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RESEARCH ARTICLE

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Life in the intertidal: Cellular responses, methylation and epigenetics

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Abstract

1. Phenotypic plasticity is essential for the persistence of organisms under changing environmental conditions but the control of the relevant cellular mechanisms including which genes are involved and the regulation of those genes remains unclear. One way to address this issue is to evaluate links between gene expression, methylation and phenotype using transplantation and common garden experiments within genetically homogeneous populations.
2. This approach was taken using the Antarctic limpet *Nacella concinna*. In this species, two distinct phenotypes are associated with the intertidal and subtidal zones. The in situ gene expression and methylation profiles of intertidal and subtidal cohorts were directly compared before and after reciprocal transplantation as well as after a common garden acclimation to aquarium conditions for 9 months.
3. Expression profiles showed significant modulation of cellular metabolism to habitat zone with the intertidal profile characterised by transcription modules for antioxidant production, DNA repair and the cytoskeleton reflecting the need to cope with continually fluctuating and stressful conditions including wave action, UV irradiation and desiccation.
4. Transplantation had an effect on gene expression. The subtidal animals transplanted to the intertidal zone modified their gene expression patterns towards that of an intertidal profile. In contrast, many of the antioxidant genes were still differentially expressed in the intertidal animals several weeks after transplantation into the relatively benign subtidal zone.
5. Furthermore, a core of genes involved in antioxidation was still preferentially expressed in intertidal animals at the end of the common garden experiment. Thus, acclimation in an aquarium tank for 9 months did not completely erase the intertidal gene expression profile.
6. Significant methylation differences were measured between intertidal and subtidal animals from the wild and after transplantation, which were reduced on common garden acclimation. This suggests that epigenetic factors play an important role in physiological flexibility associated with environmental niche.

KEYWORDS
Antarctic, antioxidants, cytoskeleton, glutathionylation, methylation-sensitive amplified polymorphism (MSAP), *Nacella concinna*, pentose shunt pathway, reactive oxygen species

1 | INTRODUCTION

Species distributions and fitness traits are significantly impacted by both biotic and abiotic factors in their immediate habitat (often called genotype–environment interactions); however, our current understanding of which genes underpin these processes and their regulation remain limited (Forsman, 2015). This is further complicated by the fact that many species exhibit flexible responses, whereby highly variable phenotypes may be expressed in the absence of genetic differentiation. This phenomenon of phenotypic plasticity frequently occurs in molluscs where the morphological characteristics of shell shape and thickness can vary considerably with environmental factors. These morphological variants (often called ecotypes) can be induced by a variety of abiotic and biotic factors including hydrodynamic stress, temperature, desiccation and predation (Harley, Denny, Mach, & Miller, 2009). Examples include *Littorina striata* in which a nodulose form is associated with wave-sheltered sites and a smooth form dominating wave-exposed sites; increased shell thickness in *Littorina obtusata* in response to the predatory green crab *Carcinus maenas* and the Antarctic species *Nacella concinna* and *Laternula elliptica*, where shell thickness varies with depth and the incidence of ice berg scour respectively (De Wolf, Backeljau, & Verhagen, 1998; Harper et al., 2012; Hoffman, Peck, Hillyard, Zieritz, & Clark, 2010; Trussell & Nicklin, 2002). In these cases, genetic homogeneity has been demonstrated across phenotypes, but in other species heritable components and genetic sub-structuring has been shown within ecotypes and linked to microhabitats. The classic examples of such phenomena include the periwinkles *Littorina saxitilis* and *Littorina fabalis* (Johannesson & Mikhailova, 2004; Johannesson & Tatarenkov, 1997), while in other species, such as *Mytilus* there has been a clear demonstration of cryptic speciation (Grant, Cherry, & Lombard, 2010). These studies emphasise the importance of understanding the genetic background of the species under study when examining phenotypic plasticity.

The current understanding of the molecular basis of phenotypic plasticity is limited, but such information is essential not only for understanding how animals function and interact with their immediate environment, but also for predicting their capacities to cope when that environment changes (Somero, 2010). For example, such genomic analyses can help decipher the cellular mechanisms underlying the competition advantage of closely related species (Lockwood, Sanders, & Somero, 2010), dissect the subtleties of multiple environmental stressors (Chapman et al., 2011) and the impact of age on the stress response (Clark et al., 2013). How these expression patterns are modulated, or more importantly fixed, is unclear in most cases but epigenetics is increasingly being revealed as a key factor implicated in gene regulation in the natural environment (Bossdorf, Richards, & Pigliucci, 2008).

In this study, the Antarctic limpet *N. concinna* was used to investigate cellular mechanisms underpinning phenotypic plasticity. This is one of the most abundant species in the Antarctic marine environment occurring in the intertidal zone and subtidally down to 100 m or more (Powell, 1951). This is not a homing limpet (Walker, 1972) and until recently two distinct ecotypes were recognised: an intertidal *polaris* form, with a taller, thicker shell and a subtidal *concinna* form with a much lighter, flatter shell that is often microscopically "scalloped" due to the grazing by other limpets on encrusting endolithic algae (Nolan, 1991). Physiological differences have also been reported between the two ecotypes, including tolerance to experimental freezing, metabolic response to air exposure, righting ability under different temperatures, thermal tolerances and wet tissue mass (Morley, Clark, & Peck, 2010; Waller, Worland, Convey, & Barnes, 2006; Weihe & Abele, 2008; Data S1 and S2). This species is a broadcast spawner with a free swimming planktonic veliger stage that lasts 1–2 months (Peck, Heiser, & Clark, 2016) and recent population genetic analyses using amplified fragment length polymorphisms (AFLPs) showed no evidence of genetic differentiation between the two ecotypes (Hoffman et al., 2010). Hence, this species displays considerable physiological and morphological plasticity against a genetically homogeneous background and represents a good candidate for the molecular investigation of phenotypic plasticity.

The aim of this study was to investigate not only the gene expression profiles underpinning the physiological flexibility of *N. concinna* to the intertidal and subtidal (15 m) zones, but also to use methylation-sensitive amplified polymorphism (MSAP) analyses to identify whether epigenetic factors may be involved in the gene expression differences. To achieve this, two experiments were carried out: a reciprocal transplant experiment in the Antarctic and a common garden experiment where collections of intertidal and subtidal animals were held in the Cambridge aquarium system for 9 months to ensure full acclimation to laboratory conditions. It was expected that there would be significant differences in the gene expression and methylation profiles of intertidal and subtidal animals in the wild and that transplantation to a different shore zone would modify these. It was also expected that the extended acclimation in a "common garden aquarium" would reset and remove any differences in gene expression and methylation between intertidal and subtidal cohorts.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Animals were collected at Rothera Research Station, Adelaide Island, Antarctic Peninsula (67°34′07″S, 68°07′30″W) during the austral

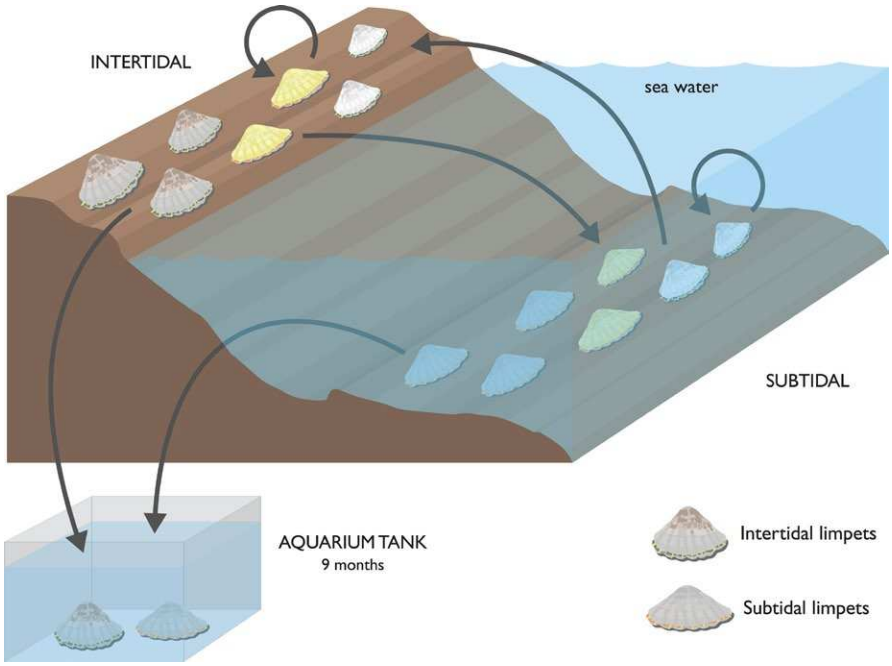

FIGURE 1 Representation of both the transplant and acclimation experimental designs

FIGURE 2 Intertidal and subtidal environments near Rothera. (a) General view of the intertidal region around Rothera base; (b) direct view of the gully where the limpets were transplanted, filled with brash ice; (c) transplanted limpets in the intertidal; (d) transplanted limpets in the subtidal at 15 m. Photos courtesy of Lloyd Peck

summer. Intertidal animals were hand collected and subtidal animals collected by SCUBA divers from 15 m. The animals produced copious amounts of mucus when detached from rocks during collection so they were held in the flow-through aquarium at Rothera under ambient sea water conditions (*c.* 0°C) for 2 days prior to sampling to disperse the mucus. To distinguish the transplanted animals, the shells were painted yellow (120 intertidal animals) and white (120 subtidal animals). Of these, 60 were returned to their original habitat and 60 were transplanted to the alternative regime (Figure 1). The intertidal animals were placed at the shore end of a long gently sloping rocky gully (*c.* 8 m long), which then dropped steeply into deep water. The subtidal animals were placed on large flat rocks at 15 m (Figure 2). One month later, two sets of transplanted animals were sampled (each of $n = 24$). Three sets of controls (each of $n = 24$) were also sampled from each zone: intertidal and subtidal animals at time point zero, intertidal and subtidal animals at 1 month (time/seasonal control) and painted animals that had been placed back into their original habitat and sampled at 1 month (manipulation controls) (Figure 1, Table 1). For each animal, the shell length, height and width were measured with vernier calipers (mm).

2.2 | Limpet migration

During the experiment, the transplanted animals started to move back to their original habitat; hence, ad hoc observations of the movement of the transplanted limpets were carried out. Painted

TABLE 1 Explanation of the experimental regimes and associated sampling

Samples	Experimental value
Subtidal controls	
Subtidal animals sampled at time 0 (STC)	Control at the start of the experiment
Subtidal animals sampled at 1 month (ST1M)	Control at the end of the experiment (temporal control)
Subtidal animals, shells painted and replaced at 15 m (STST)	Control for experimental manipulation (painted) and also time, as sampled at the end of the experiment (temporal and manipulation control)
Intertidal controls	
Intertidal animals sampled at time 0 (ITC)	Control at the start of the experiment
Intertidal animals sampled at 1 month (IT1M)	Control at the end of the experiment (temporal control)
Intertidal animals, shells painted and replaced into the intertidal zone (ITIT)	Control for experimental manipulation (painted) and also time, as sampled at the end of the experiment (temporal and manipulation control)
Transplanted animals	
Subtidal animals transplanted to the intertidal zone (STIT)	Transplant
Intertidal animals transplanted to 15 m (ITST)	Transplant
9-Month acclimation	
Intertidal animals acclimated in the aquarium for 9 months (ITA)	Common garden acclimation in the aquarium
Subtidal animals acclimated in the aquarium for 9 months (STA)	Common garden acclimation in the aquarium

Sample codes are in brackets.

shell colour was noted and animal position logged either by measuring how far they had moved from the original site they were transplanted to or by ranking their position in terms of distance moved.

2.3 | RNA extraction protocol and sequencing

RNA was extracted from the foot tissue (n = 6) for each set of control and transplanted animals using TRI reagent (Bioline) and purified on RNeasy mini-columns (Qiagen) according to manufacturers' instructions. RNA was quantified using a NanoDrop ND-1000 (LabTech International). The individuals from each treatment (n = 6) were pooled in equal amounts for RNA-Seq to produce a single library for each treatment. An initial sequencing run, using two separate libraries of pooled subtidal and intertidal control animals was performed on the Roche 454-GS-FLX Titanium platform (University of Cambridge, Department of Biochemistry Sequencing Facility) to confirm expression differences between the intertidal and subtidal cohorts before more extensive sequencing of the transplant experiment. Further sequencing was performed on all control and transplant treatments on an Illumina GAIIx at Edinburgh Genomics.

2.4 | Transplant experiment: Generation of backbone transcriptome and mapping

The sequences from the original 454 pyrosequencing run (11,688,843 reads) were assembled using Newbler (Roche), yielding 9,801 contigs with a mean length of 646 bp. These were annotated using the GenBank nr database (Benson, Karsch-Mizrachi, Lipman, Ostell, & Wheeler, 2007) using a threshold value for annotation of any matches below $1e^{-10}$. Two replicate runs from each of the six treatments were run on the GAIIx resulting in 30-bp sequences of subtidal control time 0 (STC) = 11,695,110; subtidal control 1 month (ST1M) = 4,723,402; subtidal painted control (STST) = 5,475,497; intertidal control time 0 (ITC) = 4,785,089; intertidal control 1 month (IT1M) = 3,930,267; intertidal painted control (ITIT) = 16,991,773; intertidal transplant (ITST) = 5,440,616; subtidal transplant (STIT) = 5,209,272. These were mapped onto the reference contigs using Maq (Li, Ruan, & Durbin, 2008). Three approaches were used to determine the significantly differentially expressed contigs and two of these were applied sequentially to the transplant treatments for added stringency. A normalised transcripts per million (tpm) value from the mapping onto the contig was compared to the control set by dividing the tpm value to the maximum of the control set (for ST: STIT/max(STC,STC1M,STST)). A twofold cut-off criteria was required for selection at this stage. The use the counts was used in another approach in a ratio test where the mapping for a transplant set, for example STIT, was tested against each different control set independently (i.e. STIT compared to STC, STIT compared to STC1M and STIT compared to STST) and the p-values adjusted with a selection cut-off of .01. The final results were the joint selection of these two criteria. The Bayesian modelling program BAYSEQ (Hardcastle & Kelly, 2010) was used using this replicate structure to determine the population differences with an adjusted p-value cut-off of .05. Different cut-offs were used according to the approach as the selection criteria are independent; the TPM and fold change require stricter p-value cut-offs, whereas the Bayseq is already strict, and the cut-off value was chosen to retrieve the most significant resulting genes. The final selection was an intersection of these two independent

approaches. Those upregulated transcripts, which were putatively annotated using Blast sequence similarity searching were manually verified and then further analysed using the STRING program to visualise potential protein–protein interactions using UniProtKB human identifiers (https://string-db.org/).

2.5 | Acclimation studies

A further set of intertidal and subtidal animals was collected in the austral summer of 2014. These were transported to the UK and held separately in the Cambridge aquarium for 9 months in a common garden experiment. The aquarium is a re-circulation system held at 0 ± 0.1°C, salinity at 34 psu (±1) and a 12:12 light:dark regime. The animals were held in the same aquarium tank at similar densities, but the subtidal and intertidal cohorts were kept separated. They fed on algae growing on the aquarium walls. There were no mortalities during the acclimation period. The RNAs from foot tissue were extracted as described above from six similar sized animals from the intertidal (shell size = 25.28 ± 0.30 mm *SE* mean) and the subtidal (shell size 25.4 ± 0.48 mm *SE* mean). There was no significant difference between the shells sizes of each cohort ($T = 0.21$, $p = .41$, $df = 8$). The six RNA extractions from each of the intertidal and subtidal animals were pooled to form two libraries of subtidal and intertidal RNAs. TruSeqv2 libraries were made from each of the two pools and subjected to a MiSeq 75 bp paired end read run (University of Cambridge, Department of Biochemistry Sequencing Facility). Each library was mapped to the original transcriptome backbone and analysed as described above selecting significant differential expression on fold change and *p*-value adjusted ratio test.

2.6 | Methylation studies

DNA was extracted from the foot tissue of intertidal and subtidal animals (the controls and transplanted individuals as described above: Figure 1, Table 1) using the DNeasy Blood and Tissue kit (Qiagen) according to manufacturers' instructions. The DNA concentration and quality were checked by a spectrophotometer (NanoDrop, ND-1000) and an Agilent 2200 TapeStation system. To detect methylation patterns in the different DNA samples, a modified AFLP technique using methylation-sensitive restriction enzyme (*MspI* and *HpaII*) digestion was carried out (Data S3).

3 | RESULTS

3.1 | Transplantation, migration and recapture of transplanted limpets

The aim was to sample 24 animals for each treatment, including the controls. This was possible in all cases, with the exception of the subtidal animals transplanted to the intertidal zone where it was only possible to retrieve 19 individuals from the intertidal zone. All animals were sexually mature, although individual sexes were not noted. There was no significant size difference between any of the intertidal animals sampled in this experiment (one-way ANOVA $F_{3,95} = 0.57$, $p = .636$). The subtidal animals sampled showed significant differences in size (one-way ANOVA $F_{3,90} = 6.16$, $p = .001$). There was no difference between the three sets of subtidal control animals (Tukey > 0.05), but the retrieved animals transplanted to the intertidal were significantly smaller than the controls (Tukey < 0.05). These transplanted animals (mean length 19.8 mm ± 0.9 *SE*) and also the control subtidal animals, which were sampled, painted and put back into the subtidal (23.2 mm ± 0.8 *SE*) (difference in size: $T = 2.84$; $p = 0.007$; $df = 39$) were from the same original sample of 120 painted animals and were allocated at random between the two locations. Given the problems of retrieving a full set of 24 subtidal animals from the intertidal zone after a month, the most likely conclusion was that the larger animals were either faster or more capable at returning to the subtidal zone; hence, the observed difference in size of the retrieved transplanted subtidal animals.

This species is not a homing limpet (Walker, 1972); therefore, it was surprising to see that the transplanted animals were moving back to their original habitats. This migration was relatively rapid as evidenced at the final sampling by the inability to retrieve more than 19 subtidal individuals (from an original $n = 60$) from the intertidal gully, which included sampling in 1 m of water at low tide. At several time points during the experiment, the movement of animals was catalogued. After 3 days, ranking of animals in the intertidal gully showed that the transplanted subtidal individuals had migrated further down the shore towards deeper water than the intertidal limpets (Spearman rank order [rho] correlation = 1; $p < .0001$). At 3 weeks, the measurement of distance moved along the gully showed that the subtidal animals had moved significantly closer to the deeper water than the replaced intertidal animals (*t*-test: $T = -4.30$; $p < .0001$; $df = 32$). In the subtidal at 15 m, after 2 weeks most subtidal animals had not moved off the rocks they were replaced onto, but the transplanted intertidal animals had moved significantly towards more shallow depths (*t*-test: $T = 3.08$; $p = .006$; $df = 19$). In each case, animals that were replaced in their original habitat did not travel towards the opposite zone.

3.2 | Gene expression analyses from the transplant experiment

To determine if gene expression profiles had been modified with transplantation, the transcripts upregulated in the transplanted animals were compared with control animals from both their original environments and also the control animals from the zone to which they had been transplanted. This expression profiling revealed that intertidal transplants maintained the "intertidal gene profile" despite being transplanted. A much bigger change was found with the transplanted subtidal animals, which indicated that they were re-setting their cellular machinery to cope with their new stressful intertidal environment (Table 2). These shifts in gene expression patterns are described in greater detail below.

3.3 | Intertidal transplants compared with intertidal control animals (original habitat)

There was a small difference numerically in the expression profiles in this comparison, with only 14 unique annotations in the

TABLE 2 The number of transcripts upregulated in the transplanted animals compared with control animals in both the intertidal and subtidal zones and the acclimation experiment

Transplant	Expression profile comparison	No of transcripts upregulated	Largest expression differences
Intertidal	With original environment (intertidal)	154 (33)	
Intertidal	With transplant environment (subtidal)	553 (147)	+553
Subtidal	With original environment (subtidal)	523 (123)	+523
Subtidal	With transplant environment (intertidal)	180 (39)	
Acclimation	**Comparison**	**No of transcripts upregulated**	**UniProtKB annotations**
Intertidal	With aquarium acclimated subtidal animals	469 (135)	85
Subtidal	With aquarium acclimated intertidal animals	427 (96)	59

The numbers in brackets show the number of transcripts with annotation defined using Blast sequence similarity searching.

differentially expressed transcripts (Table 2), that is the intertidal transplants maintained a very similar profile to that of the original intertidal controls.

3.4 | Intertidal transplants compared with subtidal control animals (transplant habitat)

This comparison produced a higher number of upregulated transcripts (533 with 147 annotations [Tables 2 and S1]). Of these sequences, 123 were annotated via the UniProtKB Human identifiers and entered into the STRING program. Results showed significant enrichment of functional groups ($p = 2.29e^{-12}$, with expected interactions at $p = 8.88e^{+1}$), which resulted in five main transcription modules. These comprised transcripts involved in the respiratory chain (cytochrome oxidases MT-CO3, MT-CYB), protein production (RPL ribosomal proteins), antioxidants (including glutathione genes, caspases and superoxide dismutases), protein degradation (proteasome subunit genes such as PSMB3 and LMP7) and DNA repair (polymerases and ligases, e.g. POL and LIG genes). Genes involved in actin cytoskeleton pathways were distributed around the network (Figure 3, Data S4). These are all modules, which are often associated with the classical cellular stress response. The STRING enrichments showed support for the main functional groups identified through the manually verified Blast sequence similarity searching with an additional two transcripts annotated as potential shell matrix proteins, which would not have been identified in STRING due to the human centric nature of the data (Table S1). Thus, after 1 month at 15 m, the intertidal animals still retained their original intertidal gene expression profile.

3.5 | Subtidal transplants compared with subtidal control animals (original habitat)

In this comparison, 523 transcripts were upregulated with 123 putative annotations (Tables 2 and S2). Significant enrichment was observed ($p = 6.56e^{-4}$, with expected interactions at $p = 3.20e^{+1}$) using the STRING program (Data S5). The interactions produced more diffuse gene networks compared with the intertidal analysis. There was one main node centred on PA2G4 (proliferation associated 2G4 38 kDa protein), a gene involved in signal transduction and growth regulation. The interactions with this gene produced three branches leading to two modules of ribosomal proteins (protein translation) and another centred on MAP kinase signalling and the cytoskeleton (Data S5). The STRING analysis was reflected in the Blast annotations with over 16% of the identified transcripts putatively involved in either signalling, trafficking or transport (Figure 3). In addition, 9% of annotations showed a relationship with the cytoskeleton and two transcripts putatively involved in the shell matrix (carbonic anhydrase and chitin synthase). The latter are particularly significant as a response to this new harsh environment where there is considerable mechanical stress on the shells due to brash ice and wave action and thicker shells would be needed (Figure 2). Hence, these data indicated cellular processes in transition with subtidal transplants reconfiguring their cellular machinery towards coping with the more stressful intertidal environment.

3.6 | Subtidal transplants compared with intertidal control animals (transplant habitat)

Fewer transcripts were upregulated in this comparison with only 17 unique annotations (Table 2). This indicated that the subtidal animals were changing their gene expression to that of the "intertidal expression profile" (Table 2).

3.7 | Common garden acclimation studies in an aquarium

Even after 9 months in common aquarium conditions, the expression differences between the intertidal and subtidal cohorts were not completely erased (Table 2). The annotations of the acclimated subtidal animals showed no strong functional groupings or significant protein–protein interaction networks in the STRING program (Table S3). In contrast, significant enrichment was observed in the STRING program ($p = 4.31e^{-7}$, with expected interactions at

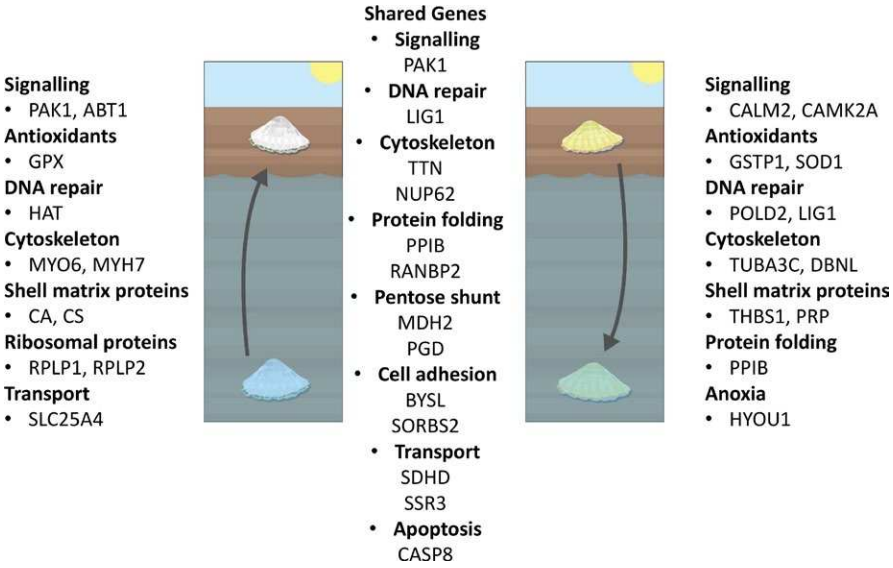

FIGURE 3 Summary of gene expression results, showing predominant functional groups in each transplanted group and shared transcripts compared with control subtidal animals. For a full set of gene IDs, see tables in Data S4, S5 and Tables S1, S2

$p = 1.14e^{+1}$) for the intertidal acclimated data. One small network of 17 genes was identified, which centred not only around transcripts putatively involved in glutathionylation (response to oxidative stress and signalling), but also included other transcripts involved combating reactive oxygen species (ROS), the pentose shunt pathway and membrane transporters representing a residue of the native intertidal gene expression profile (Figure 4, Tables 3 and S4). Thus, further validating the initial gene expression differences between intertidal and subtidal cohorts obtained from animals sampled in the wild.

3.8 | Methylation studies

ms-AFLP genotypes were generated for a representative subset of 33 individuals (controls, transplants and acclimated: 6 × ITC, 6 × STC, 6 × ITA, 4 × STA, 6 × ITST, 5 × STIT) for 172 loci. Of the 172 loci, 71 were classified as methylation-susceptible (MSL; 52 of these polymorphic) and 101 as unmethylated (NML; 45 polymorphic). There was a significant difference in ms-AFLP diversity between MSL and NML, with Shannon diversity indices of $S = 0.51 \pm 0.14$ *SD* and $S = 0.35 \pm 0.16$ *SD*, respectively ($W = 1787$, $p < .0001$). There was significant epigenetic differentiation (methylation patterns) among all groups ($\Phi_{ST} = 0.120$, $p < .001$) but no genetic differentiation across all non-methylated bands scored ($\Phi_{ST} = 0.009$, $p < .317$). Pairwise comparisons between subtidal and intertidal groups for three treatments (controls at the start of the transplant experiment, transplanted animals and 9-month acclimated individuals) revealed significant epigenetic (methylation) differentiation for C ($\Phi_{ST} = 0.131$, $p = .005$) and T ($\Phi_{ST} = 0.165$, $p = .009$), indicating epigenetic effects associated with habitat. This difference was not observed for the acclimated samples ($\Phi_{ST} = -0.004$, $p = .540$), suggesting loss of habitat-specific methylation patterns over the 9-month time-scale. There was no significant genetic differentiation for any of the pairwise comparisons (C $\Phi_{ST} = -0.058$, $p = .945$; T ($\Phi_{ST} = -0.019$, $p = .603$); 1M ($\Phi_{ST} = -0.005$, $p = .568$), substantiating previous AFLP population analyses of this species in this area (Hoffman et al., 2010).

4 | DISCUSSION

These data describe for the first time detailed intertidal and subtidal gene expression profiles and show that durable programmed alteration in gene expression plays a significant role in moulding life to the stressful intertidal lifestyle. The gene expression profiles of *N. concinna* from the intertidal and subtidal were changed on transplantation, but the extent of this change depended on the habitat of origin. The expression profile of transplanted subtidal limpets changed showing gradual acclimation to the stressful intertidal zone. In contrast, after a month spent at depths below 15 m, the expression profile of the transplanted intertidal animals largely remained the same. Although the metabolism of Antarctic benthic species is much slower than temperate animals (Peck, 2016), gene expression responses can be rapid with significant responses seen within an hour, as measured in heat shock experiments previously performed on this species (Clark, Fraser, & Peck, 2008). Hence, the lack or very slow rate of change in gene expression profiles of the transplanted animals was surprising and therefore other factors regulating gene expression must be involved. The subject of these experiments, the Antarctic limpet is a broadcast spawner and thus has the advantage that such evaluations are made on the basis that the ecotypes sampled are from the same genetic population. Therefore, the differences in expression profiles between intertidal and subtidal cohorts represented phenotypic plasticity and were not the result of genetic differentiation, as supported by current and previous genetic analyses (Hoffman et al., 2010). The intertidal and subtidal expression profiles were associated with differences in methylation patterns, which diminished in the common garden experiment over 9 months along with the dominating intertidal gene expression pattern. These data strongly suggest that epigenetic regulation (methylation) may

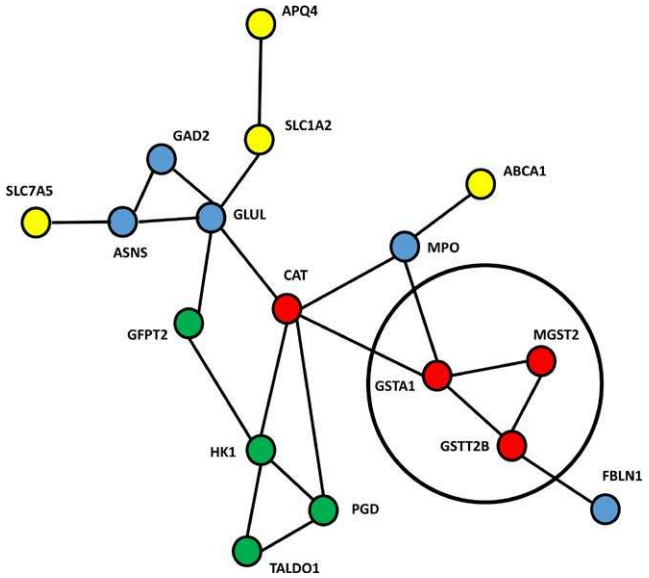

FIGURE 4 STRING output showing putative protein–protein interactions for 9-month acclimated intertidal animals. Transcripts represented by red circles are putative antioxidants; green circles: involved in the pentose shunt and carbohydrate metabolism; yellow: membrane transporters; blue: various functions: GAD2 (neurotransmission), ASNS (unfolded protein response), GLUL (glutamate metabolism), MPO: (immune), FBLN1 (cell adhesion). Transcripts involved in glutathionylation are grouped in the black circle

play a significant role in shaping the physiology of these animals to their particular habitat (intertidal or subtidal).

Physiologically, *N. concinna* shows considerable modifications according to habitat zone and depth (Morley et al., 2010; Waller et al., 2006; Weihe & Abele, 2008). In addition to the published data, further data are available showing that subtidal animals have almost double the wet body mass compared with intertidal animals (Data S2) and that the thermal limits of intertidal animals are higher. The upper lethal limit of animals warmed at 1°C/hr was 3.8°C higher in intertidal animals compared with those in the subtidal zone ($H = 23.50$, $df = 1$, $p < .001$) (Data S1). Thus, there is considerable evidence that there are defined physiological requirements for life in the two different zones, although there are clearly dependency effects. For example, the righting ability of both forms is related to shell shape, with righting being more efficient in intertidal animals (Morley et al., 2010). The intertidal animals are also better able to survive hypoxia as there is a bigger air pocket reserve in the taller shells (Weihe & Abele, 2008). Thermal tolerance can be related to the different thermal histories of the two zones, with intertidal animals regularly exposed to air temperatures in excess of the shallow subtidal waters (Clark, Geissler, et al., 2008). These data on limpets are validated by physiological differences found between intertidal and subtidal populations of other species. For example, evaluations of clam and mussel species show that intertidal animals exhibit metabolic rate depression, higher rates of anaerobic metabolism, ability

TABLE 3 Gene identifiers, names and putative functions of transcripts involved in the major network identified in the STRING program for 9-month acclimated intertidal animals

Gene ID	Gene name	Putative function
ABCA1	ATP-binding cassette subfamily A member	Membrane transporter
AQP4	Aquaporin 4	Membrane transporter
ASNS	Asparagine synthetase	Involved in the Unfolded Protein Response
CAT	Catalase	Antioxidant
FBLN1	Fibulin	Cell adhesion
GAD2	Glutamate decarboxylase 2	Neurotransmission
GFPT2	Glutamine-fructose-6-phosphate transaminase 2	Glucose flux into the hexosamine pathway
GLUL	Glutamate-ammonia ligase	Glutamate metabolism
GSTA1	Glutathione-*S*-transferase	Glutathionylation, antioxidant
GSTT2B	Glutathione-*S*-transferase	Glutathionylation, antioxidant
HK1	Hexokinase 1	Glycolysis
MGST2	Microsomal glutathione-*S*-transferase	Glutathionylation, antioxidant
MPO	Myeloperoxidase	Immune
PGD	Phosphogluconate dehydrogenase	Pentose shunt pathway
SLC1A2	Solute carrier family 1 member	Membrane transporter
SLC7A5	Solute carrier family 7 member	Membrane transporter
TALDO1	Transaldolase	Important for the balance of metabolites in the pentose shunt pathway

for air breathing and greater hypoxia tolerance compared with their subtidal cohorts (Altieri, 2006; Tagliarolo, Clavier, Chauvaud, Koken, & Grall, 2012). There are also differences between animals in the two habitats with regard to shell morphology, physiological energetics and fatty acid profiles (Freites, Labarta, & Fernández-Reiríz, 2002; Hinch, Bailey, & Green, 1986; Labarta, Fernández-Reiríz, & Babarro, 1997). However, to date, there has been little molecular data underpinning these physiological flexibilities.

The data described here clearly show considerable differences in limpet gene expression profiles related to zonation. These differences can be assigned to biochemical pathways, which correlate with these different capacities and underpin the phenotypic differences. The transplanted intertidal animals in the subtidal zone maintained an expression profile dominated by transcripts indicative of life in the intertidal where there is requirement to deal with continually fluctuating and stressful conditions, such as emersion, high UV and almost constant buffeting with brash ice. This was evidenced by upregulated transcripts putatively involved in the responses to emersion, involving anoxia and hypoxia such as anoxia-induced grl-like protein and HYOU1. Additionally, genes involved in the classical stress response were present with genes putatively involved in antioxidation and combating ROS (Figure 3, Table S1). These included transcripts with high sequence similarity to genes encoding antioxidants (GSR, GSTP1, MGST2, NXN, SOD1 and SOD2) and the pentose shunt (6-phosphogluconate dehydrogenase [PGD]). The latter is an important source of reducing cellular NADPH to combat ROS. Similar data were found in analyses of metabolic cycles in intertidal mussels where large-scale changes in physiological state and metabolite profiles were associated with regular periods of hypoxia and the switch to anaerobic metabolism associated with emersion (Gracey & Connor, 2016).

The constant production of proteins that are considered protective in the intertidal animals agrees with the previous identification of what has been termed "preparative defence" in *Lottia*, an intertidal gastropod (Dong, Miller, Sanders, & Somero, 2008) and "constitutive frontloading" in heat resilient populations of corals (Barshis et al., 2013). In the former study, candidate heat shock proteins (HSP70) were measured. High constitutive expression levels of these genes were maintained as a pre-emptive defence against extreme and unpredictable heat stress in the intertidal zone (Dong et al., 2008). Similarly, high levels of constitutive expression of the inducible forms of HSP70 were also demonstrated in *N. concinna* in response to tidal emersion, both naturally and artificially induced (Clark & Peck, 2009; Clark, Geissler, et al., 2008). In the coral study, RNA-Seq analyses revealed that the more resilient populations of coral expressed higher levels of heat shock proteins and antioxidant enzymes in addition to a series of genes involved in a wide range of functions such as apoptosis and immune responses (Barshis et al., 2013). This constant production of defence genes is energetically costly and can result in cellular trade-offs (Sorensen & Loeschcke, 2006). In the *N. concinna* intertidal expression profiles, this increased energetic requirement may be reflected in elevated levels of transcripts putatively involved in carbohydrate and fatty acid metabolism along with transcripts involved in transcription and translation (Table S3, Data S2).

Additional functional groupings were also identified in the upregulated transcripts of the intertidal transplants (Table S2). The most common of these (7% of the total) comprised cytoskeletal proteins. It has long been demonstrated that in yeast actin acts as an oxidative stress sensor (Farah & Amberg, 2007) although an increasing number of environmental studies have identified a wider range of cytoskeletal proteins, including the tubulins and collagen, as identified here (Figure 3, Table S2). These cytoskeleton genes have been shown to be upregulated in response to different types of stress, such as temperature in *Ciona* and *Mytilus* and elevated *PCO2* in *Crassostrea gigas* (Fields, Zuzow, & Tomanek, 2012; Serafini, Hann, Kultz, & Tomanek, 2011; Tomanek, Zuzow, Hitt, Serafini, & Valenzuela, 2012). The data here add to an increasing body of evidence that single-celled organisms through to higher vertebrates have incorporated the cytoskeleton within numerous signalling pathways and use the dynamic state of the cytoskeleton as an important indicator of cell health (Leadsham & Gourlay, 2008). Transcriptional analyses have showed co-expression of antioxidants and cytoskeletal proteins indicating that the cytoskeleton is a major target of ROS (Leadsham & Gourlay, 2008). Thus, upregulation of cytoskeleton transcripts can be indicators of increased environmentally induced ROS activity. A further module included transcripts putatively involved in DNA damage and repair (3% of total) (Figure 3, Table S2). This is another documented response to environmental stress and ROS accumulation, which can involve nuclear fragmentation and DNA degradation (Farah & Amberg, 2007; Galhardo, Hastings, & Rosenberg, 2007). Other transcripts of note in the intertidal transplants included those putatively involved in the shell matrix, such as thrombospondin- and proline-rich proteins (Table S1), which is not entirely surprising as the intertidal animals have thicker shells compared with subtidal animals.

Conversely, the subtidal animals transplanted to the intertidal region changed their expression profiles towards that of intertidal animals rather rapidly with an analysis of functional groupings revealing a similar profile to the intertidal animals. Overall, 2% of transcripts were putatively involved in DNA damage and repair, 4% in antioxidant activity and 9% involved in the cytoskeleton. The latter category included gelsolin, an actin depolymerising protein which has been implicated in signalling processes (Farah & Amberg, 2007) (Table S2). Also present were transcripts putatively involved in the pentose shunt (PGD which provides NADPH intermediates for combating ROS) and the shell matrix (chitin synthase and carbonic anhydrase). Interestingly, 13 annotated transcripts were shared between the subtidal and intertidal transplanted animals when their respective expression profiles were compared (Figure 3). These coded for a wide range of functions including the pentose shunt (PGD), DNA repair (LIG1) and protein folding and degradation (PPIB, RANBP2). Of particular note, was SORBS2, which assembles signalling complexes, acting as a link between kinases, such as PAK1 (also present in these annotations), with the cytoskeleton, again highlighting the potential importance of the cytoskeleton in environmental responses. Thus, the transplanted subtidal animals showed an expression profile

moving towards that of an intertidal animal, indicative of an increasing requirement to combat ROS and produce a thicker shell for defence against brash ice in the intertidal region. This also indicates that the environmental stresses in the intertidal zone (hypoxia, desiccation, temperature, ice, etc.) act as strong environmental cues on cellular expression patterns.

These molecular findings in *N. concinna*, which underpin the physiological responses to the intertidal zone are seen in other species, in particular when those species are subjected to transplantation experiments. For example, compensation of the heat shock response occurred in mussels moved between the intertidal and subtidal zones (Halpin, Menge, & Hofmann, 2004) and the transcriptional and metabolomic cyclic response to hypoxia in intertidal mussels was still observed when individuals of *M. californianus* were kept submerged for 2 weeks. These mussels continued to undergo spontaneous bouts of anaerobiosis to mimic the conditions of emersion, as they would experience in the intertidal zone (Gracey & Connor, 2016). While the animals in some transplant experiments showed complete compensation of physiology after transplantation to a different shore zone (Altieri, 2006), other studies recorded lags in compensation which, it was suggested, were due to either differences in "ecological memory" or "adaptation delay" (Freites et al., 2002; Labarta et al., 1997). This "delay" was almost certainly due to the time-scales of the experiments, as even in the temperate *M. galloprovincialis* 36–50 days were required for the animals to adjust their physiology to that of a different habitat (rocky shore vs. subtidal) (Freites et al., 2002).

This "delay" was also seen in the molecular data described here as there was a distinctive and persistent expression profile associated with life in the intertidal, even when intertidal animals were transplanted to the subtidal zone. The most parsimonious explanations for these data are either that it took the animals a long time to use their physiological flexibility and acclimate to their new conditions as has been demonstrated for Antarctic marine invertebrates in their thermal physiology (Peck, Morley, Richard, & Clark, 2014), or that the expression profiles associated with the intertidal region had become fixed in some way. The latter may potentially act via epigenetic factors, one of which is methylation. Hence, a common garden experiment was performed alongside an evaluation of genome methylation patterns.

After being held for 9 months in identical aquarium conditions, there were still some significant differences in the gene expression profiles of the intertidal and subtidal cohorts. This was surprising, given the time-scale which was more than sufficient to enable full physiological acclimation in Antarctic species (Morley et al., 2011; Peck et al., 2014), and therefore, harmonisation of the transcription profiles of both cohorts might have been expected. Although the STRING program showed no significant networks in the subtidal animals, there was still a small core of 17 genes enriched in the intertidal acclimated animals (Figure 4), which was clearly a remnant from the very extensive gene expression differences previously identified in the transplant experiment (Data S4 and S5). A critical core of an antioxidant response remained, comprising several "classical" stress response genes, in particular several members of the glutathione-*S*-transferase family, which are involved in protecting against ROS. Also present were genes within the pentose shunt pathway (PGD, SLC7D5), which generate reducing equivalents in the form of NADPH, again involved in preventing oxidative stress (Figure 4). Hence, the cellular pathways involved in combating ROS remained as the last set of genes to have their regulation pattern erased in these acclimated animals, thus indicating the importance of antioxidant protection systems in the intertidal zone. While some proteins, such as PGD identified in this study, have been shown to be redox sensitive (Wang et al., 2012), our acclimation study indicates that upregulation of these transcripts was not a rapid response to a potential stress, but more likely a durable preventive mechanism to the intertidal lifestyle, similar to the preparative defence and constitutive frontloading described earlier (Barshis et al., 2013; Dong et al., 2008). It may be that these remnants of intertidal gene expression are the result of irreversible developmental plasticity, but clearly longer common garden experiments along with expression profiling of newly developing larvae would be required to detect this. Thus, within the context of the current experiment, the question arose as to how these genes are tagged and epigenetics presented as a prime candidate for investigation.

Epigenetic changes to genomes are increasingly recognised as an important factor in species modification to local environmental conditions (Bossdorf et al., 2008). The mechanisms behind such changes include methylated cytosine residues (5-methylcytosine or 5mC); the remodelling of chromatin structure through chemical changes to histone proteins and regulation by small RNA molecules. These mechanisms are not mutually exclusive and may combine to act in a complex manner (Bossdorf et al., 2008). The best studied of these is 5mC. A relatively simple test of differential cytosine methylation between different populations, cohorts or life-history traits is the use of MSAP analysis (Reyna Lopez, Simpson, & RuizHerrera, 1997; Sun et al., 2014). While methylated cytosines have been identified in a number of invertebrates (Tweedie, Charlton, Clark, & Bird, 1997), to date little is known about how methylation affects gene expression in these taxa. The data here showed significant differences in methylation patterns between subtidal and intertidal animals, both at the start of the experiment and between the transplanted individuals, indicating that epigenetic imprinting plays an important role in the differentiation of *N. concinna* ecotypes to their respective habitats. This pattern, however, was not observed in the individuals sampled at the end of the acclimation experiment indicating that the methylation may be transient It should be noted that in this preliminary trial a very restricted proportion of the genome was assayed for methylation and therefore methylation levels were almost certainly more extensive than demonstrated here. These intriguing data demonstrate the clear need for more extensive studies especially as a small core of "intertidal profile" genes remained upregulated after 9 months. These results showing transient methylation status are

also similar to those in other species, for example in trout where salt-enriched diets can trigger short-term genome-wide methylation differences (Moran, Marco-Rius, Megias, Covelo-Soto, & Perez-Figueroa, 2013) and shellfish in which methylation is suggested to act as an immune regulatory factor (Shang, Su, Wan, & Su, 2015). While in this experiment, it was not possible to assign methylation to specific genes, there was a correlation between the extent of methylation status and the difference in gene expression profiles associated with a particular environment. The methylation levels decreased in the common garden experiment, where there was a complete absence of the natural environmental cues further substantiating the methylation: habitat correlations.

5 | CONCLUSIONS

These data, using RNA-Seq reveal the underlying complexity of response to maintaining life in the intertidal, beyond previous candidate gene approaches. These profiles indicated that elevated expression of genes associated with antioxidant responses form a constitutive defence against the harsh intertidal habitat. While the core of such a response is durable lasting many months after the environmental cue has been removed it may be reversible. Significant differences existed in the methylation patterns between intertidal and subtidal animals, which were reduced during the common garden experiment, indicating that epigenetic factors may influence the response to habitat. These data demonstrating significant phenotypic and methylation plasticity within a genetically homogeneous population are highly relevant to global evaluations of species abilities to respond to specific habitats and to climate change. Clearly, linking levels of methylation and/or other epigenetic factors, such as histone acetylation and microRNAs to specific genes and functions will provide critical data towards a more comprehensive mechanistic understanding of species cellular resilience under future climate change.

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AUTHORS' CONTRIBUTIONS

M.S.C., L.S.P. and J.I.H. conceived the study; M.S.C. and L.S.P. performed the field work and collected samples; M.S.C., J.I.H. and M.K. processed the samples; M.K. and H.H. carried out the MSAP analyses; M.A.S.T. performed the bioinformatics analyses; M.S.C. was responsible for the biological annotation and interpretation; M.S.C. wrote the paper with substantial input from L.S.P. and J.I.H.

DATA ACCESSIBILITY

All sequencing data were submitted to the NCBI SRA with the Accession Number: SRP059540.

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SUPPORTING INFORMATION

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