCA plots of scores of Component 2 against Component 1 are shown in Figure 5 separately for F₁ hybrids and intermediates. Crab and Wave are separated mainly on Component 1. The F₁ hybrids appear to lie between Crab and Wave on Component 1 and are shifted down somewhat on Component 2. They are clearly distinct from Crab but much closer to Wave. Although the Wave and F₁ ellipses overlap, the three F₁ individuals nevertheless lie outside the cloud of Wave individuals. The cloud of points for the intermediates lies between Crab and Wave on Component 1. Although the ellipses for Wave and the intermediates overlap the two clouds of points are separated.

Discussion

This study focused on comparing the proteome profiles of F₁ hybrid *L. saxatilis* bred in the laboratory with their Crab (upper-shore) and Wave (lower-shore) ecotype parents. These profiles are also compared with those of phenotypically intermediate individuals, whose ancestral status is uncertain, collected from the mid-shore where the Crab and Wave ecotypes overlap and hybridize. The proteome is analyzed over all proteins using multivariate techniques, and the proteins are also considered individually. Analytical techniques applied to the analysis of dominance are used to quantify the similarity of the F₁ and intermediates to the Crab and Wave ecotypes.

In the multivariate analysis, the distinct clustering apart of the true F₁ hybrids bred in the laboratory from the parental Crab and Wave ecotypes (Figure 4 and Supplementary Figure S2) is generally consistent with their hybrid genetic status, lying between the ecotypes for Component 1 in the PCA (Figure 5 and Supplementary Figure S3), which is consistent with what is expected of F₁ hybrids. For example, additivity of expression in proteomic data in F₁ hybrids has been observed previously in polychaetes (Blank et al. 2012). In contrast in a study of proteomic patterns of naturally occurring hybrids between the mussels *Mytilus edulis* and *Mytilus galloprovincialis* (Diz and Skibinski 2007) the two parental species were more closely similar to each other than either were to the hybrids. Thus the tendency for hybrids to be intermediate proteomically does not always occur.

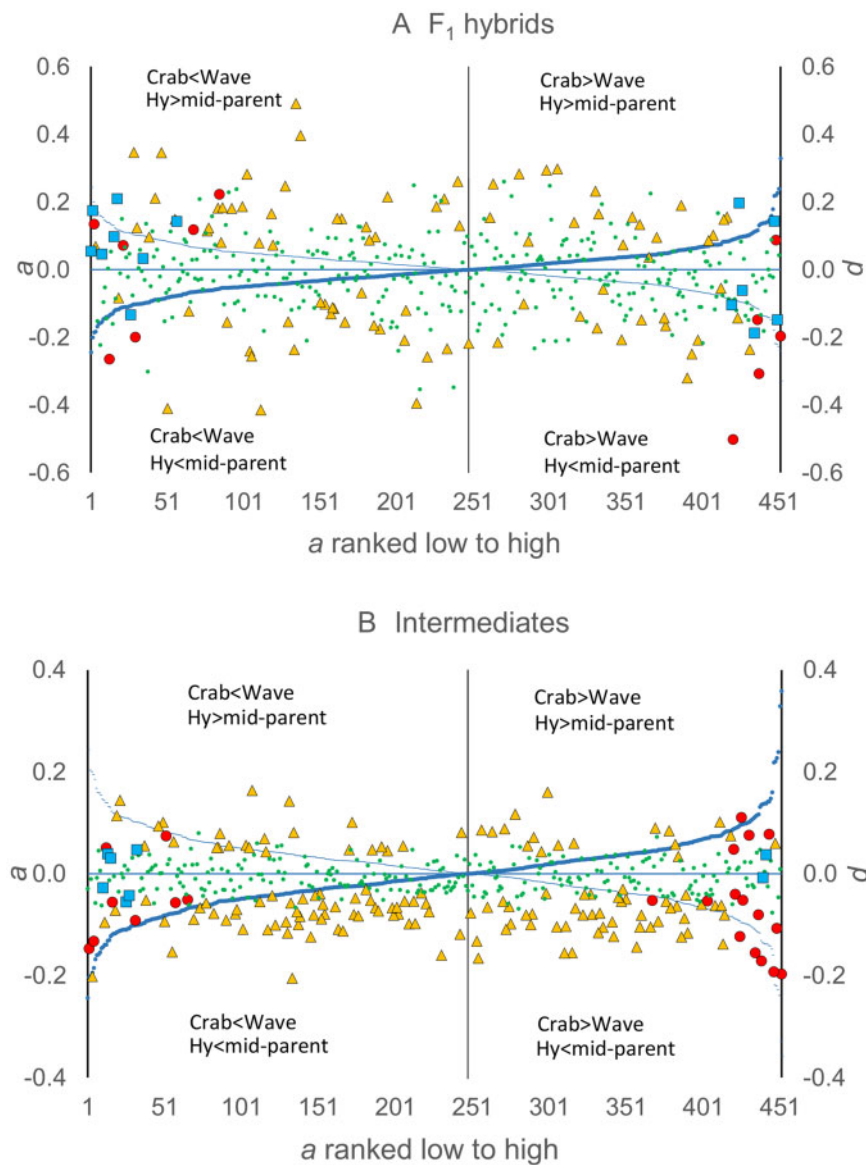


Figure 3. Values of a and d plotted for F_1 hybrids (A) and intermediates (B) for 452 protein spots. The spots are aligned on the x-axis in order of increasing a from left to right, according to rank value of a . The right-hand y-axis gives the value d . The four quadrants on the graph correspond to the patterns of Figure 2. Thus top left corresponds to Wave and high expression being dominant (Patterns 1 and 2), bottom left to Crab and low expression dominant (Patterns 3 and 4), top right to Crab and high expression dominant (Patterns 5 and 6), and bottom right to Wave and low expression dominant (Patterns 7 and 8). Descriptive statements using $<$ and $>$ symbols are given in the four quadrants corresponding to the equations at the foot of Figure 2. The value of both a (thick curved line) and $-a$ (thin curved line) are also plotted with the value of a specified by the left-hand y-axis. Points lying between these two lines show dominance; points lying above or below both lines show overdominance or underdominance, respectively. Points significant according to the SStouffer method are indicated with specific symbols. Red circles; significant with mid-parent and SNK; yellow triangles; significant with mid-parent but not SNK; blue squares; significant with SNK but not mid-parent; small green circles; not significant with either mid-parent or SNK.

The greater similarity of the F_1 hybrids to Wave on the PCA plots (Figure 5 and Supplementary Figure S3) supports our hypothesis, from Thompson et al. (2020), that hybrids resemble one parent more closely than the other. Dominance in F_1 hybrids toward the Wave ecotype might be favored by natural selection, as environmental conditions (e.g., temperature and humidity) at the mid-shore are more similar to the lower-shore (Wave ecotype) than to the upper-shore (Crab ecotype), and therefore affecting the physiological response of the organism through different protein pathways. Moreover, at the mid-shore, wave action still represents an important selective pressure and crab predation is low, and this is also reflected by a cline in shell size for the

Crab ecotype, being smaller and more similar in size to the Wave ecotype at the mid-shore (Johannesson et al. 1993; Boulding et al. 2017).

The distinct clustering of the naturally occurring intermediate snails (Figure 4 and Supplementary Figure S2) and high variation on Component 1 located between the ecotypes (Figure 5 and Supplementary Figure S3), though without such marked closer similarity to Wave, possibly suggests a high heterogeneity in protein expression, which is also in line with the heterogeneity previously detected for AFLP markers and at the shell phenotypic level (Galindo et al. 2013, 2014), with some intermediate individuals more similar to Crab, others more similar to Wave. Although, we cannot make

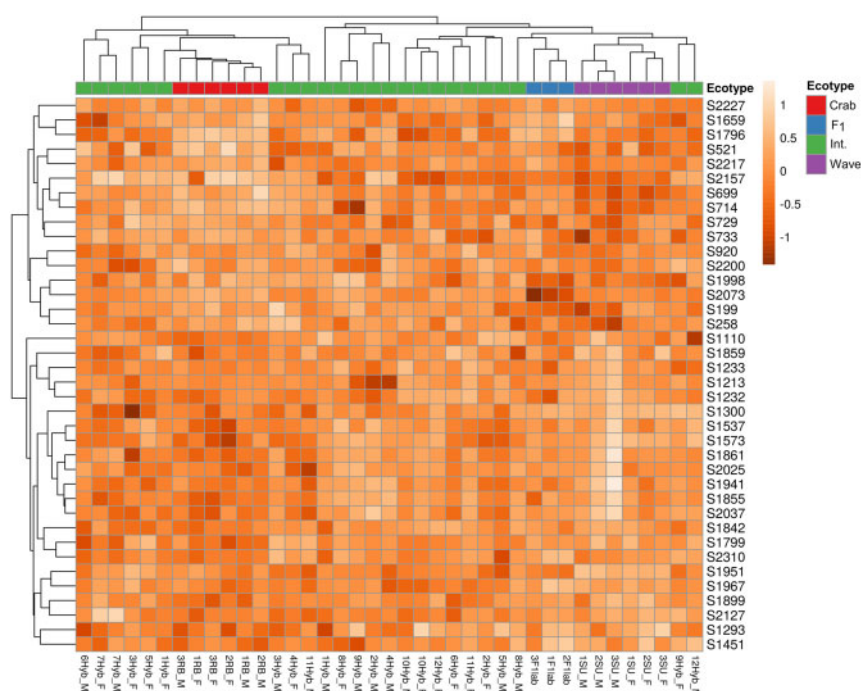


Figure 4. Hierarchical clustering and heat map of samples analyzed based on log normalized protein spot volumes of 38 candidate spots showing statistically significant differences between Crab (red) and Wave (purple) ecotypes after applying SGoF multiple testing correction. The original sample codes for the individual snails analyzed are shown at the bottom of the heat map, and the protein spot codes are shown at the right. The individual codes also indicate the sex (F or M). Clustering patterns of F₁ (blue) and intermediate snails (green) are also shown.

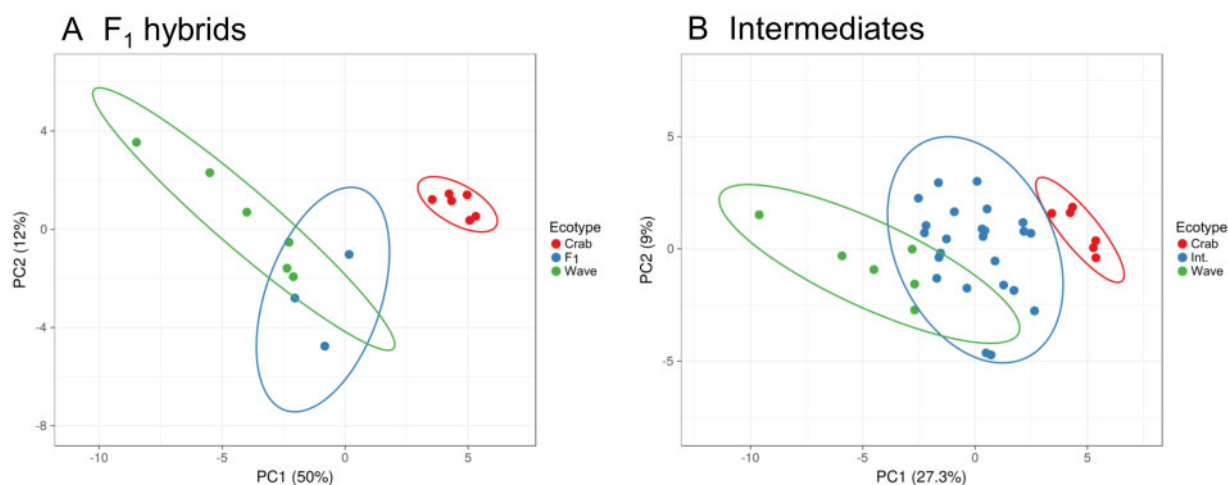


Figure 5. PCA of log normalized protein spot volumes of 38 candidate spots showing statistically significant differences between Crab and Wave ecotypes after applying SGoF multiple testing correction. Scores on PC2 are plotted against scores on PC1. The proportion of the total variance explained by a component is shown in brackets on the axes. The ellipses are such that a new observation from the same group would have a probability of 0.95 of falling within the ellipse. F₁ hybrids (A), intermediates (B).

inferences about the genetic variation of the intermediate snails studied here, the proteomic profiles should also give important information as a proxy for their genetic and gene expression variation.

Another possible explanation for why the expression of the intermediate snails lie between those of the Crab and Wave ecotype on Component 1 in the PCA plots is that these snails are adapted to the microenvironment of the mid-shore, which is different from the habitats where the ecotypes were sampled (upper and lower-shore). In this circumstance, we can suppose that their phenotypes, have been selected in this environment, but this could be the case also for Crab and Wave

ecotypes from the mid-shore that have not been included in this study. The phenotypes of these intermediates are not intermediate between Crab and Wave on any quantitative scale. Similarly, the main selective factors, crab predation in the upper-shore and wave action in the lower-shore, are not opposites on a quantitative scale. Why then should the multivariate proteomic phenotype of the intermediate snails lie clearly between Crab and Wave on the first component in the PCA plots (Figure 5 and Supplementary Figure S3)? Further studies should include individuals from the whole environmental cline in order to shed light on these questions.

In our results, it is important to highlight that there is one correspondence between the multivariate analysis and the spot-by-spot analysis of the proteomic data. The closer similarity of the F₁ hybrid snails to Wave than Crab in the PCA plots (Figure 5 and Supplementary Figure S3) is consistent with the significantly higher number of spots showing Wave than Crab dominance for the SNK method (Table 1). These results may reflect a particular general property of the Wave ecotype gene pool, but it should be noted that this generalization cannot apply to all spots studied as in contrast other spots show dominance of Crab. What would be relevant theoretically is whether the majority effect is the leading one or whether the results should be considered on a spot-by-spot basis. Still, because hybridization occurs outside of the parental micro-habitats, it is possible that natural selection is maintaining certain levels of genetic variation (Crab and Wave) within the mid-shore. On the other hand, the corresponding results for the intermediate snails are consistent with the heterogeneity previously described, showing spots where intermediates are closer to Crab and other spots closer to Wave (Figure 5, Supplementary Figure S3 and Table 1), suggesting the heterogeneity in selection found in previous studies (Galindo et al. 2013, 2014).

Two other observations made in this study are worthy of attention. The first is that some spots showed significance for the factors Sex and Sex × Ecotype. This is expected given the difference in the nature of the reproductive tissues between sexes, and despite the greater representation of muscular foot tissue in homogenates. The significant interaction implies that the difference in protein expression between sexes itself differs between the two ecotypes. This may be related to the observation that sexual size dimorphism differs between the ecotypes, the Wave ecotype showed the greatest size differences at maturity, females being larger than males (see Supplementary Figure S4 in Boulding et al. 2017; Perini et al. 2020). The second observation is the difference in results obtained with the two distinctly different methods to detect expression differences between ecotypes and the F₁ and intermediate snails (mid-parent and SNK methods). These methods both identify significant spots that are not significant using the other method (Table 1 and Figure 3), demonstrating the utility of this approach. Furthermore, two different multiple testing methods were used (SGoF and SStouffer) with similar results but also some differences (Table 1). The additional information that can be got in this approach has been noted previously (Diz et al. 2011) and might be particularly useful in identifying candidate proteins for further study. We set out in our Introduction three other hypotheses that we can test and are derived from Thompson et al. (2020). The first is that the expression of F₁ hybrids and intermediate snails is significantly closer in phenotype to one of the ecotypes supported by the current results when considered on a spot-by-spot basis. Many spots are significantly closer to either Crab or Wave, refuting the hypothesis that they are intermediate (Figures 2 and 3 and Table 1). For some spots, there is closer resemblance to Crab, for others closer resemblance to Wave. This is in accord with the second hypothesis derived from Thompson et al. (2020) that the direction of closer resemblance differs between traits. This is observed for both the laboratory-reared F₁ hybrids (Figure 3A) and the intermediate snails (Figure 3B).

The third hypothesis derived from Thompson et al. (2020) that the expression of F₁ and intermediate snails is just as likely to be closer to the ecotype with higher expression as to the ecotype with lower expression. This is supported for the F₁ hybrids where spots with dominance for both higher and lower expression are observed without any significant bias (Table 1 and Figure 3A). For the intermediate snails, there is, however, a bias with a significant excess of spots showing closer expression to the ecotype with lower expression for both Crab and Wave (Table 1 and Figure 3B). Traditional theories of the evolution of dominance might associate lower expression and

less gene product with some impairment of function and reduction in fitness. Patterns of lower expression in hybrids (reviewed in Landry et al. 2007; Ortiz-Barrientos et al. 2007), by means of gene expression (misexpression), have been previously described, for example between divergent ecotypes of whitefish *Coregonus clupeaformis* (Renaut et al. 2009) and also in a recent adaptive radiation of *Cyprinodon* pupfishes (McGirr and Martin 2020). The authors associated these phenotypes to transgressive segregation effects and linked this lower expression and misexpression to potential postzygotic barriers to gene flow. Assuming that the intermediate snails are involved in barriers to gene flow in this *L. saxatilis* hybrid zone, these might be in addition to those barriers already described in previous work (e.g., divergent selection and assortative mating; reviewed in Rolán-Alvarez et al. 2015). Lower expression in intermediate snails and its relation to barriers to gene flow needs to be studied further by measuring the fitness of F₁ hybrids and intermediate snails with specific protein expression patterns, for example by using transplant experiments in the field (Cruz et al. 2004) or measuring hybrid female fecundity (e.g., clutch size and embryo abortion) (Cruz et al. 1998; Johannesson et al. 2000, 2020). The results for both F₁ hybrids and intermediate snails show a large proportion of protein spots lying outside the range of the parental ecotypes (Figure 3), possibly representing transgressive and thus potential maladaptive phenotypes. The genetic basis of these transgressive phenotypes in hybrids and the proteins involved, also represent interesting future areas of investigation.

In comparison to genome and transcriptome analysis, less attention has been generally provided to the proteome in evolutionary ecology studies, despite proteins representing the molecular phenotype, a more direct target for natural selection (Diz et al. 2012b; Baer and Millar 2016). Further studies of the proteome of ecotypes, hybrids, and intermediate snails may thus be important in gaining knowledge of isolating barriers and speciation in *Littorina* despite the complexity of investigating the molecular genetics of a trait such as spot volume which might be a function of the joint effect of variation at many loci differing between the ecotypes, particularly modifier loci affecting the level of gene expression.

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Supplementary Material

“Supplementary material can be found at <https://academic.oup.com/cz>”.

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