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Transcriptional Profiling of Populations in the Clam *Ruditapes decussatus* Suggests Genetically Determined Differentiation in Gene Expression along Parallel Temperature Gradients and between Races of the Atlantic Ocean and West Mediterranean Sea

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Abstract: Ongoing ocean warming due to climate change poses new challenges for marine life and its exploitation. We have used transcriptomics to find genetically based responses to increased temperature in natural populations of the marine clam *Ruditapes decussatus*, which lives along parallel thermal gradients in southern Europe. Clams of the Atlantic and West Mediterranean races were collected in northern (cool) and a southern (warm) localities. The animals were kept in running seawater in the warm, southern Atlantic locality for a 15-week period. During this period, water temperature was raised to typical southern European summer values. After this period, an expression profile was obtained for a total of 34 clams and 11,025 probes by means of an oligonucleotide microarray. We found distinct transcriptional patterns for each population based on a total of 552 differentially expressed genes (DEGs), indicating innate differences which probably have a genetic basis. Race and latitude contributed significantly to gene expression differences, with very different sets of DEGs. A gene ontology analysis showed that races differed mainly in the genes involved in ribosomal function and protein biosynthesis, while genes related to glutathione metabolism and ATP synthesis in the mitochondria were the most outstanding with respect to north/south transcriptional differences.

Keywords: gene expression; bivalve; ocean warming; phylogeography; adaptation; ribosomal proteins; glutathione metabolism; electronic transport chain

Key Contribution: Hundreds of genes of the marine clam *Ruditapes decussatus* showed differential gene expression associated with race (Atlantic vs. West Mediterranean) and with the environmental temperature of the native locality after transplantation to a common warm environment.

1. Introduction

Changes in the marine environment caused by an increase in CO_2 levels in the atmosphere, mainly an increased water temperature and a reduced pH, have led to a growing interest in the physiological and molecular mechanisms that marine organisms can employ to face these changes [1–5]. In addition to traditional physiological and molecular

biology approaches, genomic techniques play an important role in studying the interactions between organisms and the changing environment [1]. In particular, studies of gene expression at the whole genome level (transcriptomics) have been especially effective in demonstrating the amount and types of genes and the molecular networks that are involved in the response to the change in seawater temperature and the adaptation to this change (e.g., [6,7]). Determining which genomic regions and genes show genetically based differential transcriptional responses is especially important in species of conservation or economic interest because it can help to achieve deep and focused research on the behavioral, physiological, cellular, biochemical, and molecular mechanisms involved.

The use of transcriptomics in the framework of population biology has several complications. The first complication is that organisms respond to environmental changes by modulating the expression of their genes (plasticity). Therefore, the observed differences among individuals collected in different locations or time points can be due to environmental differences across their ranges and temporal variation in the environment, such as seasonal variation. The second complication is that individuals from populations that are genetically differentiated can show differences in gene expression due to DNA variants in regulatory regions. Disentangling the effects of environment and genes on gene expression can be achieved by studying individuals from separate populations in one or more common environments so that the relative effect of each source of transcriptomic variation can be established [8–12].

One of the clear results that has emerged from studies of population transcriptomics in relation to temperature is that individuals native to different localities often show differences in patterns of gene expression when they are exposed to a common thermal environment in the laboratory or in the field [7,13,14]. This is expected because organisms usually show genetic differences among populations. Several studies have demonstrated that even slight changes in allelic frequencies at genetic markers among populations may be associated with differences in expression patterns [15]. These differences could affect, at least partly, their response to changes in temperature. The differential responses observed may have a genetic basis, i.e., they are caused by differences in the DNA sequence of the regulatory regions or master regulatory genes (e.g., [16]).

One additional step in discovering the genetic basis of transcriptional responses to thermal change in the sea is to know whether the populations living in contrasting thermal environments show specific adaptations to these environments. The observation of correlations of transcriptomic differences with differences in specific environmental factors of the native habitats is suggestive of adaptive responses (i.e., responses based on genetic differences caused by natural selection). Latitudinal thermal gradients in the sea are nice natural experimental settings for testing adaptation, and several studies have used them to infer the adaptive nature of some gene expression patterns [17–21].

On the other hand, population genetic studies using genetic markers have shown that many marine species harbor cryptic geographic variability at the DNA level. This variability could result in different responses to environmental change across populations. In particular, studies of intraspecific phylogenies of DNA sequences (especially mitochondrial DNA) have shown the existence of particular phylogenetic lineages that are geographically restricted, separating groups of populations across regions that have evolved independently during long time periods and are often separated by areas of abrupt genetic change. These areas have been termed phylogeographic breaks [22]. The amount and characteristics of intraspecific physiological variation across phylogeographic breaks have been rarely studied in a systematic way [23]. With very rare exceptions, gene expression variation has shared the same destiny [24]. We would expect transcriptomic differentiation to be greater across phylogeographic breaks than what is observed between populations within each phylogeographic group; however, we cannot tell because we simply have no data. Testing this hypothesis is of real interest because it can help us to better understand the process and the causes of genetic differentiation across phylogenetic breaks (e.g., neutral vs. adaptive), and it can also be useful for the adequate management of genetic resources

in commercial or endangered species [9]. Specifically, the study of the transcriptomic differences related to thermal environments across phylogeographic breaks can shed light on the different abilities of populations separated by the break to cope with increases in seawater temperature due to climate change. It can also allow for the discovery of genes and gene networks that act for this purpose but remain hidden to researchers through the effect of phylogeographic differentiation.

In this paper, we report on a study of gene expression differences among populations of the grooved carpet shell clam (*Ruditapes decusatus*), a species that shows a subdivided population genetic structure as described above. The grooved carpet shell clam (hereafter the GCS clam) is an infaunal bivalve that lives in the sandy bottoms of the Mediterranean and nearby Atlantic coasts (MEDAT) from Morocco to Norway. The species is highly appreciated as seafood, and aquaculture practice is now increasing in some countries [25]. However, most clam production still comes from harvested natural beds, especially in southern Europe and northern Africa [26–29]. The range of the species extends along wide climatic gradients that span the European and northern African coasts, making this species a good subject to study thermal adaptation. On the other hand, genetic studies using genetic markers such as mitochondrial DNA sequences, intron restriction fragment length polymorphisms (iRFLPs), and microsatellites have shown that there is a marked genetic subdivision across the well-characterized Eastern–Western Mediterranean phylogeographic break [30]. Additionally, strong genetic differentiation exists between the also well-characterized Atlantic–Western Mediterranean phylogeographic break, with some markers attaining F_{ST} distance values of over 0.3 [30,31]. As the planktonic larval phase of the GCS clam lasts for 8–15 days (Borsa and Diter, cited in Ref. [26] and Ref. [32]), allowing for an extensive gene flow, the observed amount of genetic differentiation among populations is unusual and makes this species one of the most genetically diverse organisms described thus far in the literature across the MEDAT [33,34]. As for species with similar patterns of genetic subdivision, the three genetic units (races, from now on) found in the GCS clam are thought to be the result of the population subdivision of the species during glacial periods in the Pleistocene [30,31].

In this study, we have taken advantage of the parallel temperature gradients existing on both sides of the Iberian Peninsula to disentangle the effects of phylogeography and thermal environment on differences in gene expression among GCS clam populations in response to the normal temperature increase that occurs during spring in the waters of the Mediterranean Sea and the nearby Atlantic Ocean. Clams were sampled from four localities: a cool northern locality and a warm southern locality in each marine basin. The clams were transported to one of the warm southern localities for acclimation as sea warming is the expected outcome of climate change in the near future. The clams were exposed to nearly natural conditions and experienced the natural, gradual increase in temperature that occurs during the spring in southern European waters before they were subjected to a transcriptomic analysis using an oligonucleotide microarray, with the aims of (1) quantifying the relative differences in gene expression between the races and populations living in different thermal regimes in response to a natural temperature increase, and (2) discovering functional units (genes and gene networks) that demonstrate a differential response to the same environment.

2. Materials and Methods

2.1. Clam Sampling and Experimental Setting

Adult GCS clams of an average size of 30 mm were sampled from four localities in Spain, France, and Portugal (Figure 1). These localities were chosen to represent the ATL and WMED genetic races of clams described by [30] and of the warm (south) and cool (north) environments along the parallel thermal gradients of seawater that are typical of the Mediterranean and nearby Atlantic region (MEDAT). Additionally, 20–30 animals were collected for genotyping in each locality. Clams from the AN, MN, and MS sites were transported live to the IATS facilities upon collection. Using a black marker, they were

individually labeled with a number code on the shell. The clams were placed in tanks with running, sand-filtered seawater and fed a mixture of microalgae (*Tetraselmis svecica*, *Isochrysis galbana*, and *Chaetoceros calcitrans*).
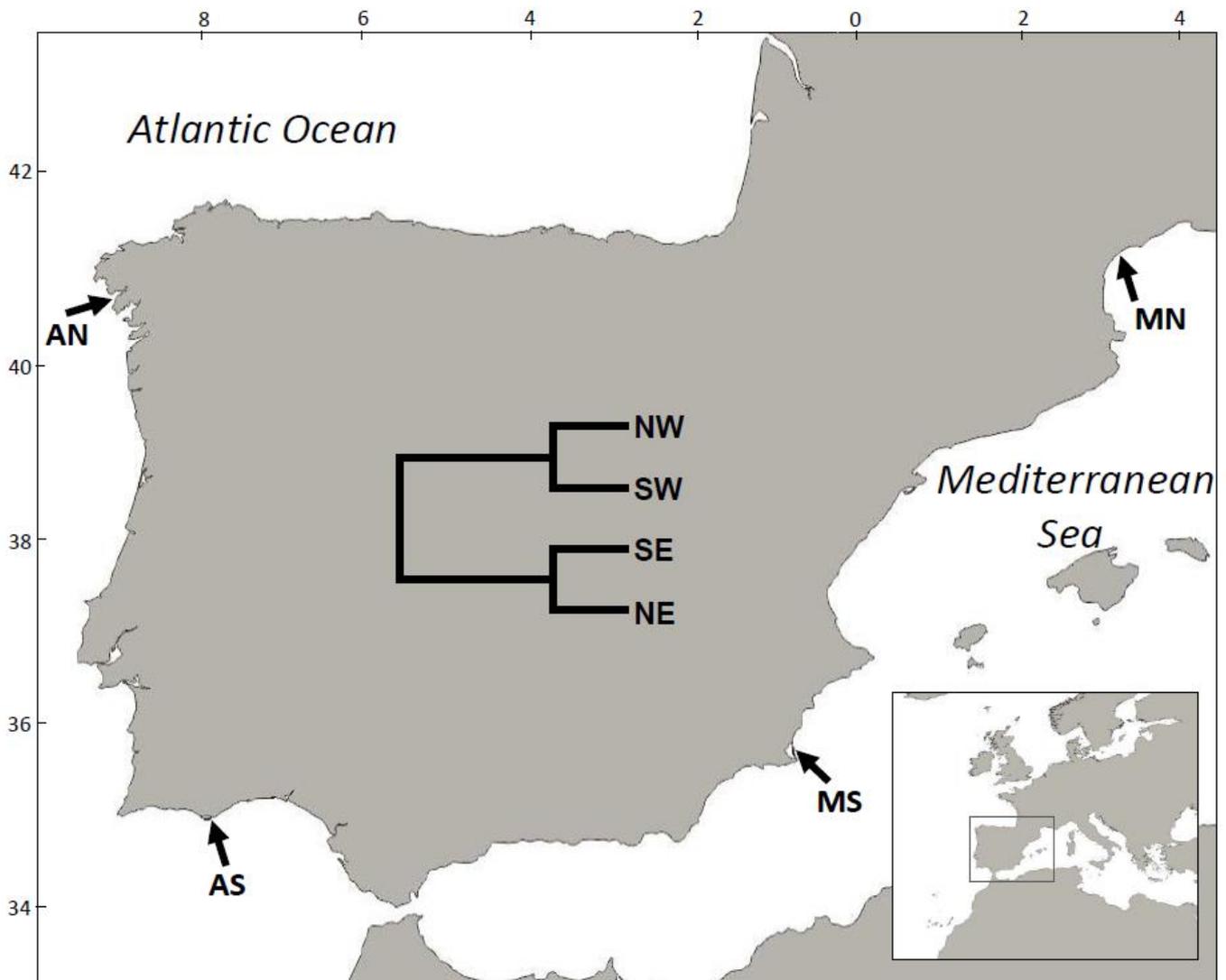

Figure 1. Map showing the localities sampled and the NJ tree (inset) based on F_{ST} values computed from 6 intron RFLP genetic markers. AN: Ría de Noia estuary (Spain); AS: Ria Formosa lagoon (Portugal); MS: Mar Menor lagoon (Spain); MN: Thau lagoon (France).

On February 2012, 15 clams from the AN, MN, and MS sites were transported live in a cool container to the Ramalhete Marine Station at the University of Algarve near Faro (Portugal). Upon arrival, the clams were placed in an outdoor 300 L PVC tank and provided with unfiltered running seawater. At this stage, 15 clams that had been collected one week before in the nearby shellfish beds of Ria Formosa were incorporated to the experiment after being individually marked. They constitute the AS sample. The seawater was pumped directly into the tank from the estuary and therefore provided the clams with the microalgae and suspended particles that constitute their usual diet in the wild. The tank was covered with a nylon mesh to avoid direct exposure to sunlight and to protect the clams from potential predators. The clams were inspected daily for mortality, and the condition of the setup was checked. Dead clams were recovered and discarded.

After a period of 15 weeks, all surviving clams were sacrificed by opening their valves with a scalpel. The gills were immediately dissected and preserved in Trizol at −80 °C. Mantle samples were preserved in 90% ethanol.

2.2. Environmental Variables

The water temperature and salinity for each sampling locality were collected from a variety of sources. For the AN site, weekly records for the period 2003–2009 from the Freixo station at −1 m were downloaded from the Instituto Tecnolóxico do Mar website (http://www.intecmar.gal/Ctd/Default.aspx (accessed on 14 May 2013)). Data for the AS site were obtained from the Estaçao Marinha de Ramalhete in Faro (Portugal). Data for the MS site were obtained from the Oceanographic Information System of the Murcia region (https://siom-murcia.hub.arcgis.com/ (accessed on 21 May 2013)). These data were recorded at −1 m in an aquaculture site located at the same latitude as the sampling site but ca. 10 miles offshore. Finally, the data for the MN site were obtained from REPHY-IFREMER (https://wwz.ifremer.fr/lerpc/Activites-et-Missions/Surveillance/REPHY (accessed on 10 September 2013)). In this case, surface (0–1 m) data from the Bouzigues station were used. For the AN, AS, and MN sites, data were obtained once a week for a three-year period immediately prior to the experiment (2008–2010). In the case of the MS site, data were available only as scattered records along the year, and the whole available series from 2004 to 2010 was used. The size of the sampled clams suggest that their age could not be more than 3 years; therefore, only data from the years 2008–2010 were used for the AN, AS, and MN sites. The temperature and salinity at the AS site were measured as described during the course of the experiment.

2.3. DNA and RNA Extraction

RNA was extracted at the University of Algarve laboratory a few days after tissue collection using Trizol, following the protocols provided by the manufacturer. DNA from the mantle samples preserved in ethanol was extracted with Chelex resin at the IATS-CSIC. The RNA and DNA quality were examined by electrophoresis in agarose gels and spectrophotometric absorbance ratios. Concentrations were measured with a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA) instrument. RNA integrity was examined with a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). With the exception of a sample from the MS site, which was discarded, all samples demonstrated RNA integrity numbers (RIN) above 7.

2.4. Intron Genotyping and Population Genetics

The polymorphism of six introns that allow for distinction between the Atlantic and West Mediterranean races of the GCS clam was scored using a simple technique based on the PCR amplification of the introns, digestion with restriction enzymes, and agarose gel electrophoresis [30].

The RFLP data were converted into genotypes. These data were pooled with data from the 12 other previously studied populations [30]. Allele frequencies, heterozygosity, and F_{ST} indices were calculated using GenePop 4.1.0 [35]. A Bayesian analysis of genetic structure [36] was carried out to confirm that the collected samples demonstrated the expected genetic profile of the ATL and WMED races. Details of this analysis can be found in [30]. A neighbor-joining tree [37] was constructed from pairwise F_{ST} comparisons among the four experimental populations.

2.5. Microarray Hybridization

RNA from 41 individuals (15 AS, 9 MS, 12 AN, and 5 MN) was hybridized to the one-color Agilent microarray previously designed from mRNA sequences obtained by RNA-seq from multiple organs and life stages of the species [38]. Eight slides were used, each containing 8 arrays. To minimize the statistical confounding effects of the slide, individuals from different populations were used to hybridize arrays in several slides so

that no slide had samples from less than 3 populations, and individuals from a population were scored together with individuals from at least 2 additional populations (due to sample size differences among populations, it was not possible to have all populations represented in all slides). Hybridizations were carried out in two batches of three slides. Six individual hybridizations that gave poor quality in the first hybridization batch were repeated in the second batch. At the end, two samples from AS, two from AN, and one from MN were discarded due to poor quality hybridizations, resulting in a total sample size of 36 clams. Normalized fluorescence data for these comparisons were deposited in the GEO database under accession GSE223000.

2.6. Microarray Data Treatment and Analysis

The fluorescence signal of the reporter probes was examined for consistency with the quality parameters, following the recommendations of the microarray manufacturer. Fluorescence data were normalized using the Lowess method prior to analysis with an in-house R script [39]. To ensure a minimum sample size in the analysis and to avoid a large variation in the sample size across probes, which could lead to increased inter-probe variance, data were filtered by choosing only those probes that demonstrated a signal in at least 75% of the clams in each population. An initial exploratory analysis of all filtered genes and samples was carried out using principal components to detect outliers. The fold change for each gene in each population was calculated as the ratio of the gene's average population signal to the total sample average.

The normalized fluorescence intensity shown by each gene was first subjected to a one-way analysis of variance in order to test for the existence of significant expression differences among populations. The model for this ANOVA was:

$$X_{ij} = P_i + e_{ij}$$

where X_{ij} represents the fluorescence value of the i individual in the j population, P_i represents the average fluorescence in population j, and e_{ij} is the error term.

A two-way analysis of variance was then carried out to test for the differential effects of the fixed factors Race (Atlantic vs. Mediterranean) and Latitude (north vs. south) on individual gene expression. For each gene, the model for this ANOVA was:

$$X_{ij} = L_i + R_j + (L{\cdot}R)_{ij} + e_{ij}$$

where X_{ij} represents the fluorescence value of an i individual in the j population, L_i represents the average effect of latitude i, R_j represents the average effect of the race j, $(L{\cdot}R)_{ij}$ represents the interaction between Race and Latitude, and e_{ij} is the error term.

For all these analyses, the TMeV software suite was employed [40]. For each gene, *p*-values from the ANOVA were used to estimate the false discovery rate by the Benjamini–Hochberg (BH) method [41]. Using mean centered data, Euclidean distances, and the complete linkage option, data were also subjected to a hierarchical cluster analysis [42] in the R environment.

2.7. Functional Analysis

The functional annotation of the sequences from which the probes were derived was carried out as described [38]. First, BLAST hits and Gene Ontology (GO) terms associated with the probes were retrieved with Blast2GO [43], which makes a rigorous evaluation of the possible GO terms associated with the BLAST hits of each probe and ascribes an index of certainty to each annotation. Valid annotations were considered if the index was higher than 0.5, using a threshold of 10–3 for BLASTx searches and default parameters in the mapping and annotation steps. The overrepresentation of particular functional GO terms (i.e., enrichment) in the groups of probes which were significant in the ANOVA sets of significant genes was examined by means of Fisher's exact test, using the DAVID platform [44] as described in [38], based on the GO terms associated with homologous

genes of the zebrafish (*Danio rerio*). The use of this species is justified because it was the species to which a BLAST analysis of the clam sequences produced the highest number of hits with annotations [38]. We did not intend to use overrepresentation tests as a touchstone for the implication of differentially expressed genes and their associated GO terms in the functional aspects discussed in the text; therefore, we examined overrepresented terms with associated *p*-values of slightly more than 0.05 when they were supported by high values of other parameters, such as fold enrichment or count numbers.

2.8. Neutrality Tests

The testing of neutral or selective patterns of gene expression variation among populations was carried out for each gene using the "expression variance and evolution" model for quantitative trait evolution developed by Rohlfs and Nielsen [45] with the program EVE, which was developed by the same authors.

3. Results

3.1. Environmental Variables

The seawater temperature values recorded at the sampling sites in the three years prior to the study are summarized in Figure 2a. The average T values were lower in the northern populations (14.7 ± 0.3 °C in AN, and 16.4 ± 0.5 °C in MN) when compared to their southern counterparts in the same basin (18.9 ± 0.4 °C in AS, and 19.5 ± 1.0 °C in MS). The average values at the Mediterranean sites were higher than at the Atlantic sites. Maximum values were detected in August in all populations; these values were over 27 °C in all but the AN population, which had a maximum recorded temperature of 19.9 °C. The minimum values did not show a clear geographic pattern; however, they were never lower at the southern sites than at the northern sites within the same marine basin. The general picture is that the clams living in southern populations were exposed to higher temperatures during longer periods of time over the year than those living in the north, which was assumed in the experimental hypothesis.
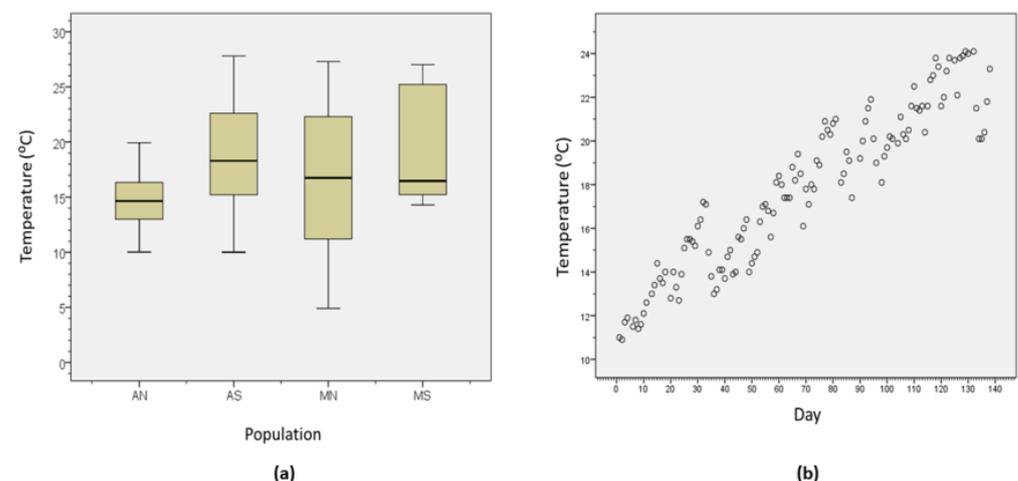

Figure 2. (**a**). Seawater temperature variation at the four sampled localities in the three years prior to the experiment. (**b**). Daily average seawater temperatures registered during the experimental period.

The average salinity values (PSU) in the three years prior to the experiment were 31.9 ± 5.4 for AN, 35.8 ± 1.7 for AS, 37.5 ± 1.9 for MN, and 37.8 ± 1.1 for MS. These values showed the expected pattern of higher salinity in the Mediterranean locations.

The temperatures of the seawater in the experimental setup increased steadily from 13.8 °C to 24.1 °C during the course of the experiment (Figure 2b), falling in the range recorded in the previous 3 years in AS (Figure 2a). This indicates that during the experiment, the temperatures did not depart from the usual range experienced by clams in this locality in recent times. On the other hand, they were higher than those experienced normally by

some populations, such as the clams from the AN site. The salinity varied between 31.0 and 36.5 PSU, with no specific temporal pattern.

3.2. Population Genetics

The allelic frequencies for the six genetic markers analyzed in the four studied populations are provided in Table S1 (Supplementary Tables and Figures). A Bayesian analysis of the population structure of the four samples specific to this study, pooled with the eleven samples studied by [30], ascribed the AN and AS samples to the Atlantic race and the MN and MS samples to the West Mediterranean race, as expected (Figure S1, Supplementary Tables and Figures). Based on the four samples, the F_{ST} was 0.13 ($p < 0.001$) and varied between 0.046 and 0.227 for the pairwise comparisons (Table S2, Supplementary Tables and Figures). The neighbor joining tree based on the pairwise F_{ST} comparisons separated the populations into two groups according to their race, which reflects the higher average differentiation between the Atlantic and the Mediterranean basins compared to the within-basin population differentiation (Figure 1).

3.3. Gene Expression Variation across Populations (One-Way ANOVA)

Our filtering procedure rendered expression values for 11,025 probes. The results of the exploratory principal components analysis did not reveal extraordinary departures from the general group of samples (Figure S2, Supplementary Tables and Figures). Significant differences ($p < 0.05$) in the expression level across populations were detected at 2268 probes by the one-way ANOVA, 418 (3.8%) of which were significant at 5% FDR (Table 1 and Supplementary Results). A cluster analysis of this set of 418 probes, using Euclidean genetic distances among samples, showed that individuals sampled in the same locality clustered together (Figure 3) and that individuals sampled in different localities formed separate clusters. The clams from the MN site were the most transcriptomically divergent and were grouped in the most basal branch of the tree. The samples from the AN site showed the least intrapopulation transcriptomic divergence (shortest branch lengths). Downregulated genes were more frequent than upregulated genes in all populations except MN (Figure 4a).

Table 1. Results of ANOVA on gene expression across clam populations. FDR: false discovery rate.

ANOVA Type	Factor (Levels)	No. of Significant Genes at $p < 0.05$ (FDR)	No. of Significant Genes at FDR = 0.05
One-way	Population (MN, MS, AN, and AS)	2287 (0.24)	418
			412
Two-way	Race (ATL; WMED)	1897 (0.29)	337
(Race × Latitude)	Latitude (North; South)	1553 (0.36)	104
	Interaction	1406 (0.39)	9

A cluster analysis of significant genes showed that the populations featured characteristic expression patterns for particular groups of genes. Specifically, genes demonstrated differential expression across populations grouped in four main gene clusters (Figure 3). Cluster A comprised 111 genes that were overexpressed mainly in AN. Enrichment tests based on their associated GO terms indicated that this cluster was enriched for genes involved in purine nucleoside metabolism and/or electron carrier activity, such as cytochrome c oxidase subunit IV, methylthioadenosine phosphorylase, ATP synthase, or adenylate kinase. Cluster B comprised 54 genes that were more highly expressed in AS and showed no significant GO term enrichment. Cluster C included 213 genes overexpressed in MN and was enriched for genes related to ribosomal activity, peptide biosynthesis and glutathione metabolism. Finally, cluster D comprised 39 genes which were overexpressed in MS and was enriched for genes related to cellular respiration, such as cytochrome c oxidases and NADH dehydrogenases.
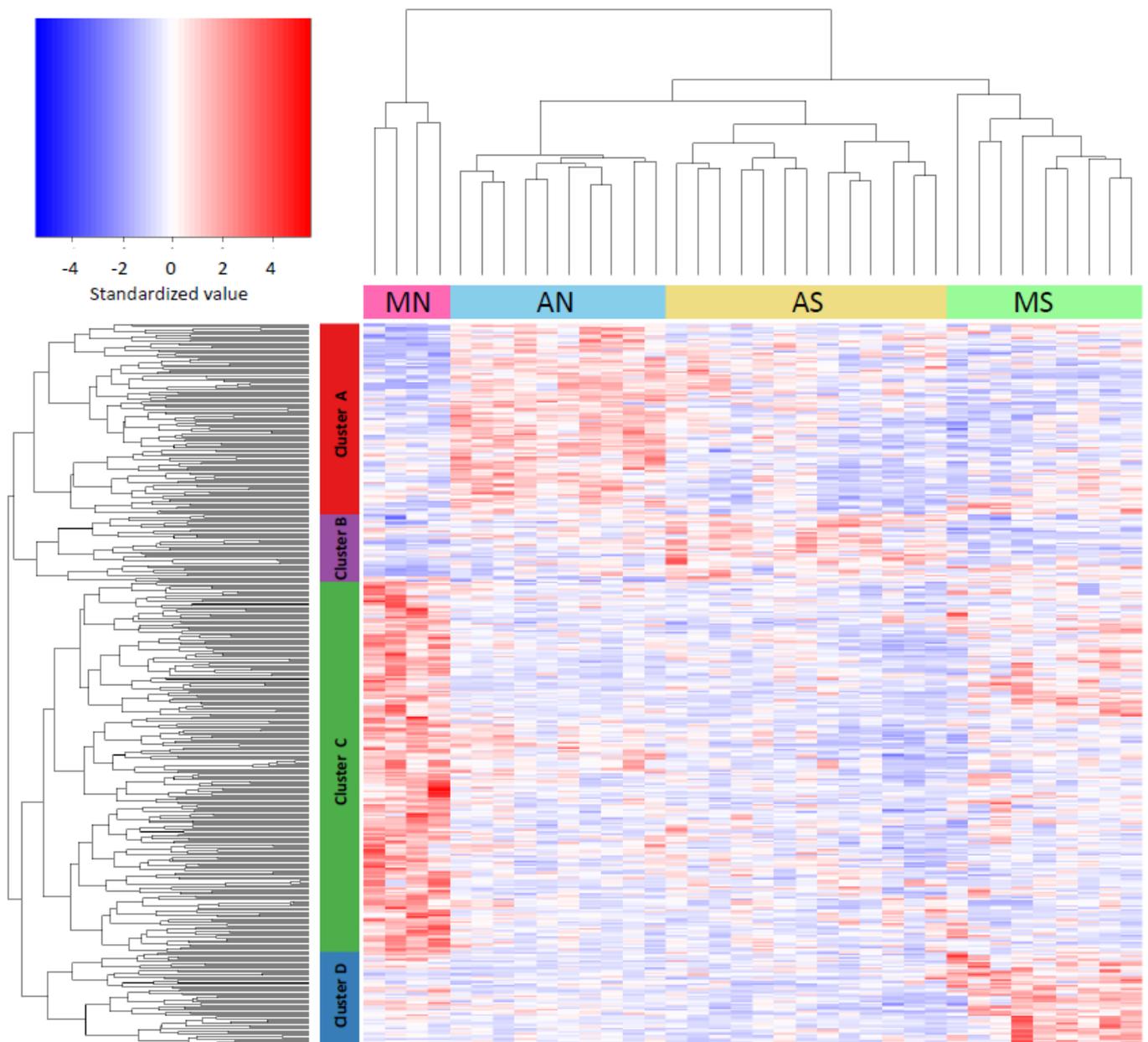
Figure 3. Heatmap of normalized gene expression and cluster analysis of distances between populations and genes, based on the set of genes that were significant (5% FDR) in the one-way ANOVA.

3.4. Gene Expression Differences Associated to Race and Latitude (Two-Way ANOVA)

The results of the two-way ANOVA (Race × Latitude) are shown in Table 1. This analysis detected significant differences (5% FDR) at 413 probes. Of these, 278 probes (50%) were significant in the one-way ANOVA. The genes significant in the two-way ANOVA included 337 probes significant for Race (87%), 104 for Latitude (25%), and 9 for their interaction (2%). Some probes were significant for the two factors or for a combination of factors and interaction (Figure 5a). Specifically, twenty-nine probes that showed significant signal differences between the races also showed significant differences for the latitude. Similarly, six probes that exhibited significant Race × Latitude interaction also showed significant effects for one of the two factors (three probes) or both (three probes). In total, 35 out the 413 significant probes were significant for some combination of race, latitude, and interaction (Figure 5a).

Figure 4. Bar charts showing the number of upregulated (blue) and downregulated (orange) genes according to the populations and type of statistical test. (**a**) One-way analysis of variance. (**b**) Two-way analysis of variance, Race factor. (**c**) Two-way analysis of variance, "Latitude only" gene set. Numbers for pooled samples of races and latitudes are also given when appropriate (**b**,**c**).
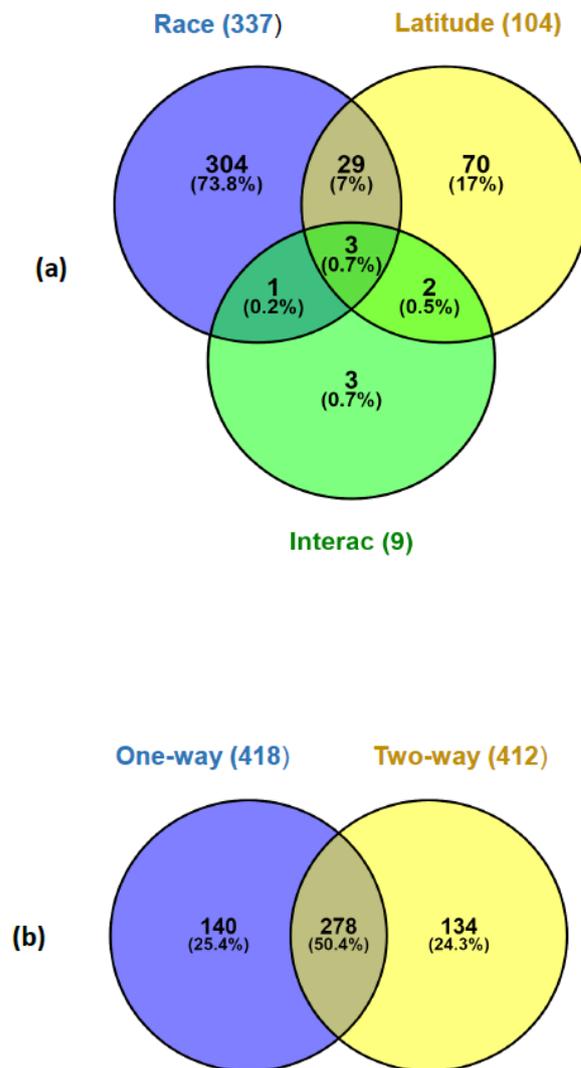
Figure 5. Venn diagrams showing the number of DEGs (FDR = 0.05) of gene expression data. (**a**) two-way ANOVA (Race × Latitude). (**b**) Comparison of the DEGs between one-way and two-way ANOVAs.

The 337 probes significant for the Race factor in the two-way ANOVA (Figure 5a) are candidates for expression differentiation caused by the genetic divergence that occurred during one or more episodes of Atlantic–Mediterranean population subdivision in the Pleistocene. In this group of genes, the average signal intensity was higher in the Atlantic populations than in the Mediterranean populations for 107 probes (32%) and higher in the Mediterranean populations for 230 probes (68%) (Figure 4b).

Using BLAST, GO terms could be obtained for 173 race-significant genes against the Swis-sprot database and for 200 using BLAST against the *Danio rerio* genome (Supplementary Results). The list of GO terms in the Cell Component category indicates that DEGs code for proteins located in the cytoplasm, the nucleus, the membranes, and the extracellular space. Within the cytoplasm, DEGs mainly coded for proteins of the cytosol, the cytoskeleton, the mitochondria, and the Golgi system, with other parts in lesser numbers. This diversity of cell locations parallels a large number of GO terms (169) associated with a very diverse set of functions in the Biological Process category, the most frequent being Translation and other functionally related terms, proteolysis, oxidation-reduction, and the regulation of the transcription, transport, and metabolic processes. Fisher's exact test indicated an over-representation of 44 GO terms at $p < 0.05$ in this set of genes, although this term collection

is highly redundant (Figure 6). The term that grouped the highest number of probes was Protein metabolic process (46 probes). Nineteen additional overrepresented terms were related to protein synthesis and ribosomal function, comprising 22–34 probes. The remaining overrepresented terms were related to cell homeostasis, development, differentiation, carbohydrate metabolism, extracellular part, peptidase activity, and the immune system (in decreasing number of probes). The KEGG pathway dre03010:Ribosome was also enriched. The heatmap in Figure S3 (Supplementary Tables and Figures) shows the between-race variation for the subset of 105 genes associated with the significantly enriched GO terms. These genes include a large number of ribosomal proteins and other proteins related to protein biosynthesis, fourteen of which were more highly expressed in the Mediterranean populations and seven in the Atlantic. Five protease DEGs (cathepsin and other proteases) were more highly expressed in the Mediterranean populations and only one in the Atlantic populations. The five DEGs coding for proteins of the mitochondrial electron transport chain (cytochrome oxidases and NAD dehydrogenases) and the functionally related ATP-synthase F1 subunit were more highly expressed in the Atlantic populations.
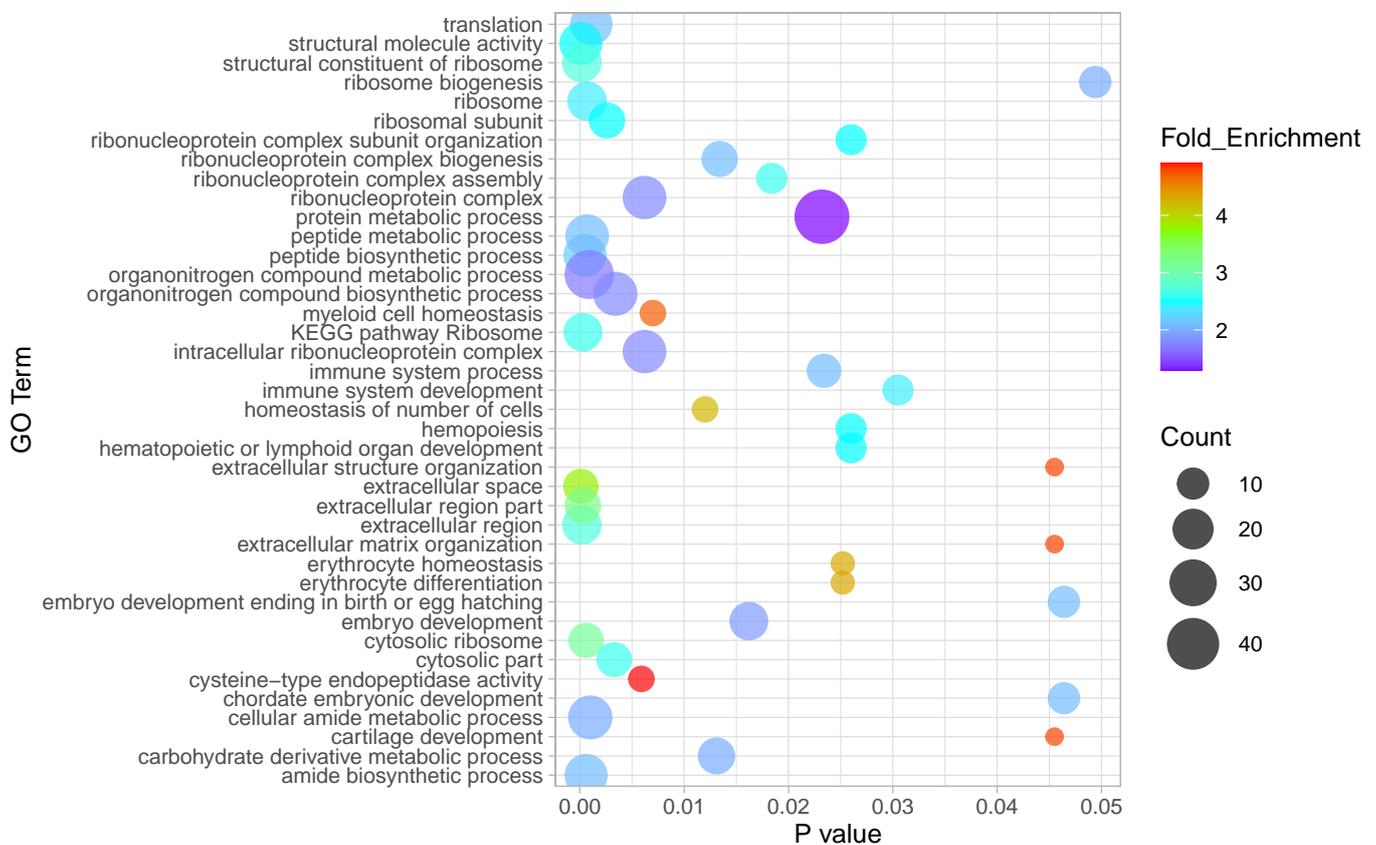

Figure 6. Overrepresented biological process GO terms ($p < 0.05$) in the set of genes which were significant for the Race factor in the two-way ANOVA.

Of the 104 genes significant for latitude in the two-way ANOVA, 32 were also significant for Race, and two were significant for the Race × Latitude interaction (Figure 5a). The remaining 70 genes, which showed significant transcriptomic differentiation for latitude but not for race, are candidates for adaptive gene expression variation related to temperature differences between the northern and southern populations. We will refer to them as the "latitude-only" gene set. The average signal was higher in the northern populations for 63 probes (88%) in this gene set and higher in the southern samples for only 7 probes (Figure 4c). When the latitudinal expression change was considered separately within each race (i.e., AN compared with AS and MN compared with MS), this pattern remained the same with only one exception (gene #4479, with no BLAST hit (Supplementary Results).

These results indicate that after transplantation to the warm waters of southern Portugal, the clams native to the cool northern environments had a higher transcription rate than the clams native to the southern environments for most of these genes. We found 13 GO terms overrepresented in this group of genes at $p < 0.10$ (Figure 7). The term Cellular processes involved the largest number of genes (24), with an average fold enrichment of 1.19X. The highest enrichments were found for the terms transferase activity and glutathione transferase activity (23X and 27X, respectively). Three other enriched terms (GO:1901564, GO:0006790, and GO:0043603) included genes that code for the enzymes of glutathione metabolism. The three significantly enriched KEGG pathways in the latitude-only gene set were drug metabolism via cytochrome P4502, the metabolism of xenobiotics via cytochrome P450, and glutathione metabolism. The genes responsible for the significance of these three pathways gave BLAST hits to glutathione S-transferase (GST) omega (two cases) and the GST-rho of zebrafish (genes #3702, #12815, and #8274 in the Supplementary Results). The three genes were upregulated in the northern populations exposed to southern temperatures (Figure S4, Supplementary Tables and Figures). The results of BLASTX against databases other than Ensemble zebrafish revealed an additional two genes in the latitude-only gene set (genes #8304 and #4170, Supplementary Results), with hits on GSTs of other species. Another gene present in this gene set coded for glutathione synthetase, an essential protein for the provisioning of reduced glutathione in the cell (gene #4887, Supplementary Results). All these results underscore the importance of upregulation of glutathione-related genes in northern populations.
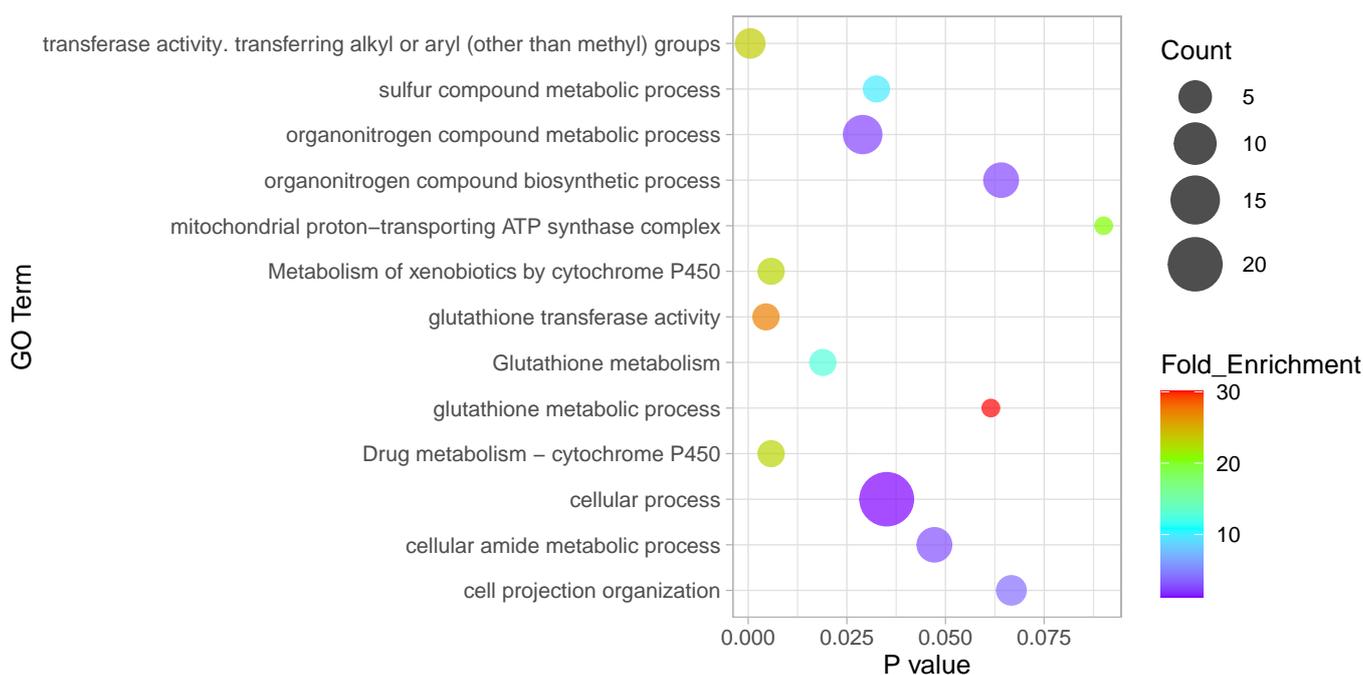

Figure 7. Overrepresented biological process GO terms ($p < 0.10$) in the set of genes which were significant only for Latitude in the two-way ANOVA.

The GO term mitochondrial proton-transporting ATP synthase complex also showed a *p*-value < 0.10 and a very high fold enrichment (20X) in the latitude-only gene set (Figure 7). This term was associated with two genes coding for the subunits of the FoF1-ATP-synthase enzyme (ATPK) (genes #5665 and #7588 in the Supplementary Results and Figure S4). Two other genes in the latitude-only gene set, which demonstrated no high-confidence BLAST against any zebrafish gene and were therefore missed in the GO term overrepresentation tests, seemed to correspond to ATPK as well, as indicated by their similarity to the *Drosophila melanogaster* ATPK in the SwissProt database (genes #1210 and #10018 in the Supplementary Results). Their presence in the latitude-only gene set

provides additional support to the overrepresentation of ATP synthase complex in this group of genes.

Nine probes provided significant results for the Race × Latitude interaction (Table 1). The patterns of variation at these probes showed a clear distinctiveness of the MN population. A set of seven probes showed a higher average signal in MN (Figure S5, Supplementary Tables and Figures), five of which showed no BLASTX match and three of which corresponded to genes coding for spermatogenesis-associated protein 1 (SPAT-1), stress response protein nhaX, and protein-L-isoaspartate (D-aspartate) O-methyltransferase (PCMT-1). An additional two genes demonstrated the lowest expression value at MN and corresponded to T-complex protein 1 subunit alpha, Nucleolar complex protein 3 homolog, prohibitin-2 (2 probes), and Eukaryotic translation initiation factor 2-alpha kinase 4.

3.5. Test of Selection on Gene Expression Variation

The results of the EVE selection test are reported for each gene in the Supplementary Results. For the null hypothesis of a higher variance among populations than what was predicted by the phylogeny, the tests were significant at $p < 0.05$ for 235 genes. However, none were significant after a multiple test correction using the BH procedure ($p > 0.10$ in all cases).

4. Discussion

Taking together the results of the one-way and two-way ANOVAs, 553 genes showed differences in gene expression among localities after the transplantation of the clams to the experimental facility and their gradual acclimation to a common environment for a period of ca. 3 months. This represents 5% of the genes included in the microarray. The number of DEGs was estimated by applying a significance threshold of a 5% FDR to account for multiple testing, which is quite a stringent threshold. Gene expression differences probably occur in a higher number of genes. The number of differentially expressed genes in our study at a single-test significance threshold of $p < 0.05$ was 2287 (20%), and this can be considered well above the real number since multiple testing would result in a large fraction of false positives. Therefore, assuming that the probes contained in the microarray are representative of the whole genome, we can conclude that an important number of genes (at least 5% but less than 20%) showed differences in expression across populations of *R. decussatus* clams. Previous studies in animals have reported highly variable fractions of genes showing differences in gene expression among populations: to cite a few diverse examples, 0.1% in a marine snail [46], 7.8% in humans [47], and 12% in the killifish [7]. These studies are only loosely comparable since they differ in critical aspects, such as the technique used (microarray or RNA-seq), the number of replicates, the sample sizes, the statistical significance threshold selected, and whether the organisms had been acclimated in the laboratory before performing the study or were analyzed immediately after being sampled in the field. Thus, the number of DEGs found among populations of the GCS clam is within the range of values observed in other animal species.

The one-way and the two-way ANOVAs rendered similar numbers of DEGs among populations (418 and 412 genes, respectively). The similarity of these numbers hides the fact that only 278 probes (50%) were significant in both analyses (Figure 5B). A total of 140 genes (24%) that were significant in the one-way ANOVA were not significant in the two-way ANOVA. This suggests that although the factors race and latitude captured a substantial amount of the interindividual variation in gene expression, the variation at one-quarter of the DEGs could be due to other factors. Conversely, 134 probes that were significant in the two-way ANOVA were not significant in the one-way ANOVA, suggesting that the partition of the variance performed by the two-way ANOVA resulted in a statistical power increase that led to the detection of many new DEGs.

Our study was based on a "common garden" design, addressed to detect genes whose expression at the population level was potentially affected by genetic differences between populations. With the logic of this design, the observed gene expression differences between

clams sampled in different localities but kept under a common environment for a long time are interpreted as innate differences in gene expression. There are two potential sources of innate differences. One is the existence of differences in the allelic composition of the populations at the genomic regions responsible for transcription regulation. These genetic differences among populations would have been produced either by the classic neutral population genetic mechanisms of mutation, genetic drift, and gene flow limitation or by natural selection. The second potential source of innate differences is the action of epigenetic memory mechanisms elicited by the local native environments, which could still be acting after the acclimation to the experimental setting [48]. For example, differences among populations in DNA methylation patterns potentially involved in epigenetic regulation have been shown in in another bivalve, the oyster *Crassostrea gigas* [49,50]. Specific studies will be necessary to determine the existence and extent of epigenetic differences that influence the transcription patterns associated with the response to seawater warming and those observed between the two clam races.

Irrespective of whether epigenetic factors play a role, the most obvious source of innate transcriptional differences in the studied clams is genetic drift that results in the genetic differentiation of transcriptional regulatory genomic regions. Genetic drift should have had a principal role as the two races involved in this study appeared as a result of the species subdivision in the Pleistocene [30]. Moreover, the present-day limitation of gene flow at the Almeria–Oran front probably also contributes to the maintenance of the genetic differences between the two races, as it appears to happen in other species (e.g., [30,51]. An additional source of transcriptional differentiation due to genetics is isolation by distance between populations within the Atlantic and the Mediterranean marine basins. Previous studies with microsatellites showed that the amount of genetic differentiation (F_{ST}) among pairs of Atlantic populations reached 0.08, and values for two West Mediterranean populations on the Spanish coast reached 0.04 [31]. These values indicate that the gene flow within each basin is relatively limited, which allows for an important effect of genetic drift at intermediate to high geographic distances in the long term.

If the expression differences among populations were the result of genetic drift alone, the pattern of overall gene expression differences among populations would match the pattern of genetic relationships derived from the population genetic data, as they appear in the phylogenetic tree shown in Figure 1. In other words, the two Atlantic and the two Mediterranean populations should be transcriptionally more similar to each other than to any population of the other race. The cluster analysis of the DEGs detected by the one-way ANOVA departed slightly from this expectation. Although the samples from the two Atlantic populations clustered together, the MN population appeared basal to the other three, suggesting that this population was transcriptomically more differentiated (Figure 3). Results of the other analyses also indicate some differences in the MN population. For example, contrary to the other three populations, the MN population showed a higher proportion of upregulated genes than downregulated genes (Figure 4). Moreover, genes that demonstrated significant interactions in the two-way ANOVA showed a clearly distinct level of expression in the MN population (Figure S5, Supplementary Tables and Figures). These observations suggest that factors other than the historical population split and isolation by distance have influenced the pattern of gene expression differences across the clam populations observed in this study, and that those factors are, at least in part, specific to the MN population. The clams collected in that population experienced a high mortality from the very moment of their capture in the lagoon of Thau, while mortality in the clams collected from the other localities was very low. This means that the health status of the MN clams was already compromised at the moment of sampling. We can only speculate about the causes of such health impairment. Harsh winter conditions in the lagoon could have played a role, as the temperatures recorded in the lagoon in the winter were much lower than in any other population (Figure 2), and we have observed a winter mortality of clams maintained in captivity at the IATS-CSIC facilities due to similarly low seawater temperatures while performing other studies (unpublished results). The

effect of disease seems less probable, as there were no significantly enriched GO terms related to disease response in the MN-specific DEG group. Alternatively, the pattern of the transcriptomic differentiation of MN population could be due to a different genetic makeup of the population. Some support for this explanation comes from the population genetic data. Allele C at locus TBP has been previously recorded in clam populations from the Adriatic and Aegean seas only [30]; however, in the present study, it appeared in the MN clams at a very low frequency (Table S1, Supplementary Tables and Figures). This points to the presence of some individuals of Eastern Mediterranean ancestry among the MN clams, which could be native or introduced. More detailed genetic studies of the clam populations in southeast France are necessary to clarify this point.

In spite of the particularities detected in the transcription profiles at the MN population, the results point to a large fraction of DEGs not being affected by these local features. Actually, the results showed that the observed patterns of transcription variation among clams at three-quarters (413) of the 552 detected DEGs can be explained by two single factors: the race the clams belong to and the latitude of the locality from which they were sampled. Ninety per cent (373 out of 413) of the significant genes in the two-way ANOVA provided significant results for only one of the two factors, and only 38 genes showed significant variation for both factors and/or their interactions. Overall, the Race factor seems to have had a stronger effect on transcriptomic differentiation among populations, as it explained the variation observed at 337 genes (62% of the 552 detected DEGs), while the sampling latitude explained only one-third of the variation (104 genes, or 19%).

4.1. Transcriptional Differences between Races

Previous work using intron polymorphisms and microsatellites showed that populations of the GCS clam from the Atlantic and the western Mediterranean were differentiated in allele frequencies at a large fraction of the scored genes and can therefore be considered different races, which were called ATL and WMED, respectively [30,31]. A third race comprising populations from the Aegean and Adriatic seas (AEGAD) was also detected [30]; however, populations of this race were not included in this study. Our initial hypothesis was that the population subdivision should have resulted in the accumulation of nucleotide differences along the whole genome, including the regulatory regions of coding genes, and therefore should result in differences across populations in the transcription levels of many genes.

The results of the two-way ANOVA showed statistically significant expression differences between the Atlantic and West Mediterranean populations for 337 genes (3%), confirming the previous hypothesis. This result constitutes the first transcriptomic characterization of the two clam races and also (to our knowledge) of any marine species exhibiting genetic or phylogeographic differentiation across the Mediterranean Sea and the nearby Atlantic Ocean. Although other studies of population transcriptomics have included samples from regions separated by phylogenetic breaks, specific characterization across the breaks was not properly addressed in those studies. Increased transcriptomic differentiation between Atlantic populations and one single Mediterranean population separated by a phylogeographic barrier was observed in the seagrass *Zostera marina* [24]. However, to our knowledge, no other transcriptomic screening addressed specifically to a phylogeographic system has been carried out thus far.

The results prompt several questions regarding the clam races. One of the most interesting questions is: to what extent do the observed differences in transcription result in differences in the clams' physiology? We have approached this question by performing the functional characterization of the differentially expressed transcripts with the Gene Ontology (GO) system and through enrichment tests of the GO categories. This approach has several limitations, the most important being that GO annotation in bivalve mollusks (as well as non-model invertebrates in general) is quite underdeveloped. Only 40% of the DNA sequences used to design the microarray probes could be associated to a known coding sequence in other organisms by BLAST family tools, and only a fraction of these

had associated GO terms [38]. It is also well-known that the transcript levels are only loosely related to the amount of protein in the cells [52]; therefore, transcription differences between individuals or groups are only indicative of potential physiological differences. For all these reasons, the conclusions based on transcription differences and GO analyses should be taken with great care and will require independent testing in other clam samples by additional studies [53].

If the transcriptional differences were generated by a random, genome-wide variation resulting from mutation and genetic drift after the Pleistocene split of the species in the Atlantic and Mediterranean subpopulations, it would be expected that many genes scattered over the whole genome and with different functions would show expression differences. Our results fit this expectation. GO terms associated with the race-related set of DEGs show that this gene set was functionally very diverse, with proteins pertaining to nuclear, cytoplasmic, membrane, cytosolic, and extracellular parts and showing a large variety of molecular functions.

Similarly, random differentiation would result in ca. 50% upregulated genes in each marine basin. However, 68% upregulated genes were observed in the Mediterranean, which departs substantially (although not dramatically) from this expectation. GO term enrichment tests indicate that some GO categories were significantly overrepresented in the set of race-associated DEGs. The most outstanding were a group of GO terms associated with a total of 45 genes that code for proteins involved in the translation machinery (Supplementary Results). Nineteen of these genes code for ribosomal proteins, and most of them (14) were upregulated in the Mediterranean race. Other genes involved in translation were eukaryotic translation factor 3 and NSA2 ribosome biogenesis homolog. These results suggest that protein synthesis routes could demonstrate important differences in the two races, although it should be considered that the regulation of the expression of ribosomal proteins is only shallowly understood in metazoans. Moreover, these proteins may have other functions aside from their role in ribosome assembly [54].

In addition to the GO terms related to protein biosynthesis, another 18 terms were significantly enriched in the race-associated DEGs. These can be summarized in three categories: development, cell differentiation, and extracellular space. These terms are associated with some of the ribosomal proteins and translation-related proteins, clearly demonstrating the large influence that the differences in the transcription levels of ribosomal and protein-biosynthesis-related proteins between the clam races might have on other aspects of their biology aside from protein synthesis. However, these terms include many other proteins as well. The significant enrichment for the carbohydrate derivative metabolic process is especially interesting from the physiological perspective. This term involves 15 DEGs, some of which code for proteins of the mitochondrial energy production processes, such as adenylate kinases, cytochrome c oxidase subunit IV, ATP synthase subunits, or NADH dehydrogenase. Other DEGs code for enzymes involved in the post-translational modification of proteins, such as glycosilations and aminations (glutamine-fructose-6-phosphate transaminase 1), and others play a role in the biosynthesis and transport of glycosilated lipids (phosphatidylinositol glycan anchor biosynthesis, class X, and GM2 ganglioside activator). The GO term enrichment tests also showed enrichment for terms related to proteins of the extracellular spaces. Six DEGs induced significance of the cysteine-type endopeptidase activity GO term. Cysteine type endopeptidases have a role in the catabolism of proteins in the lysosomes. These proteins have important roles in many aspects of cell biology, such as tissue growth, cell differentiation, signaling, or immunity. The GO terms and the genes summarized above are just a fraction of the whole set of GO terms associated with genes that are significantly differentiated in expression between the two races. The large number of enriched GO terms suggest that many cell and physiological functions can be affected by differential transcription levels between the two clam races and might therefore result in physiological differences between them. The physiological differences between the clam races is a topic that should be examined in detail in future studies.

The great majority of observed expression differences between races should be the result of genomic differences accumulated during the separation of the Atlantic and Mediterranean lineages during the Pleistocene due to genetic drift. However, we cannot exclude the possibility that the environmental changes that took place during the glacial periods had an influence on the transcription rates of clams in their Atlantic and Mediterranean refuges. The fact that a fraction of the genes that were found to be significant for the Race factor in the two-way ANOVA were also significant for the Latitude factor suggest that their expression may be influenced by temperature differences between the northern and southern localities in present times, but this response could have been modelled by differences in temperature between the two glacial refuges (see next section).

4.2. Transcriptional Differences Associated to Latitude

In the two-way ANOVA, the northern clams showed increased transcription of 104 probes compared with the southern clams. As many as 34 genes were also significant for race, but 70 were significant for latitude only, and they are candidates for a transcriptional response to temperature change. The GO term enrichment tests showed a clear overrepresentation of two types of enzymes: glutathione transferases (GST) and ATP synthase subunits (ATPK). GST is a family of enzymes that is well-known for their participation in glutathione-based xenobiotic detoxification phase II mechanisms [55–57]. They also neutralize the secondary metabolites produced during oxidative stress [56]. Temperature increase is a source of oxidative stress; accordingly, several studies have seen GST expression related to an increase in environmental temperature in the gills of marine bivalves [58,59]. Some authors have indicated that temperatures of 25 °C or higher are stressful for the GCS clam [60]. In the present study, this temperature was approached but never reached. A lack of differential stress experienced by the northern and southern clams is also suggested by the absence of heat-shock proteins in the group of DEGs. Therefore, it is probable that the northern clams have simply higher transcriptional levels, either constitutively or as a plastic response to temperatures that are less frequent in their normal habitat. In addition to the enrichment of the GST, other data support the involvement of glutathione-based xenobiotic detoxification phase II mechanisms. Specifically, two other probes with BLAST hits to enzymes related to glutathione metabolism were significant for the Latitude factor: glutathione peroxidase (gene #1975) and glutathione synthase (gene #4887) (Supplementary Results).

ATP synthase (ATPK) is a multimeric enzyme with the essential role of coupling the electron transport chain with the synthesis of ATP in the cellular respiration process inside the mitochondria [61]. Due to its position in the proton transfer enzyme network, it also has a role in protecting against oxidative stress. Several studies have shown a relationship between ATPK expression and environmental temperature in aquatic organisms, including bivalves. Increased expression of ATP synthase has been found in the muscle of cold-acclimated carps (*Cyprinus carpio*) [62,63]. Li et al. [64] reported an increased expression of ATPK at both high and low stressful temperatures in the razor clam *Sinonovacula constricta*. In the mussel *Geukensia demissa*, ATPK expression increased in the autumn in parallel to a decrease in the water temperature [65]. Wijers et al. [66] found increased amounts of ATPK in human skeletal muscle cells taken from biopsies of human males exposed to a cold temperature for three days. All these studies suggest that the expression of ATPK is frequently upregulated in response to a decrease in environmental temperature. These studies support the view of the observed higher expression of ATPK in northern clams is a result of constitutive expression associated to their cool native environment.

GSTs and ATPKs represent only a few of the 107 genes that demonstrated differential expression between the northern and southern clams. While only enriched terms related to oxidative stress response and ATPK were found in the latitude-only set of genes, it is worth noting the presence in it of several genes coding for proteins of the mitochondrial electron transport, such as NADH dehydrogenases or cytochrome b, which interact intimately with ATPK and were upregulated in the northern populations. All together, these observations

strongly suggest that the upregulation of some electron transport chain proteins is constitutive in the GCS clams living in cool environments. This conclusion is in line with studies of Antarctic fishes in which the upregulation of oxidative chain genes was described and explained as an adaptation to the cold environment [67].

A fraction of the genes which were found to be significant for the Latitude factor in the two-way ANOVA were also significant for the Race factor, indicating expression differences between races. It is possible that these genes respond to the temperature differences between northern and southern localities in present times, and this response could have been modeled in the past by differences in temperature between the two glacial refuges. This possibility makes sense in light of the different opportunities for the clam populations to move latitudinally during glaciations. Movement of the Mediterranean populations from north to south in response to the advance of the cold wave southwards would have been restricted by the presence of the northern African coasts. However, the populations in the Atlantic could have moved more to the South along the west coast of Africa, staying in their optimal, warmer environment. These differences could have resulted in natural selection operating in the Mediterranean population to cope with the cooler environment, favoring the action of natural selection to modulate transcriptional levels in genes related to thermal response. These differences could have remained until present times, giving rise to our observations.

4.3. Potential Adaptive Role of the Observed Differential Expression

A recurrent topic of research regarding transcriptomic variation among individuals and populations is whether the differential expression is caused by the accumulation of neutral polymorphisms in the genome or if they are due to adaptive changes in the DNA, driven by natural selection. Several methods of performing tests for adaptive transcription differences have been proposed, but all methods seem to have pitfalls [68]. Previous studies of differences in gene expression among conspecific populations have used comparisons between DNA and quantitative genetic variation with F_{ST}/Q_{ST} ratios [69], phylogenetic contrasts [7], and, more recently, the partition of variance [45]. Variance contrast tests were developed to deal with study designs similar to the one used in this work, based in population sampling. In this study, we adopted this approach using the EVE (Expression Variance and Evolution) software [45]. We found no clear evidence for selection because although more than 200 genes were significant for the test, correcting for multiple testing resulted in no gene demonstrating a significant deviation from the neutral expectation. This result should be taken with great care for several reasons. Firstly, the test was based on the partition of phenotypic variance according to the phylogeny, assuming a complete split of taxa [45], and the effect of gene flow among taxa was not considered. Moreover, even if the tests were robust with respect to deviating from the model assumptions, the power of these tests is probably low in this particular case because of the small number of populations scored. We know of only one application of EVE to intraspecific populations in which significant results were obtained [70]. That study differed from ours in three ways: a slightly larger number of populations, the use of RNA-seq instead of a microarray, and the fact that the samples compared were not kept in a common environment before the expression study and were therefore influenced by environmental differences among the sampled habitats; this probably resulted in an increased gene expression variation due to plasticity, which could confound with the genetically based variation.

In the absence of powerful neutrality tests, correlations with environmental parameters provide an alternative means to search for candidates to adaptive evolution. Our factorial sampling scheme (race × latitude) allowed for the separation of the effects of genetic differentiation between races from the effects of the latitudinal thermal gradients operating in the study area. The set of 70 genes that showed latitude-related differential expression but not differences in expression between races constitute a set of candidate genes for response to thermal variation. As these latitude-associated differences have been observed after a long period of acclimation to a common environment, the conclusion is

that they were caused by genetic differences in the expression of the genes which were driven by adaptation to the thermal characteristics (and possibly other factors associated to temperature) of the habitats from which the clams were sampled.

An alternative explanation for the observed latitude-related differences in gene expression is that they are due to neutral genetic differences between the north and south populations in each basin. Molecular dating suggests that the differentiation between the Atlantic and West Mediterranean races was due to historical separation in the Pleistocene, no earlier than 400,000 years before present (YBP) and as far as 2 million YBP [30]. Therefore, the clam populations of the two basins were evolving independently over one or more long periods of time in the past, resulting in highly differentiated races. Moreover, the present-day gene flow is probably limited between them due to the Almeria–Oran Oceanographic Front (AOF) barrier. As a consequence of this independent evolution in the two basins, it would be expected that, by chance, many genes that show gene expression differences between the north and south populations in one basin would show differences in the opposite direction in the other basin. However, the observation of only 6 out of 70 genes that demonstrate contrasting patterns of expression between the north and south in the two basins argues against this possibility and supports an adaptive role of the observed latitude-related expression variation in the two parallel thermal gradients that occur at both sides of the Iberian Peninsula.

5. Conclusions

We have shown that populations of *R. decussatus* constituting the two races separated by the Atlantic–West Mediterranean phylogeographic break and that span parallel latitudinal gradients in temperature show clear differences in gene expression in a remarkable portion of their genes after a relatively long period of acclimation to a warm, Atlantic environment. A large fraction (3/4) of these differences can be explained by the simple, direct effects of two factors: the race the clams belonged to and the latitude at which they were sampled. The differentiation patterns were different for each of these causal factors. In the case of racial expression differences, most genes showed higher expression in the Mediterranean, but a significant fraction (40%) showed the opposite pattern. This observation is concordant with an explanation based on the random differentiation of populations due to genetic drift during historical isolation caused by sea level changes during the Pleistocene, although the additional implication of natural selection cannot be discarded. An important fraction of the DEGs between races are related to the protein biosynthesis machinery and ribosomal function, suggesting that the two races could show important differences in their functional aspects regarding adaptation to global warming and aquaculture. Additional research will be required to find out if the observed differences between races are a response of the Mediterranean race to their transplantation to Atlantic waters or were already present in their native habitats. In the case of the genes that are significant for latitude, the observation of a higher expression of almost all significant genes in clams coming from the northern localities, which usually experience lower temperatures than those in the south, independently of their Atlantic or Mediterranean genetic background suggests that we have recorded a constitutive expression response to the N–S temperature gradient that may have a genetic basis. The analysis of GO term enrichment in the set of genes whose expression was correlated with latitude uncovered that glutathione metabolism and energy production in the mitochondria are key metabolic routes that should be explored in the quest to understand the adaptation of grooved carpet shell clams, and possibly bivalves in general, to ocean warming.

Figure S3: Heatmap showing gene expression variation across samples for the 105 genes which were significant for the Race factor in the 2-way ANOVA and contributed to the associated enriched GO terms, Figure S4: Heatmap showing gene expression variation across samples for the genes which were significant for Latitude only in the 2-way ANOVA and contributed to the enriched GO terms, Figure S5: Average expression in populations for the 9 genes which were significant for Race × Latitude interaction in the 2-way ANOVA.). Supplementary Results (csv format).

Author Contributions: Conceptualization, C.S.; methodology, C.S., M.M., R.B.L., D.C. and L.B.; software, C.S., M.M. and D.C.; validation, C.S., M.M., D.C. and L.B.; formal analysis, C.S., M.M. and D.C.; investigation, C.S., M.M. and D.C.; resources, C.S., M.M., R.B.L., M.L.C., T.P. and L.B.; data curation, C.S., M.M. and D.C.; writing—original draft preparation, C.S.; writing—review and editing, C.S., M.M., R.B.L., D.C., T.P. and L.B.; visualization, C.S. and D.C.; supervision, C.S. and L.B.; project administration, C.S., M.L.C. and L.B.; funding acquisition, C.S., M.L.C., T.P. and L.B. All authors have read and agreed to the published version of the manuscript.

Institutional Review Board Statement: Not applicable. According to the Spanish law, which is transposed form the European Union directives, studies with marine invertebrates do not require to be supervised by the Ethics Committee of the research institutions, with the only exception of Cephalopods (octopuses and the like), which is not the case.

Data Availability Statement: Raw data are available at the GEO database under accession GSE223000.

Conflicts of Interest: The authors declare no conflict of interest.

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