


## ORIGINAL ARTICLE

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# 31° South: The physiology of adaptation to arid conditions in a passerine bird

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## Abstract

Arid environments provide ideal ground for investigating the mechanisms of adaptive evolution. High temperatures and low water availability are relentless stressors for many endotherms, including birds; yet birds persist in deserts. While physiological adaptation probably involves metabolic phenotypes, the underlying mechanisms (plasticity, genetics) are largely uncharacterized. To explore this, we took an intraspecific approach that focused on a species that is resident over a mesic to arid gradient, the Karoo scrub-robin (*Cercotrichas coryphaeus*). Specifically, we integrated environmental (climatic and primary productivity), physiological (metabolic rates: a measure of energy expenditure), genotypic (genetic variation underlying the machinery of energy production) and microbiome (involved in processing food from where energy is retrieved) data, to infer the mechanism of physiological adaptation. We found that the variation in energetic physiology phenotypes and gut microbiome composition are associated with environmental features as well as with variation in genes underlying energy metabolic pathways. Specifically, we identified a small list of candidate adaptive genes, some of them with known ties to relevant physiology phenotypes. Together our results suggest that selective pressures on energetic physiology mediated by genes related to energy homeostasis and possibly microbiota composition may facilitate adaptation to local conditions and provide an explanation to the high avian intraspecific divergence observed in harsh environments.

## KEYWORDS

aridity, birds, energetic physiology, microbiome, natural selection, target enrichment

## 1 | INTRODUCTION

Arid environments provide ideal ground for investigating the mechanisms of adaptive evolution in endotherms. The environmental stressors associated with elevated aridity, i.e., increasing levels of daytime heat and dryness, pose challenges that may need to be compensated through physiological performance. Thus, there is a growing interest in the role of physiology in adaptive evolution to arid environments (Boyles, Seebacher, Smit, & McKechnie, 2011; MacManes, 2017; Sabat, Cavieres, Veloso, & Canals, 2006).

Birds inhabit desert regions across all of the world's continents. They typically have high body temperatures and the highest mass-specific metabolism of all terrestrial vertebrates (Bicudo, Buttemer, Chappell, Pearson, & Bech, 2010). At face value this suggests they should be poor candidates for inhabiting an ecosystem in which they must experience daily air temperatures far beyond their normal body temperatures, face food shortages due to low primary productivity, and withstand low and unpredictable water availability.

Nevertheless, the results of compelling comparative physiological studies indicate that birds have evolved physiological mechanisms to cope with these extreme conditions, including to reduce cutaneous water loss (e.g., Muñoz-García, Ro, Brown, & Williams, 2008; Williams & Tieleman, 2005), control blood flow as to retain or dissipate heat (e.g., Tattersall, Andrade, & Abe, 2009) and lower energetic demands (e.g., Sabat et al., 2006; Smit & McKechnie, 2010; Tieleman, Williams, & Bloomer, 2003; Williams & Tieleman, 2001). Despite the efforts to understand energy economy in arid biomes, there is still a knowledge gap pertaining to the adaptive mechanisms (phenotypic plasticity and adaptive genetic changes) that allow energetic physiological phenotypes to match the prevailing environmental conditions.

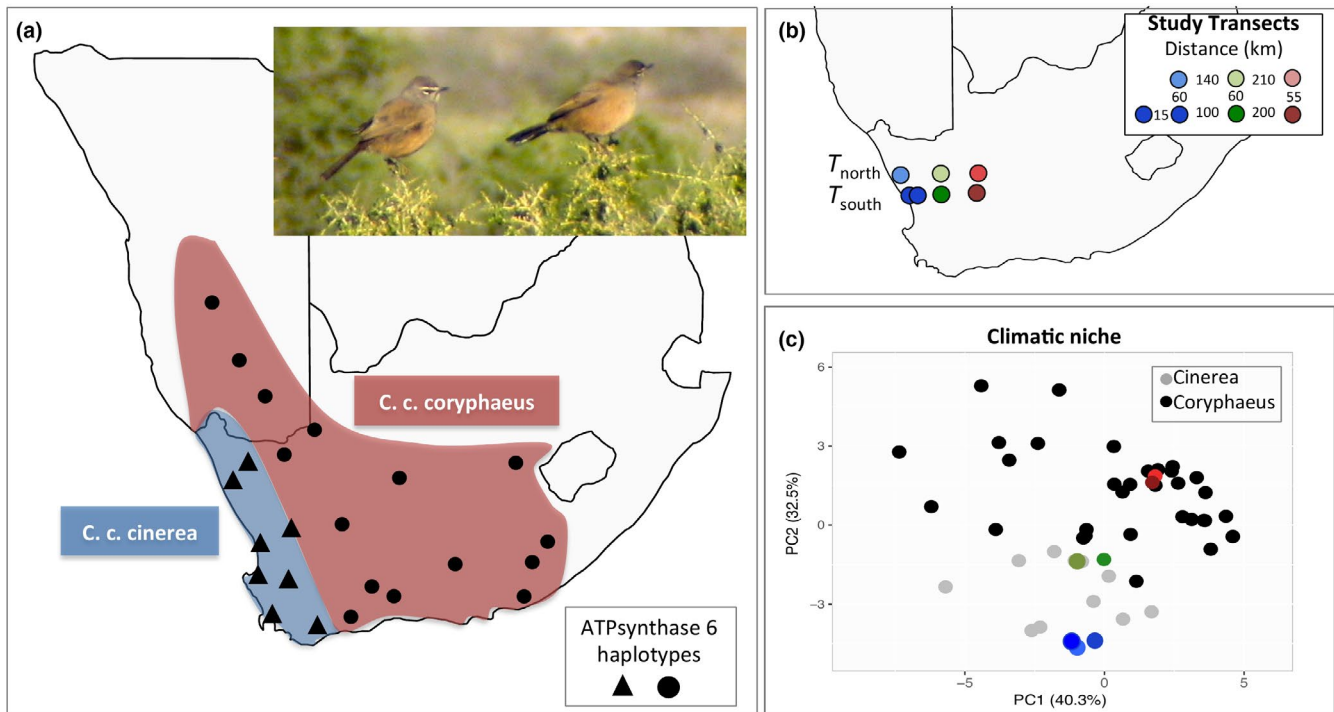
Given that environmental spatial heterogeneity should maintain genetic differentiation among populations (Hedrick, 2006; Lande, 1976), temporal change may favour phenotypic plasticity (Chevin, Lande, & Mace, 2010; Schlichting & Pigliucci, 1998), both mechanisms can potentially result in local adaptation, we hypothesised that it might be possible to untangle the physiological conundrum of life in deserts, through combining ecological, physiological and genetic data, from an avian species that spans mesic to desert habitats. One such example is the Karoo scrub-robin (*Cercotrichas coryphaeus*), a passerine whose distribution range spans the highly seasonal semi-arid and arid zones of southern Africa. The Karoo scrub-robin (hereafter: scrub-robin) has two subspecies recognized according to plumage colour (Collar, 2005): *C. c. cinerea*, a greyish form restricted to a coastal semi-arid environment, and *C. c. coryphaeus*, a brownish form that inhabits the inland Karoo desert (Figure 1a). Critically, each subspecies occupies a different climatic niche (Ribeiro, Lopes, & Bowie, 2012), with *cinerea* inhabiting a region where rain falls in winter and mean annual thermal amplitude seldom exceeds 20°C, while *coryphaeus*' habitat mostly receives erratic rains during the summer, and has mean annual thermal amplitudes exceeding 30°C as a result of cold winter temperatures. This climatic and plumage concordance is mirrored through fixed mitochondrial types (Ribeiro,

Lloyd, & Bowie, 2011; amino acid changes in the ATPase6 gene that is part of the pathway responsible for energy production) despite extensive nuclear gene flow (Figure 1a), suggesting that different energetic physiology phenotypes may have higher fitness in different conditions. Taken together this information suggests this species may be an excellent model with which to assess the roles of genetic determination and plasticity in facilitating adaptation to local conditions, with a particular emphasis to temperature, an environmental factor that affects all levels of biological organization: from aerobic metabolism at the cellular level, to driving biogeographic patterns, through modifying whole organism metabolic rates (Schulte, 2015).

An organism's metabolic rate reflects the biochemical process of transforming food into energy, that is then used to ensure basic cellular functioning, thermoregulation, digestive activity and locomotion (Lovegrove, 2006). Thus, in a fasted endotherm in its rest phase and under thermoneutral conditions, it is possible to quantify the minimum energy spent on homeostasis, i.e., basal metabolic rate (BMR; Lovegrove, 2006). Variation in BMR has a strong genetic component (Nilsson, Åkesson, & Nilsson, 2009; Rønning, Jensen, Moe, & Bech, 2007; Tieleman et al., 2009; Wikelski, Spinney, Schelsky, Scheuerlein, & Gwinner, 2003) with both mitochondria and nuclear genomes being implicated in adaptive response to extreme environmental challenges (Pichaud, Ballard, Tanguay, & Blier, 2012; Tieleman et al., 2009; Welch et al., 2014). Alongside the genetic component, BMR is also affected by environmental factors such as ambient temperature (Jetz, Freckleton, & McKechnie, 2008; McKechnie & Swanson, 2010; White, Blackburn, Martin, & Butler, 2007). In addition to the genetic and plastic components of energetic metabolism, one other element has been suggested as potentially relevant: the gut microbiome. In fact, there is a growing appreciation that the microbial community that lives in the gastrointestinal tract of animals may affect the metabolic traits of hosts (Alberdi, Aizpurua, Bohmann, Zepeda-Mendoza, & Gilbert, 2016; Sommer & Bäckhed, 2013; Tremaroli & Bäckhed, 2012). Unlike the host genome, the microbiome can change rapidly (Bletz et al., 2016; David et al., 2014), thus potentially allowing for the preservation of beneficial interactions, which may be key for the host's metabolic function and energy balance.

Thus, to assess whether energetic phenotypes (measured as metabolic rates) are adaptive and discover the underlying mechanism (plasticity, molecular variation and/or modulation by microbiota) we combined field physiological experiments to measure metabolic rates with avian genome screening (target enrichment approach) and gut microbiome profiling (metabarcoding) to ultimately contemplate an understanding of the mechanisms that allow birds to occupy these challenging arid environments.

We predicted that, should the rate at which birds expend energy (metabolic rate) confer fitness advantages in different environmental conditions, then: (a) metabolic rates should be associated with environmental conditions (i.e., lower in populations from more arid regions) and; (b) if the gut microbiome plays a role in this system, it would co-vary with environmental change, possibly implying a functional role in energy balance; (c) variation in genes underlying



**FIGURE 1** Details about the study system—the Karoo scrub-robin. (a) Distribution of the Karoo scrub-robin. Depiction of the two subspecies (*C. c. cinerea* and *C. c. coryphaeus*) range and coincidence with distribution of two mitochondria haplotypes (ATP synthase subunit 6 nonsynonymous substitutions) as reported in Ribeiro et al. (2011). (b) Diagram of our sampling design: two transects ( $T_{\text{North}}$  and  $T_{\text{South}}$ ) across the subspecies boundary, mitochondrial divide and climatic gradient. Geographic distance between sampling sites, as estimated with the great circle method. (c) Representativeness of our sampling sites in regards to the climatic niche of the Karoo scrub-robin: the first two principal components of 19 bioclimatic variables explained ~73% of the variation. GPS coordinates for species wide distribution from Ribeiro et al. (2012). Populations at the extreme of our transects occupy different portion of the climatic niche (Tukey's HSD,  $p < .01$ , after significant ANCOVA, controlling for the effect of latitude and longitude) [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

energetic metabolism would be associated with environmental change, contrasting with variation in noncoding regions of the genome that should mostly be affected by gene flow; and (d) metabolic rates would be correlated with functional genetic background, i.e., variation in genes underlying energy production pathways.

## 2 | MATERIALS AND METHODS

### 2.1 | Study system: sampling sites and climatic variation

During the summer (November–December) of 2015 and winter (June–July) 2016, we captured 96 scrub-robins in seven sites (Coastal<sub>North</sub>, Coastal<sub>South1</sub>, Coastal<sub>South2</sub>, Central<sub>South</sub>, Central<sub>North</sub>, Inland<sub>South</sub>, Inland<sub>North</sub>; summer: 47, winter: 49) along two transects (Figure 1b) which concomitantly cross (a) the subspecies divide, (b) the ATP synthase subunit 6 break (Ribeiro et al., 2011), and (c) the climatic niche of the species (Figure 1c). We captured individuals using spring traps baited with mealworms (*Tenebrio molitor*). Immediately after capture, we weighed the birds and collected a blood sample from the brachial vein that was preserved in RNAlater. Birds were then kept in cages for no longer than 48 hr, until the physiological experiment, with food provided *ad libitum*.

Permits for capturing the birds were issued by the Northern Cape Department of Environmental Affairs (ODB 2665 & 2666/2015) and CapeNature (0056-AAA008-00051) in South Africa. The Animal Ethical Committee at the Nelson Mandela University (South Africa) approved all experiments (A15-SCI-ZOO-005).

We used climatic variables and a proxy of primary productivity (normalized difference vegetation index [NDVI]) as surrogates for environmental selection pressures because precipitation, temperature and NDVI have been correlated with variation in metabolic rates in birds (Jetz et al., 2008; Swanson & Vézina, 2015). We used GPS coordinates of each sampling location (obtained with a handheld device; Garmin) to extract climatic data from WORLDCLIM database (30 arc-second resolution; [www.worldclim.org](http://www.worldclim.org); Hijmans, Cameron, Parra, Jones, & Jarvis, 2005) and NDVI (the proxy for primary productivity) from USGS-LandDAAC-MODIS data set hosted by United States Geological Survey (250 m resolution; [https://lpdaac.usgs.gov/dataset\\_discovery/modis](https://lpdaac.usgs.gov/dataset_discovery/modis)). We performed a principal component analysis (PCA) to summarise the climatic variation, using only climatic variables with cross-correlation coefficient value (Pearson correlation  $r$ ) value  $\leq 80\%$ . The two principal components were designated climatePC1 and climatePC2, for the sake of clarity. We performed all analyses using R-packages RASTER (Hijmans, 2017) and STATS (R Core Team, 2018).

## 2.2 | Energetic physiology: basal metabolic rate and metabolic scope

To assess energetic physiology phenotypes we measured basal metabolic rates (BMR; energy required by an endotherm to maintain energetic homeostasis under resting conditions at thermoneutrality) and metabolic expansibility (ME; the capacity to elevate the metabolic output from a basal to maximum level, so as to meet the thermoregulatory demands of cold conditions).

We excluded from the metabolic experiments juvenile birds (as assessed by plumage), birds showing any sign of body moult, and those showing body mass loss exceeding 5% while in captivity. This left 70 birds remaining: Coastal<sub>South1</sub>: 8, Coastal<sub>South2</sub>: 8, Coastal<sub>North</sub>: 8, Central<sub>South</sub>: 12, Central<sub>North</sub>: 10, Inland<sub>South</sub>: 17, Inland<sub>North</sub>: 7. To quantify BMR we measured gas exchange rates ( $O_2$  consumption and  $CO_2$  emission) of individual birds in post-absorptive conditions throughout the bird's resting-phase (night), using a regularly calibrated open-flow respirometry system (FoxBox-C Field Gas Analysis System, Sable Systems). Gas exchange was recorded using EXPEDATA v.1.2.6 (Sable Systems). BMR was determined as the lowest 10 min mean  $VCO_2$  at thermoneutrality (Londoño, Chappell, Castañeda, Jankowski, & Robinson, 2014). Full details of experimental procedures are in Appendix S1, Methodology.

Metabolic expansibility was estimated as the ratio between  $M_{sum}$  (maximum metabolic capacity) and BMR obtained for the same individuals:  $ME = M_{sum}/BMR$ . We used  $M_{sum}$  values (maximum thermogenic capacity, obtained as maximum  $VCO_2$  at low helix temperatures) from Ribeiro, Prats, Pattinson, Gilbert, and Smit (2018) and calculated ME for 61 adult scrub-robins: Coastal = 21, Central = 18 and Inland = 22. Briefly, as reported in Ribeiro, Prats, et al. (2018),  $M_{sum}$  was measured as the bird's  $O_2$  consumption and  $CO_2$  production while exposed to a HelOx atmosphere (79% helium + 21% oxygen). The birds were exposed to a sliding cold exposure protocol by reducing HelOx temperature by 3°C every 10 min. Trials ended when (a)  $VCO_2$  started to decline indicating peak thermogenic metabolism was reached, and (b) body temperature— $T_b$  < 34°C.

### 2.2.1 | Phenotype-environment association

To test whether environmental variation was a significant predictor of the energetic phenotype (BMR and ME), we used generalized linear models (GLM). BMR and ME were the dependent variables (normality and homogeneity of variance tests in Appendix S1, Methodology), and body condition ( $M_{b-scaled}$ ; estimation procedure in Appendix S1, Methodology; Peig & Green, 2012), sex and environment (NDVI, climatePC1) were the predictor variables. Due to the lack of morphological sexual dimorphism in scrub-robins, we determined sexes using a molecular method (Appendix S1, Methodology). To assess the effect of region (three levels as defined from climatePC1: Coastal, Central, Inland) and season (two levels: summer and winter) in phenotypic variation, we implemented a GLM. Finally, to test for within-region seasonal differences in basal metabolism and metabolic expansibility, while controlling for the effect of body condition, we used a one-way

analysis of covariance (ANCOVA); upon significance, post-hoc Tukey's HSD tests were carried out to identify significant pairwise differences between populations. All statistical analyses were performed in R v3.3.2 (R Core Team, 2018) using MASS package and plots produced with GGPLOT2 package (Wickham, 2016). We accepted  $p \leq .05$  as a significant difference for all statistical tests.

## 2.3 | Gut microbiome composition

### 2.3.1 | Amplicon sequencing, sequence processing and data filtering

From the 96 birds handled, we collected 18 individual faecal pellets ( $N_{Coastal} = 6$ ,  $N_{Central} = 7$ ,  $N_{Inland} = 5$ ) using single-use filter-paper bags that lined cloth bags in which captured birds were placed. All samples were collected in summer 2015. Faecal samples were preserved in soil/faecal DNA MiniPrep kit lysis buffer (ZymoResearch) and DNA extracted following the soil/faecal DNA MiniPrep kit instructions. A negative control proceeded in the workflow with all other extracts as to control for contamination. The DNA extracted was used in a dual indexed PCR approach to target the V3–V4 variable region of the bacterial 16S rRNA gene (465 bp) using the primer pair Bact-341F and Bakt-806R (Hansen et al., 2012) with Illumina Nextera overhang adapters (Illumina Inc.). PCR products were pooled in equimolar proportions and sequenced on an Illumina MiSeq platform with 250PE chemistry. Details about DNA extraction, PCRs and library preparation are provided as Appendix S1, Methodology.

### 2.3.2 | Sequence processing

Post sequencing, raw reads for the 16S rRNA amplicon were processed using a similar approach to the UPARSE pipeline (Edgar, 2013) with VSEARCH v2.1.2 (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). Briefly, (a) paired reads were merged, quality filtered and lengths trimmed to >400 bp; and (b) barcodes, adapters and primers were trimmed. Filtered reads were then clustered into Operational Taxonomic Units (OTUs) in USEARCH v9.0.2132 with default parameters (Edgar, 2010) and chimeras were additionally removed (Edgar, 2016). OTU taxonomic assignment was performed using the software LCACLASSIFIER v.2.0.4 and the SILVA database (Lanzén et al., 2012) with 50 database matches per OTU. Unassigned OTUs, eukaryotic OTUs and samples with fewer than 9,000 reads were removed from the OTU table prior to statistical analyses.

### 2.3.3 | Microbiome-environmental association

We estimated Shannon-Wiener's and Simpson's indexes to quantify bacterial community diversity by region using the R-package PHYLOSEQ (McMurdie & Holmes, 2013; R v3.4.3, R Core Team, 2018). Differences in richness were assessed using an ANOVA after confirming normality (Shapiro-Wilk test). Prior to multivariate analyses, samples were subsampled randomly to 9,871 reads (we contend is close enough to

the minimum 10,000 reads; Faith et al., 2013) and log + 1 transformed. Differences in bacterial community composition were inspected with a principal coordinate analysis using a Bray-Curtis dissimilarity matrix in R-package PHYLOSEQ. To test whether climate (measured as climatePC1) or primary productivity (assessed as NDVI) were shaping the diversity in gut microbiome we used multiple matrix regression approach (MRM; Lichstein, 2006). We implemented the tests at individual-level and tested significance by permutation ( $n = 9,999$ ) using R-package ECODIST (Goslee & Urban, 2007; R v3.3.2; R Core Team, 2018). Briefly, we compared the observed dissimilarity matrix (Bray-Curtis dissimilarity) against a matrix for climate (Euclidean distance along climatePC1) or NDVI (Euclidean distance between NDVI value at time of sampling) effects while controlling for the effect of geographical distances (distance in Km). Taxonomic-based pairwise comparisons of microbial communities between each of the three geographical regions were tested in R-package METACODER (Foster, Sharpton, & Grünwald, 2017) with Wilcoxon rank-sum test followed by a Benjamini-Hochberg correction for multiple testing (Benjamini & Hochberg, 1995).

## 2.4 | Avian genomics

### 2.4.1 | Target enrichment, sequence processing and data filtering

We extracted DNA for 96 samples (Coastal<sub>South1</sub>: 14, Coastal<sub>South2</sub>: 9, Coastal<sub>North</sub>: 13, Central<sub>South</sub>: 20, Central<sub>North</sub>: 12, Inland<sub>South</sub>: 21, Inland<sub>North</sub>: 7) using DNeasy Kit (Qiagen) or KingFisher Duo Prime System (ThermoFisher Scientific) and built genomic libraries slightly adapting the protocol by (Carøe et al., 2018; details in Appendix S1, Methodology). We enriched our libraries for protein coding regions (2,889 exons associated with energetic metabolism, water economy, heat tolerance, mitochondria biogenesis, egg shell features) and non-coding regions (2,122 intergenic regions of 200 bp) using a custom myBaits target capture Kit (Arbor Biosciences). These libraries were sequenced on the Illumina HiSeq 2000 platform to generate 80 bp single-end reads. Prior to downstream analysis, we first filtered the raw data to remove adapters and low quality bases and reads using Trimmomatic (Bolger, Lohse, & Usadel, 2014). The reads were then mapped to the *Oenanthe oenanthe* genome with BWA-mem (Li, 2013); we used this species as reference because it is the closest (Muscicapidae family) currently available relative to *Cercotrichas* with an annotated genome. Mapped reads were further filtered as to remove multi-mappers and low quality mapping reads using SAMtools (Li et al., 2009) and duplicates with Picard tools (<https://broadinstitute.github.io/picard/>). The resulting BAM files were used to estimate genotype likelihoods or for genotype calling when site depth >15× (hereafter hard genotypes) in ANGSD (Korneliussen, Albrechtsen, & Nielsen, 2014). See Appendix S1 for full details.

### 2.4.2 | Population genetic structure

To minimize the potential effect of selection on measures of genetic structure, we restricted this analysis to the intergenic regions.

We assessed population genetic structure following two methodological approaches: (a) non-model-based methods using a principal components analysis (DPCA) and sparse non-negative matrix factorization (sNMF); and (b) model-based methods testing models with 1–6 clusters ( $K$ ), using NGSadmix (Skotte, Korneliussen, & Albrechtsen, 2013; 16,104 single nucleotide polymorphism—SNPs; minor allele frequency—MAF >1%) and fastSTRUCTURE (Raj, Stephens, & Pritchard, 2014). Discriminant analysis of PCA (DPCA; Jombart, Devillard, & Balloux, 2010, implemented with adegenet R package), sNMF (performed in LEA package in R) and fastSTRUCTURE were applied to hard genotypes. Because rare alleles can affect inference of population structure, we retained only sites with MAF >1%. In addition, we used genotypes likelihoods to estimated pairwise  $F_{ST}$  (among seven populations) using a two-dimensional site frequency spectrum (SFS) with ANGSD (Korneliussen et al., 2014). For comparative purposes we also ran fastSTRUCTURE with our gene data set. Details on model parameterization are provided in Appendix S1.

### 2.4.3 | Isolation by distance and isolation by ecology

The degree of genetic differentiation between populations, if any, translates the relative effect of drift and gene flow (Lenormand, 2002), which in turn are affected by geographical distances and selective factors. Because disentangling the relative effects of geographic distance (isolation by distance; Slatkin, 1993) and environmental factors (isolation by environment; Wang & Bradburd, 2014) is essential for understanding local adaptation (Sexton, Hangartner, & Hoffmann, 2013), we tested for isolation by distance and isolation by environment using a Mantel test (Mantel, 1967) and multiple matrix regression (MRM; Lichstein, 2006) approach, respectively. We implemented the tests at individual- and population-level, and tested significance by permutation ( $n = 9,999$ ). Genetic distance between individuals ( $D_{xy}$ ) was calculated from genotype posterior probabilities as the average number of nucleotide differences as implemented in ngsDist (Vieira, Lassalle, Korneliussen, & Fumagalli, 2016). At population-level, genetic distance between populations was estimated as  $F_{ST}/1-F_{ST}$ . Environmental distance between individuals was estimated as the Euclidean distance between points along the climatePC1. Geographical distance between sampling points was calculated as the great-circle distance, i.e., the shortest distance between two points on the surface of a sphere in kilometres. All analyses were performed in R using packages VEGAN (Oksanen et al., 2017), ECODIST (Goslee & Urban, 2007) and SP (<https://github.com/edzer/sp/>).

### 2.4.4 | Genotype-environment associations

Although isolation by environment (IBE) can indicate a role of the environment in shaping genetic variation, it cannot pinpoint the loci that exhibit deviations from patterns of neutral evolution. Thus, we proceeded to find a genotype-environment association using latent factor mixed models (LFMM v1.3; Frichot, Schoville, Bouchard, & François,



2013) using the R package LEA (Frichot & François, 2015). Specifically, we applied this method to investigate for correlations between allele frequencies with climatic variables (climatePC1), while controlling for the effect of population structure. Although our analyses of population structure did not indicate any clear clustering, we decided to run the model with two latent factors (clusters;  $K = 2$ ), as a conservative approach to control for any subtle pattern in allele frequencies. To also avoid any false positives caused by rare alleles, we used only SNPs with MAF >5%. For each  $K$ , we ran 10 repetitions with 100,000 iterations each, which were performed after discarding the initial 50,000 steps as burnin. The median Z-scores of the 10 runs were converted into  $p$ -values and those adjusted for a false-discovery rate threshold of 1%. We further investigated the function of the top 25% SNPs significantly correlated with climatic variables. We utilized our scrub-robin consensus sequence (built for bait design, details in Appendix S1) obtained using the *Oenanthe oenanthe* annotated genome as a reference, to extract and translate the coding regions containing the LFMM outliers into amino acid sequences. And then used Blast2GO (Conesa et al., 2005) to retrieve functional annotations by comparison with birds sequences in the nr database (8,792 bird species). Only hits with an  $e$ -value <1.0E-5 and percent identity >80% were retained and used to assign GO categories to the matching coding regions.

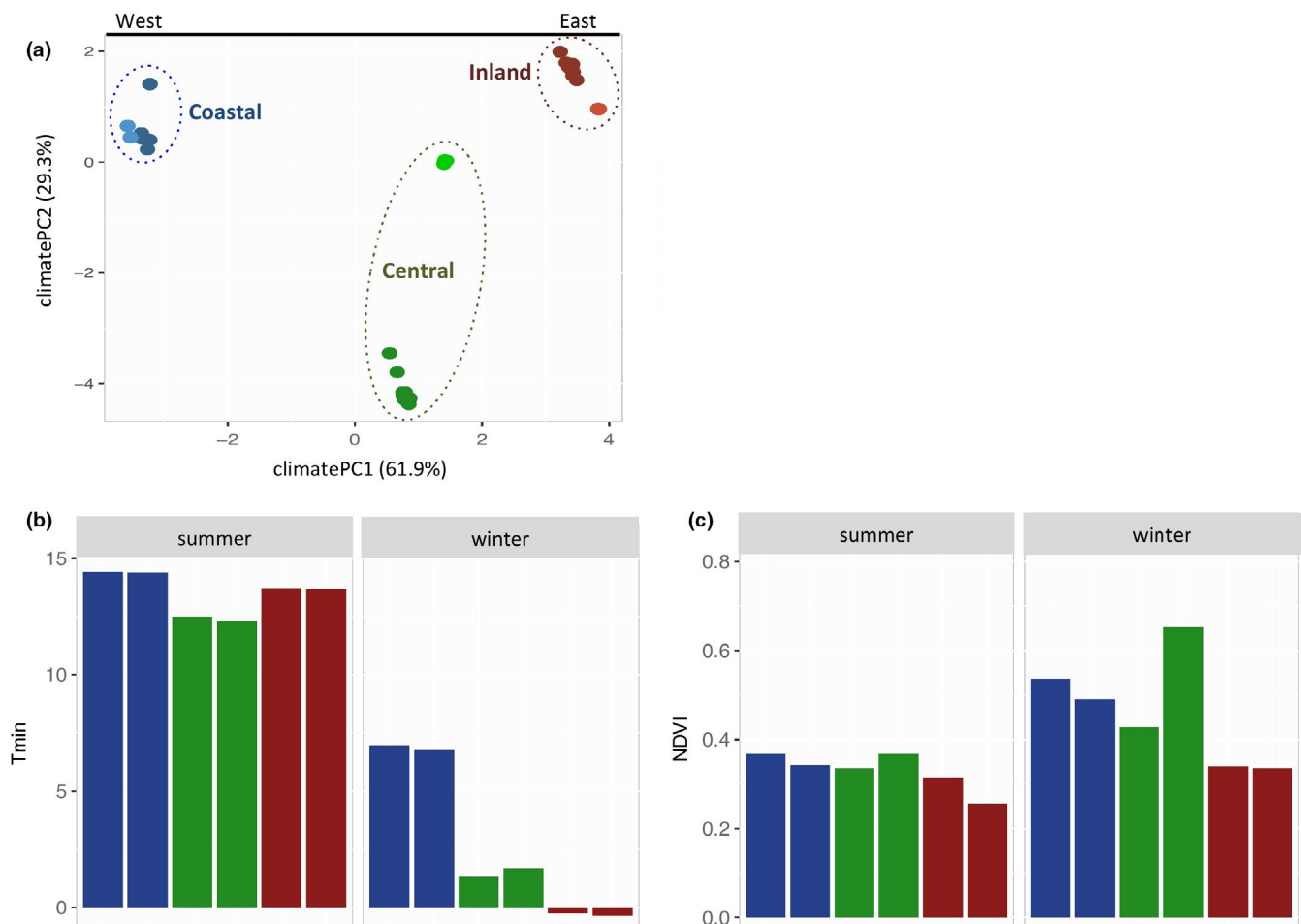
## 2.5 | Phenotype-genotype association

We defined an “adaptive genotype” by applying a PCA implemented in ngsTools (Fumagalli, Vieira, Linderroth, & Nielsen, 2014) on the genotype likelihood of SNPs significantly associated with climate (from LFMM) and extracting PC1 values (henceforth snpsPC1) for each bird. The snpsPC1 was then used as a predictor of energetic phenotypes (BMR and ME) in a GLM. Statistical analyses were performed in R v3.3.2 (R Core Team, 2018) using MASS package and plots produced with GGPlot2 package (Wickham, 2016). We accepted  $p \leq .05$  as a significant difference for all statistical tests.

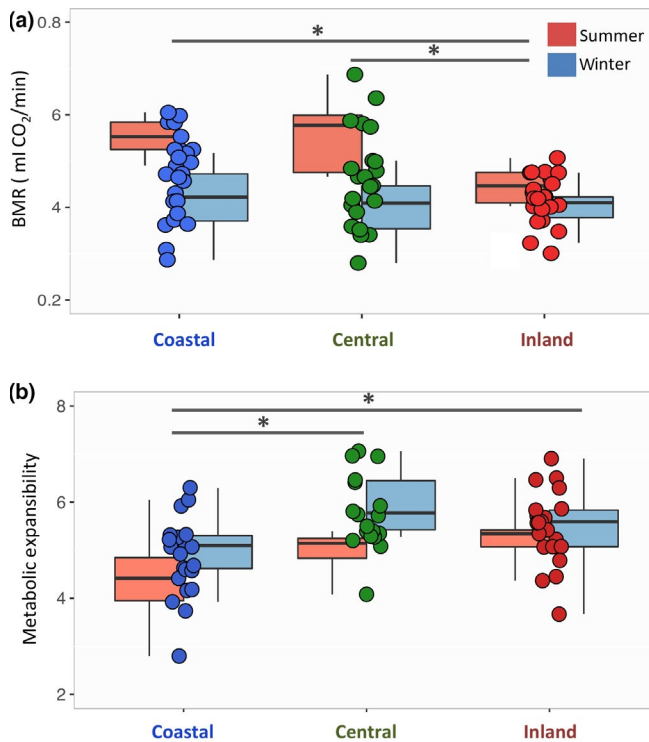
## 3 | RESULTS

### 3.1 | Environmental variation: climate and primary productivity variation across the transect

We found a clear environmental heterogeneity across the transect. The first two principal component analysis axis, implemented with 14 noncollinear climatic variables, explained 91.2% of the variation in the data (Figure 2a). While PC1 (climatePC1) explained 61.9% of variation, PC2 (climatePC2) only explained 29.3%. Therefore, we retained



**FIGURE 2** Environmental variation across the study transects. (a) Climatic variation using 14 noncollinear bioclimatic variables. (b) Variation in minimum temperature— $T_{min}$  (°C). (c) Seasonal change in primary productivity (NDVI) in the year of sampling [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



**FIGURE 3** Phenotypic variation in energetic phenotypes across regions and between seasons. (a) Basal metabolic rate (BMR): Coastal and Central populations had larger summer BMR than Inland birds (post-hoc Tukey's HSD,  $p_{\text{Coastal-Central}} > .1$ ,  $p_{\text{Coastal-Inland}} < .001$ ,  $p_{\text{Central-Inland}} < .001$ ). Overall, BMR was lower in winter than in summer (GLM:  $t_{\text{winter}} = -8.266$ ,  $p < .001$ ). (b) Metabolic expansibility (ME): Central and Inland birds had greater ME than the Coastal population (GLM:  $t_{\text{Central}} = 3.763$ ,  $p < .001$  and  $t_{\text{Inland}} = 3.244$ ,  $p = .002$ ) and ME increased in winter (GLM:  $t_{\text{winter}} = 2.917$ ,  $p = .005$ ). Regional significant differences are annotated with asterisk [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

climatePC1 for further analyses because it is related to temperature. Mean minimum temperature in summer is similar in all populations, but decreases dramatically in winter to subzero values in the Inland regions and  $\sim 2^{\circ}\text{C}$  in the Central area (Figure 2b). Regarding primary productivity, while there was a winter increase in the Coastal and Central regions, NDVI did not change much in the Inland region (Figure 2c).

### 3.2 | Energetic phenotype versus environment

Our analyses revealed the association of physiological phenotypes (BMR and ME) with environmental variables. Variation of BMR was associated with NDVI and climate (Table S1). Overall, BMR was lower in winter than in summer (GLM;  $p < .001$ ). Across regions, BMR was similar in winter (ANCOVA,  $F = 0.337$ ,  $p > .1$ ), yet there were regional differences in BMR in summer (Figure 3a; Table S2): both Coastal and Central populations had higher summer BMR than Inland birds (post-hoc Tukey's HSD,  $p_{\text{Coastal-Central}} > .1$ ,  $p_{\text{Coastal-Inland}} < .001$ ,  $p_{\text{Central-Inland}} < .001$ ). Within-region, BMR decreased in winter in Coastal and Central birds (ANCOVA;  $F_{\text{Coastal}} = 39.070$ ,  $p < .001$ ;  $F_{\text{Central}} = 21.180$ ,  $p < .001$ ) but not in the Inland population (ANCOVA:  $F_{\text{Inland}} = 2.656$ ,  $p = .118$ ).

Metabolic expansibility varied with climatic variation and NDVI (Table S1). We found significant regional differences in ME (Figure 3b; Table S2): Central and Inland birds had greater ME than the Coastal population (GLM;  $p < .01$ ). Across regions, ME increased in winter (GLM;  $p = .005$ ). The post-hoc comparisons revealed that in winter only Central populations significantly increased ME compared to Coastal birds (post-hoc Tukey's HSD,  $p_{\text{Coastal-Central}} = .001$ ,  $p_{\text{Coastal-Inland}} > .1$ ,  $p_{\text{Central-Inland}} > .1$ ). In addition, within region ME was only seasonally flexible for the Central population (ANCOVA:  $F_{\text{Central}} = 7.768$ ,  $p = .014$ ;  $F_{\text{Coastal}} = 2.871$ ,  $p > .1$ ,  $F_{\text{Inland}} = 0.216$ ,  $p > .1$ ).

### 3.3 | Gut microbiome versus environment

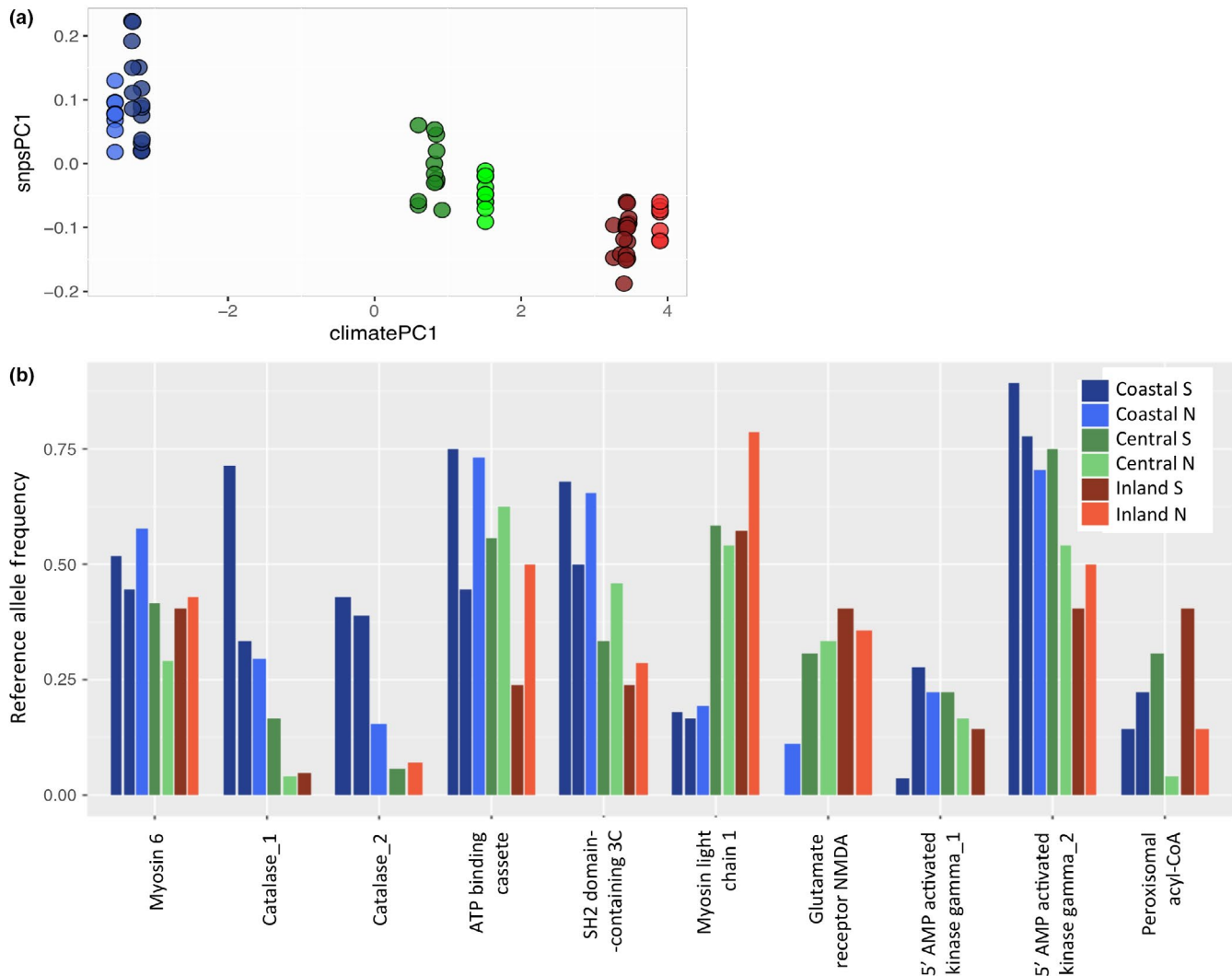
The sequences obtained from 18 individual faecal samples targeting bacterial 16S rRNA gene allowed the identification 802 different operational taxonomic units (OTUs) representing 167 bacterial genera. The diversity of gut microbiota as measured with Shannon-Wiener and Simpson indices was not significantly different across regions ( $F_{\text{Shannon-Wiener}} = 0.153$ ,  $p > .1$ ;  $F_{\text{Simpson}} = 0.325$ ,  $p > .1$ ; Figure S2a). In addition, the principal coordinate analysis revealed no apparent geographical structure in gut microbiota diversity (Figure S2b).

We found no association between climate or NDVI with microbiome dissimilarities, while controlling for the effect of geographic distances: MRM-Climate, coefficient-climate =  $-0.084$  ( $p > .1$ ) and coefficient-geography =  $0.035$  ( $p > .1$ ); MRM-NDVI: coefficient-NDVI =  $0.058$  ( $p > .1$ ) coefficient-geography =  $-0.077$  ( $p > .1$ ). In fact, both climate and NDVI explained a very low portion of the overall variance in gut microbiome diversity ( $R^2_{\text{climate}} = 0.003$ ,  $R^2_{\text{NDVI}} = 0.003$ ). Although we found birds from the three regions to have similarly diverse gut microbiota, when inspecting the taxonomic composition of the communities we found regional enrichment for some bacteria (Figure S3). Namely, while scrub-robins from the Coastal population had highest proportions of gut bacteria from the genera *Kocuria*, *Tepidimonas*, *Pseudomonas*, *Rhizobium* and *Anoxybacillus* (Figure S3), the microbiota of Inland birds were enriched for several genera of the Order Rhizobiales, an unknown *Coxiellaceae* genus, and the genera *Sphingomonas* and *Leucobacter* (Figure S3).

### 3.4 | Genotype versus environment

Our sequence capture enrichment assay yielded genetic information for  $98.2\% \pm 2.34$  (mean  $\pm$  SD) of the total 876,500 sites targeted. The median on-target depth per sample was 68 $\times$ . Overall, genetic diversity in the intergenic regions was two-fold of that observed in coding-regions—genetic diversity indexes are reported in Appendix S1, Results.

From the total of 1,211 SNPs identified in coding-regions (considering only SNPs with MAF  $> 5\%$  and present in at least 94 individuals), the LFMM analysis classified 94 SNPs, located in 63 genes, as putatively adaptive and associated with climatePC1 (heavily influenced by temperature seasonality; Figure 4a). The top 25% SNPs associated with climate were located in the following genes (Table S3): myosin



**FIGURE 4** Adaptive variation across the gradient. (a) Genotypic variation for 94 candidate SNPs as identified by LFMM was summarized as *snpsPC1*. (b) Variation in allele frequency of the top 10% candidate SNPs across transects. SNP frequencies for each population were estimated using the allele in the reference genome to polarize the data. Absence of a column denotes the lack of the reference allele in the respective population [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]

light chain and the troponin (part of the muscle contraction machinery), calcium voltage-gated channel auxiliary subunit gamma 1 (plays a role in excitation-contraction coupling process that initiates muscle contraction), 5'AMP-activated kinase subunit gamma and alpha (metabolic switch by which cells sense changes in energy status), the ATP synthase subunit beta (catalytic core of complex V of OXPHOS pathway) and the catalase (protection from oxidative damage). Often, Coastal and Inland populations represent the extremes of SNP allele frequency (Figure 4b). In contrast, frequencies in Central populations were sometimes intermediate (e.g., 5'AMP activated kinase gamma) and in other instances similar to one of the extremes (e.g., Myosin light chain 1, Figure 4b).

### 3.5 | Phenotype versus genotype

The first principal component of a PCA on the genotype likelihood of SNPs (henceforth *snpsPC1*) significantly associated with climate

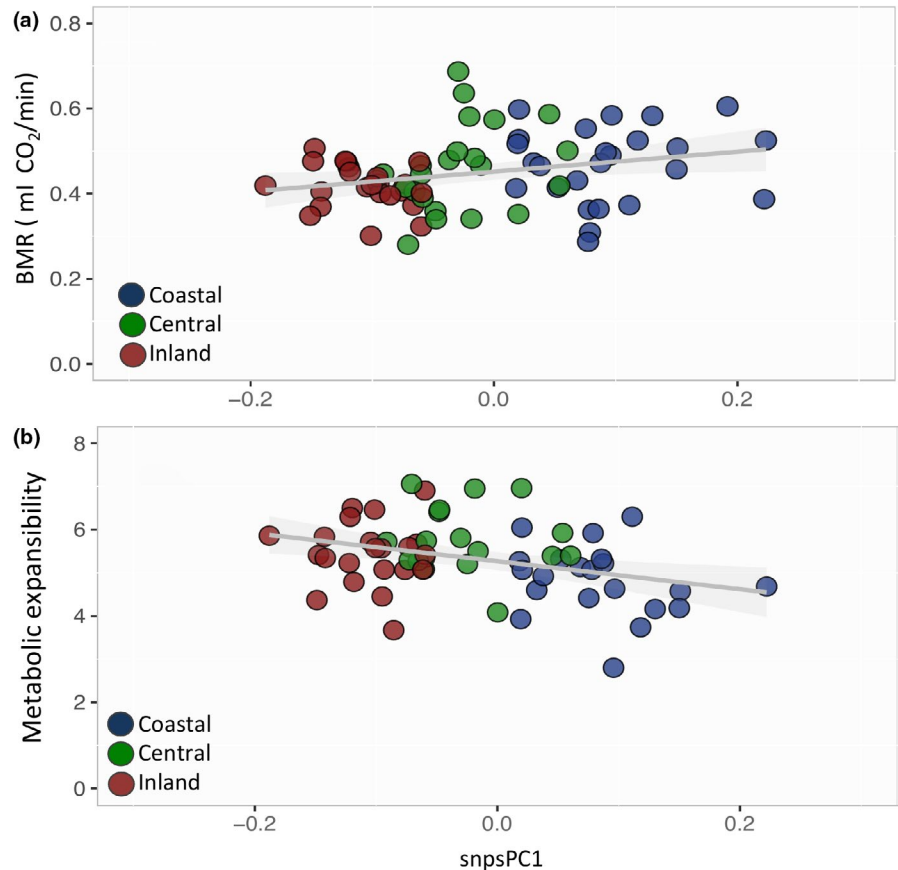
(from LFMM) was a significant predictor of variation in BMR (GLM;  $p = .011$ ; Figure 5a) as well as in ME (GLM;  $p = .006$ ; Figure 5b), while including body condition and sex as covariates in the model.

## 4 | DISCUSSION

Our understanding of the physiological traits that contribute to tolerance to arid conditions has improved greatly over the last decade (Sabat et al., 2006; Smit & McKechnie, 2010; Williams & Tieleman, 2002). Nevertheless, knowledge on how those traits came to exist, and their role in enabling adaptation to local conditions in such harsh ecosystems is still poor. We, therefore, combined environmental, phenotypic, microbiome and genomic data to address this and revealed that: (a) energetic phenotypes were associated with environmental features, with scrub-robins living in the most arid region having the lowest BMR and wider metabolic expansibility; (b) only



**FIGURE 5** Phenotype-genotype association: variation of energetic phenotype with different putatively adaptive genotypes as summarized by snpsPC1. (a) Association of BMR with adaptive genotypes (GLM,  $t_{\text{snpsPC1}} = 2.604$ ,  $p = .011$ ) and (b) Association of Metabolic Expansibility with adaptive genotypes (GLM,  $t_{\text{snpsPC1}} = -2.848$ ,  $p = .006$ ). Regression line fitting a GLM: Phenotype  $\sim M_b^{\text{scaled}} + \text{Sex} + \text{snpsPC1}$  [Colour figure can be viewed at [wileyonlinelibrary.com](http://wileyonlinelibrary.com)]



a few components of gut microbiota changed across the environmental transect; (c) a small list of genes (94 SNPs in 63 genes) are putative targets of natural selection despite overall low differentiation; and (d) variation in putatively adaptive SNPs genes explains the differences observed in energetic phenotypic traits suggesting a modular but simple basis for adaptation to arid conditions.

#### 4.1 | Energetic metabolism

The intraspecific variation in the energy expended to maintain basic function (BMR) as well as in the energetic capacity to deal with cold stress (ME) supports the adaptive role of energetic metabolism. Scrubrobins living in the most arid region (lower NDVI, larger temperature amplitude) had a lower BMR, and this trait was not seasonally flexible, i.e., low phenotypic flexibility (Figure 3). These findings support previous work that attributed selective advantages to low maintenance energy phenotypes (BMR) in birds living in arid environments (Williams & Tieleman, 2005). Although we strongly believe that our results were not affected by regional differences in life-history traits such as breeding time, as we found no evidence of reproduction (e.g., active brood-patches in females, nesting or chick provisioning), we cannot fully reject the idea. It may be possible that the temporal proximity of our experiments (start: middle November) to the end of the energetically demanding breeding activity (end: late October–early November) had some carryover effects in the Coastal birds, and hence increased BMR.

When comparing the BMR values obtained here with the allometric expectations for an 18–20 g passerine (Londoño et al., 2014) the Inland population stayed below the expected values both in winter (68%) and summer (74%) as opposed to Coastal and Central populations which approached the above mentioned expected values (~95%). The selective advantages attributed to a reduced BMR include a lower overall energy and water demands, and low endogenous heat production (Williams & Tieleman, 2005). Such traits may be favoured in the scrub-robin populations living in the most arid region (Inland) because a reduced metabolism not only decreases heat load and hence the risk of hyperthermia in summer, but also reduce overall energy demands, hence the need to obtain food in an area of low productivity. This pattern was also reported for bird species inhabiting the Kalahari desert (Smit & McKechnie, 2010), a region adjacent to the Karoo desert. BMR was revealed to be a flexible phenotype in the Coastal and Central populations. These short-term changes in BMR are thought to arise from adjustments in the mass of metabolically active organs: mass of thermogenic muscles in winter (Zheng, Liu, & Swanson, 2014), and/or mass of reproductive organs in spring/summer of reproductive organs (Vézina, Salvante, & Williams, 2003). Although the striking increase in BMR during summer in Coastal and Central populations cannot be explained by hypertrophy of thermogenic muscles (Ribeiro, Prats, et al., 2018) we cannot rule out the possible effect of metabolic costs of maintaining reproductive organs. However, we find it unlikely, because the breeding

season of subspecies *cinerea* (Coastal morph showing increase in summer BMR) typically occurs between September–October and we collected data from 11–18 November 2015. Moreover, we did not observe any sign of breeding activity (e.g., active brood patches) at the time of our study.

Metabolic expansibility reflects the capacity of an organism to rapidly adjust its metabolism to meet the energetic challenge of thermoregulation under cold conditions (measured as the ratio of maximum thermogenic capacity and basal metabolism). Given the absence of regional differences in maximum thermogenic capacity (Ribeiro, Prats, et al., 2018), the wider metabolic expansibility observed in populations experiencing lower winter temperatures (Central and Inland populations; mean minimum temperature in winter  $<2^{\circ}\text{C}$ ) inescapably stems from the lower BMR. Thus, as opposed to northern-temperate climates, where highly variable climates select for higher maximum thermogenic capacity (Swanson, Zhang, & King, 2014), subtropical and highly variable climates should select for lower BMR.

Living in arid environments poses energetic constraints because food is not only scarce, but unpredictable. In the southwestern arid-zone of Africa, insectivorous birds such as the Karoo scrub-robin face dramatic spatial and temporal changes in food abundance as a consequence of unpredictable pulses of rainfall followed by dramatic droughts (Dean & Milton, 1999; Lloyd, 1999). Thus, maximizing energy intake from food items is of utmost importance, and indeed the major role of the gut microbiome (Valdes, Walter, Segal, & Spector, 2018). Our findings that a few bacterial genera were differentially enriched in different environments may indicate functional shifts associated with energetic demands. For instance, the gut microbiota of Inland birds was enriched for genera *Sphingomonas*, chemoheterotrophic bacteria that can survive conditions of nutrient stress and metabolizes a wide variety of carbon sources (Balkwill, Fredrickson, & Romine, 2006), and several genera from the Order Rhizobiales, which include bacteria capable of nitrogen fixing. The presence of the latter bacteria may contribute to nitrogen budget as the Karoo scrub-robin has been found to supplement its diet with plant items (Ribeiro, Smit, & Gilbert, 2018), which are low in nitrogen. Although our integrative approach would have benefited from larger sample sizes, we contend our results provide solid-ground to further explore whether the observed bacterial turnover have functional consequences, namely in energy homeostasis (e.g., Debebe et al., 2017).

## 4.2 | Energetic metabolism and local adaptation

Disentangling the effects of demography from local adaptation can be particularly challenging in systems where isolation by distance coincides with the environmental gradient (Nadeau, Meirmans, Aitken, Ritland, & Isabel, 2016). Thus, to accurately detect signatures of local adaptation using genetic-environment associations and reduce the risk of false positives it is fundamental to control for possible effects of demographic processes such as differential migration and thus gene flow. In our study, there was no sign of isolation by distance (see Appendix S1, Results), population structure was undetectable

to shallow (pending on the use of hard genotypes or genotype likelihoods; Appendix S1, Results) and there was no indication of population demographic expansion as revealed by the site frequency spectrum (Figure S6) and Tajima's D values (Table S4). These findings were further corroborated in a species-wide study (Ribeiro et al., 2011) where putatively neutral microsatellites and introns also revealed the lack of population structure and extensive gene flow. However, as a conservative approach, our association analysis incorporated neutral population structure ( $k = 2$ ). If anything, this would lead to an underestimation of putative adaptive SNPs. Thus, we contend that the signatures of genetic local adaptation were not confounded with footprints of neutral processes.

Local adaptation emerges when a spatially heterogeneous environment generates differential pressures, and depends on the balance among drift, selection and gene flow (Lenormand, 2002; Yeaman & Otto, 2011). While extensive gene flow has been thought to erase any locally adapted genotype/phenotype (Lenormand, 2002), when considering temporal heterogeneity, intermediate levels of gene flow can have beneficial effects on local adaptation (Blanquart & Gandon, 2011; Blanquart, Gandon, & Nuismer, 2012). Because temporal unpredictability is a well known feature of the arid Nama Karoo, where our Inland populations occur (Dean & Milton, 1999), it was not unexpected to uncover potential adaptive-loci in the presence of extensive gene flow as suggested by the lack of isolation by distance pattern, the low to undetectable population structure and low degree of genetic differentiation among populations.

Being aware that footprints of selection associated with spatial climatic variation do not necessarily imply causality, it is compelling that several of the putative adaptive SNPs (Table S3) localize in genes encoding machinery responsible for skeletal fibre twitching (myosin, troponin and calcium channel) as this is the principal mechanism that birds use as a heat source in response to cold stress (shivering thermogenesis; Hohtola, 2004). This finding is simultaneously surprising and fascinating, because Inland birds did not increase winter thermogenic capacity through shivering (Ribeiro, Prats, et al., 2018; measured as whole organism  $\text{O}_2$  consumption/ $\text{CO}_2$  production under cold conditions), suggesting that natural selection may be favouring genotypes that have a high efficiency of fibre twitching without altering  $\text{O}_2/\text{CO}_2$  rates. Furthermore, the potential role of 5'AMP activated kinase in allowing local adaptation is noticeable. This enzyme maintains the intracellular energy balance through decreasing energy demand by switching off ATP-consuming pathways, and increasing energy supply by switching on ATP-generating pathways (Carling, 2004). Catalase is another enzyme that may be facilitating local adaptation, as it is essential to convert hydrogen peroxide, highly toxic reactive oxygen species which result from electron transport chain in mitochondria, into water and oxygen and thereby mitigates its toxic effects (Bai & Cederbaum, 2001). These reactive oxygen species increase in domestic chicken (*Gallus gallus*) during heat stress conditions and consequently affected mitochondria function by disrupting its membrane (Azad, Kikusato, Hoque, & Toyomizu, 2010).

Although Janzen's perspective about the evolutionary effects of climatic variability on physiological tolerances (Janzen, 1967) was originally proposed to explain biodiversity patterns in the tropics, it also offers a useful conceptual corollary with which to understand intraspecific divergence in arid-zones. If climatic variability determines population physiological tolerances, and this in turn affects dispersal among populations, then adaptation to local conditions is facilitated. Our results at the intraspecific level support the idea that divergent selection between climatic environments is strong enough to maintain a nonrandom distribution of genotypes underlying key fitness-associated traits (here energetic phenotype) in a genomic background of low differentiation (e.g., Schweizer et al., 2015). Yet, we contend that future work including a "common garden experiment" or "egg transplantation" is fundamental to test for genotype-by-environment interactions while accounting for the possible effects of phenotypic plasticity.

Ultimately, our findings highlight the need for an intraspecific approach when modelling species physiological responses to climate change or forecasting adaptive shifts, rather than assuming species-specific responses (e.g., Moran, Hartig, & Bell, 2016) and may provide an explanation to the high avian intraspecific divergence in harsh environments (Botero, Dor, McCain, & Safran, 2013): local adaptation in energy maintenance-related traits, at least in arid environments.

To conclude, we believe our study exemplifies how it is only within an integrative and detailed framework, that one can begin to pinpoint the factors underlying phenotypic adaptation, and provide candidates for further functional testing under controlled conditions.

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## AUTHOR CONTRIBUTIONS

A.M.R., and M.T.P.G. conceived the study. A.M.R., N.B.P., and B.S. collected field physiology data. L.P. produced and analysed the microbiome data. L.D. was involved in the data sequencing. Y.D., and G.Z. provided access to the *Oenanthe oenanthe* genome (B10K consortium). A.M.R. designed the target capture with input from R.R.F., performed all the molecular work, and analysed the genomic data with input from

R.R.F., and M.T.P.G. A.M.R. analysed physiological data with input from B.S. A.M.R. wrote the paper with contribution from all coauthors.

## DATA AVAILABILITY STATEMENT

Genomic data (avian target capture and gut microbiota) are deposited in NCBI BioProject database: accession PRJNA492976. Geographic location, sex, body mass, tarsus-length, environmental, physiological and summary statistics of genomic data used in this study are provided as Appendix S2 "GeographicalEnvironmentalPhysiologicalGenomicData.xls".

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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