Evolutionary Biology, Genetics and Genomics

Gene expression plasticity followed by genetic change during colonization a high-elevation environment

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Reviewed Preprint

Revised by authors after peer review.

About eLife's process

Reviewed preprint version 2
June 30, 2023 (this version)

Reviewed preprint version 1
May 30, 2023

Posted to bioRxiv
February 21, 2023

Sent for peer review
February 20, 2023

Abstract

Phenotypic plasticity facilitates organismal invasion of novel environments, and the resultant phenotypic change may later be modified by genetic change, so called “plasticity first”. Herein we quantify gene expression plasticity and regulatory adaptation in a wild bird (Eurasian Tree Sparrow) from its original lowland (ancestral stage), experimentally implemented hypoxia acclimation (plastic stage) and colonized highland (colonized stage). Using a group of co-expressed genes from the cardiac and flight muscle, respectively, we demonstrate that gene expression plasticity to hypoxia tolerance is more often reversed than reinforced at the colonized stage. By correlating gene expression change with muscle phenotypes, we show that colonized tree sparrows reduce maladaptive plasticity that largely associates with decrease hypoxia tolerance. Conversely, adaptive plasticity that is congruent with increase hypoxia tolerance is often reinforced in the colonized tree sparrows. Genes displaying large levels of reinforcement or reversion plasticity (*i.e.*, 200% of original level) show greater genetic divergence between ancestral and colonized populations. Overall, our work demonstrates that gene expression plasticity at the initial stage of high-elevation colonization can be reversed or reinforced through selection-driven adaptive modification.

eLife assessment

This study provides **useful** information on the evolution of gene expression levels and plasticity in tissues impacted by hypoxia during colonization of a high-altitude environment. Unfortunately, the evidence for the conclusions is **incomplete** because of the low sample size available.

Introduction

Understanding the interactions between organisms and their environments is becoming increasingly important as environmental change and human activity change the original

distribution of many species (Visser 2008; Wingfield et al. 2011; McCairns et al. 2016; Oostra et al. 2018). While some organisms can easily cope with environmental change, others run the risk of local or global extinction. An organismal response to environmental change partially depends on its ability of phenotypic change (Pfennig et al. 2010; Scoville & Pfrender 2010; Murren et al. 2015; Fox et al. 2019). For organisms that have recently colonized a new environment, phenotypic change may involve two stages. At the early stage, species may rapidly change their phenotype through plasticity without involving genetic changes. Under a persisting strong selective pressure, selection may bring genetic change to canalize phenotypic changes. Consequently, phenotypic plasticity has been considered to play an important stepping stone for genetically based evolutionary change at a late stage, so called "plasticity first" hypothesis (*i.e.*, West-Eberhard 2003; Schwander & Leimar 2011; Levis & Pfennig 2016; Corl et al. 2018).

Under this hypothesis, plasticity may serve adaptive evolution in two alternative ways (Ho & Zhang 2018; Ho et al. 2020). On the one hand, if plasticity changes the phenotype in the same direction as adaptive evolution does, genetic variation can strengthen the change towards an optimal phenotypic value in the new environment (reinforcement plasticity). On the other hand, if the plastic change works in the opposite direction as that driven by adaptive evolution, the subsequent genetic change would have to revert the initial plastic response (reversion/reduction plasticity). As previous studies on morphological and physiological traits have revealed mixed evidence for the reinforcement and the reversion of plasticity (Pigliucci et al. 2006; Lande 2009; Pfennig et al. 2010; Moczek et al. 2011; Levis & Pfennig 2016; Corl et al. 2018), it remains unclear if and how genetic change acts to reinforce or reverse plasticity in the wild species (*i.e.*, Campbell-Staton et al. 2021). The potential challenges are how to quantify phenotypic change relative to plasticity and adaptive evolution, and how to define what genes are involved in the phenotypic change, especially as many phenotypes are likely polygenically mediated (Novembre & Barton 2018; Pritchard et al. 2010).

Comparison of gene expression provides a potential approach to quantify the relative contributions of phenotypic plasticity and genetic variation since gene expression can bridge an organisms' genotype to its cellular biology and, by extension, higher order physiological processes (Wray et al. 2003; Carrol 2008; Morris et al. 2014). While entire transcriptional program is orchestrated by the gene regulatory network, it is possible to trace the genes that are co-expressed or underpin the phenotypic change (Fukao et al. 2011). In addition, gene expression evolves in a stabilizing manner, where regulatory elements accumulate mutations that keep gene expression at an optimal level for physiological functions (Coolon et al. 2014; Gilad et al. 2006; Hodgins-Davis et al. 2015). Herein we used gene expression data to quantify regulatory plasticity and adaptation, as well as the relevant genetic changes in a group of wild birds that have recently colonized a highland environment, the Eurasian Tree Sparrows, *Passer montanus* (hereafter referred as tree sparrows).

The tree sparrow is a human commensal that has spread over a wide variety of habitats in the Eurasian continent, and that has been successfully introduced to Australia and North America (Widmann 1889; Graham et al. 2011). This species colonized the Qinghai-Tibet plateau approximately 2600 years ago (Qu et al. 2020), possibly concurrently with the introduction of barley agriculture in the highland. The Qinghai-Tibet plateau is a harsh environment with an average elevation of 4500 m above sea level (m a.s.l.) where only a few endemic animals can survive. For mammals and birds living at high elevations, hypoxia is one of the strongest selective pressures that drive physiological changes (Barve et al. 2016; McClelland & Scott, 2019). Compared to the lowland ancestral population, highland tree sparrows have evolved physiological and gene expression change for hypoxia tolerance (Sun et al. 2016; Qu et al. 2020), but how these changes relate to plasticity and adaptive evolution are largely unknown.

In order to explore this, we acclimated a group of lowland birds to an experimentally implemented hypoxic condition similar to that where we collected highland birds (*i.e.*, 3200 m a.s.l). We could assess gene expression plasticity and regulatory adaptation in hypoxia tolerance by studying tree sparrows collected a) in their original lowland environment (representing the physiologically ancestral stage), b) shortly after their exposure to the new environmental stimulus (representing the ancestral plasticity at the initially plastic stage), and c) after having adapted to the highland environment (the colonized stage). Using the framework described in Figure 1, we quantified gene expression change stemming from hypoxia acclimation and highland colonization and defined genes with the expression plasticity being reinforced or reversed at the colonization stage. We test whether these genes increase genetic variation between ancestral and colonized populations using a permutation test. We use this framework in four datasets including groups of co-expressed genes associated to hypoxia tolerance and those correlated to muscle phenotypes for the cardiac and flight muscle, respectively. These independent comparisons congruently show that gene expression at the plastic stage is more often reversed than reinforced at the colonized stage, and the selection-driven genetic change depends on the magnitude of reinforcement and/or reversion plasticity.

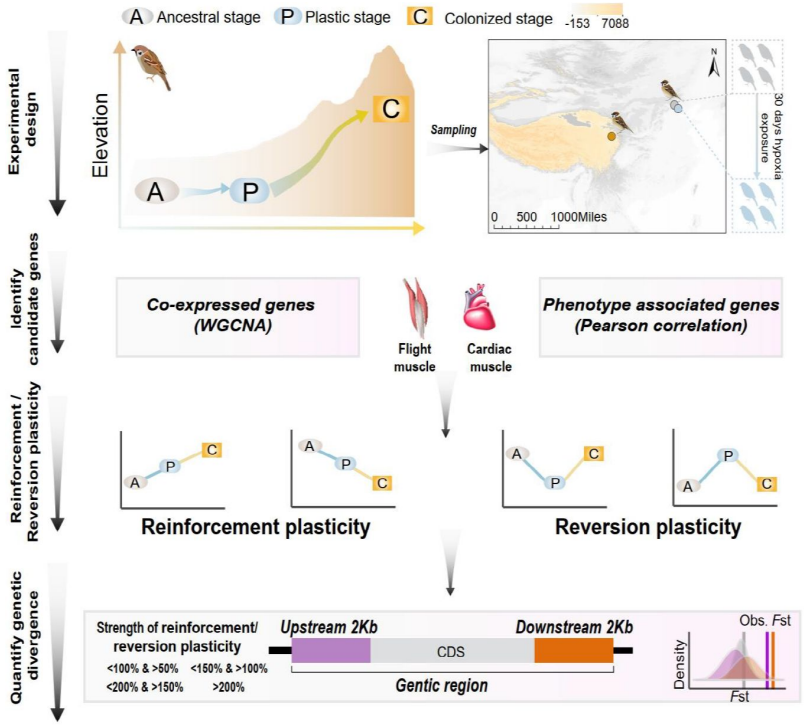

Figure 1.

A schematic representation of quantifying gene expression plasticity and genetic change in relevant genes. **Experimental design**, transcriptomic data of the flight and cardiac muscle were collected from tree sparrows obtained in their original lowland habitat (ancestral stage), experimental exposure to the hypoxic condition (plastic stage), and colonization to a high-elevation environment (colonized stage). **Identify candidate genes**, co-expressed genes that display correlation with hypoxia tolerance were identified by a WGCNA analysis and muscle phenotype associated genes were identified by correlating gene expression levels and each of the muscle phenotypes. **Reinforcement/reversion plasticity**, the genes with reinforcement or reversion plasticity were defined by comparing expression changes between the ancestral and plastic stages (plastic change), and between plastic and colonized stages (evolved change). The strength of reinforcement/reversion plasticity is measured by a range of thresholds, *i.e.*, <100% and >50%, <150% and >100%, <200% and >150%, and >200%. **Quantify genetic divergence**, genetic divergence (*i.e.*, F_{ST}) between lowland and highland populations was estimated for the genic region, 2kb up-stream and down-stream regions of the candidate genes. The empirical F_{ST} values were compared with permuted F_{ST} distributions generated from 100 random samplings from the genic background. A threshold of $P<0.05$ was used to determine statistical significance.

Results

Co-expressed genes involved in hypoxia tolerance generally reverse gene expression plasticity

We sequenced transcriptomic data of the cardiac and flight muscles from the lowland tree sparrows that had been kept under the hypoxia acclimation in this study (n=7), and re-analyzed transcriptomic data in the lowland and highland tree sparrows collected by Qu et al. (2020)(n=14, Supplementary Table 1). We focused on the flight and cardiac muscles because they affect thermogenic capacity and oxygen transport and delivery and are thus critical for the highland adaptation (Scott et al. 2015; Storz et al. 2010). Considering that response to environmental change often involves complex coordinated biological response, such as the co-regulation of genes underpinning physiological functions, we analyzed the resultant expression data for all individuals using weighted gene correlation network analysis (WGCNA, Langfelder & Horvath 2008). Weighted correlation network is a system biology method for identifying groups of highly co-expressed genes (modules), summarizing module-level expression and identifying important genes within modules (hub genes). Consequently, these co-expressed genes can capture expression-stage correlations due to the hypoxia-dependent expression. We identified six and nine modules of co-expressed genes with their expression changes that are significantly associated with the three stages (*i.e.*, ancestral, plastic and colonized stages) for the flight and cardiac muscle, respectively (Figure 2a and Supplementary Figure S1). Across these modules, we identified 2413 and 508 hub genes based on the gene significance function (GS>1st quartile and P<0.05) for the flight and cardiac muscles, respectively (Figure 2b-c).

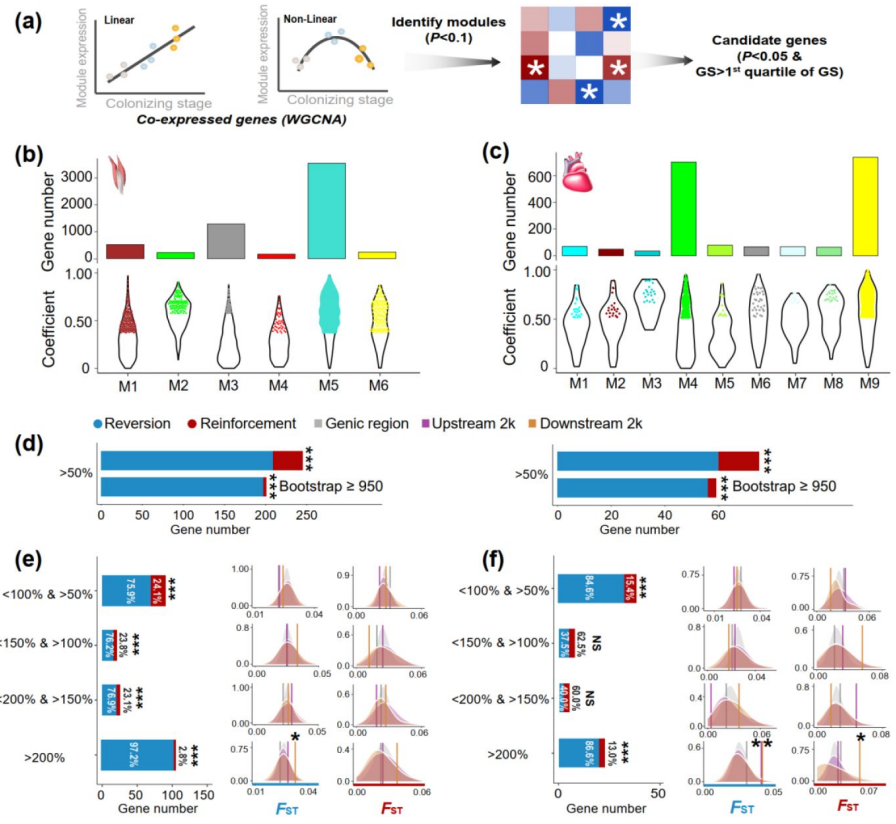

Figure 2.

The WGCNA analysis identified co-expressed genes and reinforcement and reversion of expression plasticity. (a) Pearson linear correlation is used to identify genes with expression reinforcement and nonlinear regression to identify genes with expression reversion. Regulatory modules were identified as branches of the resulting cluster tree via the dynamic tree-cutting method and highly correlated modules (P<0.1) were merged. We used "GS > 1st quartile of GS" and "P<0.05" to identify important genes within modules (hub genes). (b) and (c) Six and nine modules respectively were identified to be associated with different stages for the flight and cardiac muscles (P<0.01). Within each module, colored dots show the genes with the expression levels significantly associated with the stages (gene

significance function>1st quartile and P<0.05). (d) Frequencies of genes with reinforcement and reversion plasticity (>50%) and their subsets that acquire strong support in the parametric bootstrap analyses (≥ 950/1000). (e) and (f) Left, genes with expression plasticity being reinforced (red) or reversed (blue) at the colonized stage identified for the flight (e) and cardiac muscles (f), respectively. There are more genes showing reversion plasticity than those showing reinforcement plasticity. Two-tailed binomial test, NS, nonsignificant; ***, $P < 0.001$. Right, the F_{ST} values (vertical lines) significantly increase in the 2kb up-stream and/or down-stream regions of the genes having the magnitude of reinforcement/reversion plasticity > 200%. Vertical lines, the empirical F_{ST} values; shades, permutated F_{ST} distributions generated from 100 random samplings. NS, non-significant, *, $P < 0.05$; **, $P < 0.01$.

Using these candidate genes we examined how gene expression plasticity changed across the three stages. We compared the gene expression levels between the birds at the ancestral stage and those at the plastic stage, as well as between the birds at the ancestral stage and those at the colonized stage. We attributed genes showing the same direction of change to the reinforcement group and those showing opposite direction of change to the reversion group as described in Ho and Zhang (2018). Using a threshold of >50% increase/reduction of gene expression between two comparisons, we attributed 36 and 15 genes to the reinforcement group and 209 and 60 genes to the reversion group for flight and cardiac muscle, respectively. Interestingly, we found fractions of genes with reversion plasticity are larger than those of genes with reinforcement plasticity (Figure 2d). To confirm the robustness of this observation with respect to random sampling errors, we carried out a parametric bootstrap procedure as described in Ho and Zhang (2019), which aimed to identify genes resulting from genuine differences rather than random sampling errors. Bootstrap results also confirmed that genes exhibiting reversing plasticity significantly outnumber those exhibiting reinforcing plasticity (Figure 2d).

We then explore whether this result is robust with different magnitudes of plasticity by using a range of thresholds of <150% and >100%, <200% and >150%, and >200% to attribute gene to reinforcement and reversion groups. These different categories consistently showed an excess of genes with reversion plasticity (six out of eight comparisons, two-tailed binomial test, P<0.05, Fig. 2e-f). To further confirm the robustness of the discovered pattern against the arbitrariness of threshold selection, we also adopted different categorizations according the magnitude of plasticity (*i.e.*, 20%, 40% and 60% bin settings along the spectrum of the reinforcement/reversion plasticity), and these analyses showed similar results (Supplementary Figure S2). Altogether, these results suggest that high-elevation colonization of tree sparrows generally reverses gene expression plasticity.

If the reinforcement and/or reversion gene expression plasticity in hypoxic tolerance were the target of selection in the high-elevation environment, we would expect to see an increase in the genetic divergence between the ancestral and colonized populations. To evaluate this we searched for genomic signatures of selection using resequencing data from 12 lowland and 11 highland tree sparrows generated from a previous study (Qu et al. 2020). Significantly elevated genetic divergence (*i.e.*, F_{ST}) within the candidate genes beyond the genic background would provide evidence for selection acting on this group of co-expressed genes (*i.e.*, polygenic adaptation). We thus calculated average F_{ST} for the genes with reinforcement or reversion plasticity using all SNPs included in the genic region, 2kb up-stream and down-stream regions, respectively. We considered the up-stream and down-stream regions because gene regulation may refer to genetic variation in nearby non-coding regions. By comparing to the F_{ST} distributions generated from 100 random samplings of the same number of SNPs from the genic background (see Methods), we found that the empirical F_{ST} values significantly increased in the genes with the levels of reinforcement/reversion plasticity reaching above 200% ($P < 0.05$, Figure 2e-f). In all cases, the empirical F_{ST} values increased significantly in the 2kb up-stream and/or down-stream regions of the candidate

genes, but not in the genic regions. This observation thus suggests that selection-driven genetic change is more targeted on the non-coding regions and depends on the magnitude of reinforcement/reversion plasticity.

Gene expression and muscle phenotype analyses show adaptive plasticity and maladaptive plasticity

The evolutionary outcome of the phenotypic plasticity for a population colonizing a novel environment depends on the direction of plastic change with respect to the local optimum of novel environment. If the plastic change is close to the local optimum, *i.e.*, adaptive plasticity, natural selection likely reinforces adaptive plasticity. Conversely, if the plasticity moves the phenotypic change away from the local optimum, *i.e.*, maladaptive plasticity, natural selection should reduce or reverse the reaction norm and restore the phenotypic change back to the original ancestral values (Campbell-Staton et al. 2021). In order to investigate phenotypic plasticity and its evolutionary consequence, we analyzed two datasets that included gene expression and muscle phenotypes collected from the lowland ancestral and highland colonized tree sparrows (Qu et al. 2020).

Based on the correlation analysis between gene expression and muscle phenotypic values of the ancestral and colonized tree sparrows, we categorized the direction of expression-phenotype correlation as positive or negative regulators for the muscle phenotype-associated genes ($P < 0.05$, Figure 3a). We identified 2037 and 1866 muscle phenotype-associated genes in the flight and cardiac muscle, respectively (Figure 3b-c). Out of these genes we found 328 positive regulators and 1709 negative regulators for the flight muscle, and 843 positive regulators and 1023 negative regulators for cardiac muscle (Supplementary Figure S3). We then characterized the differences in gene expression observed for the ancestral-plastic groups and the ancestral-colonized groups as congruent or incongruent with increased hypoxia tolerance (see Methods). Because the muscle phenotypic changes observed in the colonized tree sparrows have been shown to enhance oxygen delivery and improve metabolic capacity in the highland animals (Giordano 2005; Storz et al. 2010; Scott 2011; Scott et al. 2015), we deemed that the gene expressions in which the direction of plastic change matched the expectation for increase hypoxia tolerance as putatively adaptive, while those that opposed this expectation were considered to be putatively maladaptive (Figure 3a). Using a threshold of >50% of plasticity change, we found that the tree sparrows displayed more genes with maladaptive plasticity than genes with adaptive plasticity (two-tailed binomial test, flight muscle, 198 vs. 48, $P<2.2e^{-16}$, cardiac muscle 186 vs. 166, $P<0.05$). These results are robust with random sampling errors as our parametric bootstrap analyses also revealed similar results (Figure 3d).

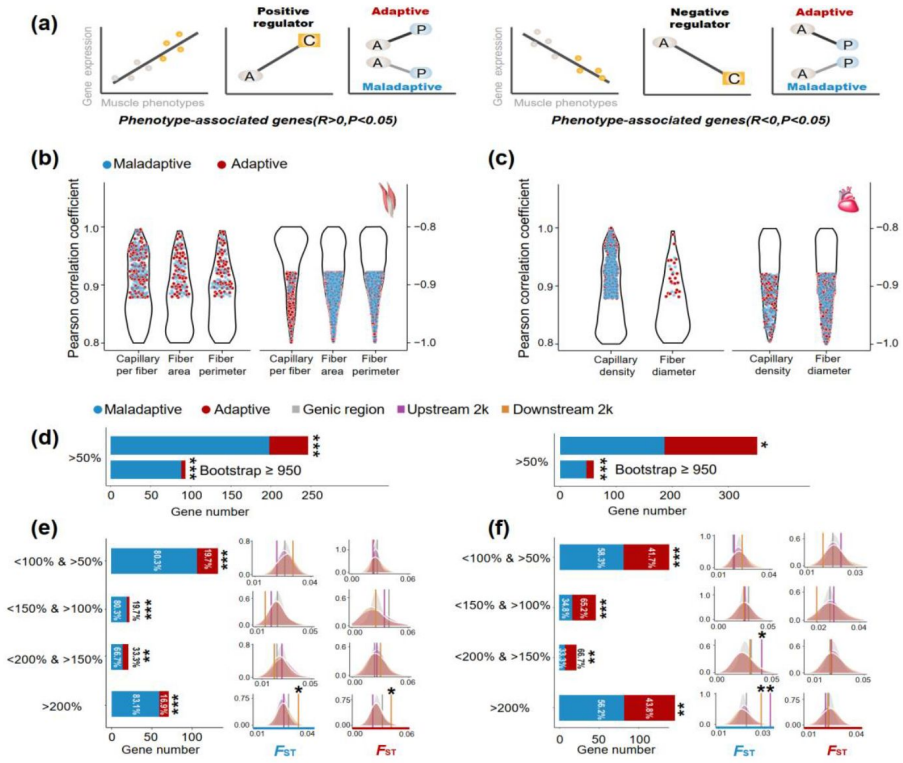

Figure 3.

Genes with expression levels associated with muscle phenotypes and adaptive and maladaptive plasticity. (a) Gene expression profiles were correlated with muscle phenotypes. The direction of gene expression plasticity at the plastic stage matched the expectation for positive regulator (increased expression) and negative regulators (decreased expression) in the colonized tree sparrows was considered to indicate adaptive plasticity. Conversely, those that opposed this expectation were considered to be maladaptive, *i.e.*, the direction of gene expression plasticity at the plastic stage showed decreased expression for the positive regulators and increased expression for negative regulators. A, ancestral stage; P, plastic stage; C, colonized stage. (b) and (c) Gene expression profiles were correlated with three flight muscle phenotypes (b, capillary per fiber, fiber area and perimeter) and two cardiac muscle phenotype (c, capillary density and fiber diameter). Red dots show genes with adaptive gene expression plasticity and blue dots show genes with maladaptive gene expression plasticity. (d) Frequencies of genes with adaptive and maladaptive plasticity (>50%) and their subsets that acquire strong support in the parametric bootstrap analyses (≥ 950/1000). (e) and (f) Left, candidate genes were classified to four categories with different magnitudes of adaptive or maladaptive plasticity. More genes show maladaptive plasticity than those show adaptive plasticity. Two-tailed binomial test, **, $P < 0.01$; ***, $P < 0.001$. Right, the empirical F_{ST} values significantly increased in the 2k up-stream and/or down-stream regions of the genes having adaptive/maladaptive plasticity <200% and >150% (cardiac muscle) and >200% (flight and cardiac muscles). (e) Flight muscle, (f) Cardiac muscle. Vertical lines, the empirical F_{ST} values; shades, permutated F_{ST} distributions generated from 100 times sampling. *, $P < 0.05$; **, $P < 0.01$.

We subsequently examined expression-stage relationship using the thresholds (<150% and >100%, <200% and >150%, and >200%) and bin settings (20%, 40% and 60% bin settings) as above-mentioned to explore the magnitudes of adaptive and maladaptive plasticity. We found that the fractions of genes with maladaptive plasticity were larger than those of genes with adaptive plasticity (two tailed binomial test, $P<0.01$, Figure 3d). This observation is robust with different thresholds and bin settings for the flight muscle, but slightly sensitive to bin setting for the cardiac muscle, indicating by more than 50% of comparisons supporting an excess of genes with maladaptive plasticity (Supplementary Figure S4). These results suggest that gene expression plasticity induced by hypoxia exposure at the early stage may be mainly maladaptive for muscle physiological performance in highland environment.

The observed adaptive and maladaptive gene expressions in the tree sparrows suggest that selection may have acted to reinforce and/or reverse gene expression plasticity. We then quantified the genetic divergence of these genes between ancestral and colonized tree sparrows by testing for statistically significant increase of F_{ST} as compared to the F_{ST}

distributions generated from 100 random samplings (see Methods). We found that the empirical F_{ST} values were significantly larger in the genes within the <200% and >150% category (cardiac muscle, P<0.05) and >200% category (flight and cardiac muscle, P<0.05), but not in those within other categories with low levels of reinforcement and reversion plasticity. Additionally, we found that genetic divergence between the ancestral and colonized tree sparrows tends to increase in the 2kb up-stream and down-stream regions of these genes (P < 0.05, Figure 3e-f). These results suggest that selection on regulatory adaptation (*i.e.*, genetic divergence) may only be expected if large strength of selection is needed to bring initial plasticity toward adaptive optimum of the novel environment.

Discussion

An understanding of phenotypic plasticity and its consequence for adaptive evolution is important in evolutionary biology but remains challenging (Ghalambor et al. 2007; 2015; Campbell-Staton et al. 2021; Kenkel & Matz 2017; Rivera et al. 2021). Using an integrative approach of field-based studies, experimentally implemented acclimation experiment, multiomic and muscle phenotypic data, our study demonstrates the importance of gene expression plasticity in facilitating initial survival and population persistence at early stage of colonization. In particular, our results provide novel insight into an issue of active debate within evolutionary biology (*i.e.*, reinforcement vs. reversion plasticity) by showing that the evolutionary consequence of plasticity depends on the adaptive plasticity (reinforcement) or maladaptive plasticity (reversion). We also demonstrate that natural selection drives genetic divergence when the magnitude of reinforcement and/or reversion plasticity becomes intense, providing novel insights into the mechanism of plasticity first and gene as followers (Schwander & Leimar 2011).

Our gene expression and sequence analyses provide several lines of evidence in support for gene expression plasticity contribution to the high-elevation colonization of tree sparrows. First, we show that co-expressed genes and muscle phenotype associated genes change either the intensity or the direction of their expression profiles, and that gene expressions at the plastic stage are more often reversed than reinforced at the colonization stage. Second, by combining gene expression and muscle phenotype, we show that selection operates intensely on maladaptive plasticity during the initial stage of responding novel environment (Ghalambor et al. 2007; 2015; Ho and Zhang 2018; Campbell-Staton et al. 2021). Third, the genes with intensified levels of reinforcement and reversion expression plasticity show large genetic divergence (F_{ST}) in their up-stream and down-stream regions between the ancestral and colonized populations, suggesting that selection has driven genetic changes in the noncoding regions after the tree sparrows had colonized the novel environment.

These observations thus support that plasticity serves as a stepping stone in a successful colonization of a new environment and that genetic changes that evolve at a late stage will modify the plastic change to an optimal phenotype in the new environment (Corl et al. 2018; Schwander & Leimar, 2011). It is interesting to think about why phenotypic change at the plastic stage sometimes differs from those at the colonized stage. A likely reason is that initially plastic change allows organisms to survive upon a sudden environmental shift but the fitness is much reduced compared to that after a long-term colonization to the novel environment (Fischer et al. 2016; Huang & Agrawal 2016; Leonard & Lancaster 2020; Kuo et al. 2023). Thus, the overall physiological states of the organisms right after an environmental shift may result in low fitness. To coordinate the phenotype values to close the organismal fitness, a genetic change may be required modifying plastic change of phenotype to approach the optimum to the novel environment (Storz 2021). Thus, the consequence of

genetic change on plasticity is dependent on the cost of plastic change (Ho and Zhang 2018; Corl et al. 2018).

A limitation of our study is that we could only consider hypoxic condition in the acclimation experiment, while also other factors associated with high-elevation environments, *e.g.*, low temperature and UV radiation, may be of importance. Although we assume that hypoxia is the dominant selective force in the Qinghai-Tibet Plateau (see also Sun et al. 2016; Qu et al. 2020), the use of a single environmental stimulus makes our conclusions more conservative. Also, it is reasonable to assume that plastic change in organisms is time-dependent and that our results cannot catch gene expression occurring at later stage of plastic response (*i.e.*, after one month). Since we could only run the acclimation experiment for one month it is likely we have not identified all genes related to hypoxia tolerance. Despite this, we could observe a clear pattern of reinforcement and reversion plasticity and the associated genetic changes. Our sample sizes are rather small (n=12 for the flight muscle and n=9 for the cardiac muscle), mostly because of the logistical challenge of keeping tree sparrows in the necessary common garden experiments. In order to compensate for this, we utilized comparative transcriptomics to generate the four independent datasets (a group of co-expressed genes and a group of phenotype-associated genes for the flight and cardiac muscles, respectively). As the analyses of these four independent datasets show similar results, we believe the conclusions drawn from our study are robust.

Despite some limitations, our study demonstrates an easy implemented framework for quantifying phenotype plasticity and how to relate this to genetic change. Short-term acclimation response of lowland populations has been found to be different from the populations adapted to the high-elevation environments. For example, people newly exposed to high-elevation environment increase their red blood cell as compared to people at sea level, which is likely mediated by *HIF* and *EPO* genes in hypoxia response cascade (Erzurum et al. 2007; Peng et al. 2017). This physiological change, however, is less activated in native people at high-elevation when compared to people at sea level, demonstrating an example of reversion of phenotypic plasticity (Storz 2021). Together with previous studies, our work shows that short-term acclimation response and long-term adaptive evolution in hypoxia response may affect different evolutionary pathways. Our work provides understanding how plasticity facilitates species to invade new habitats and survive environmental change. Such an understanding is important, in particular when numerous species are being introduced to new regions of the globe through human intervention and spread as invasive species (Visser 2008).

Materials and Methods

Sampling

We generated transcriptomic data from hypoxia-acclimated lowland tree sparrows by exposing five lowland tree sparrows to hypoxic condition for 30 days using a hypoxic chamber (Qu et al. 2020). The oxygen content in the chamber was set to 14% of the oxygen content (simulating the oxygen concentration at 3200 m a.s.l., 70% of ~20%, latter of which is the content of oxygen content at sea level), which is similar to condition where we collected the highland tree sparrows (Qinghai Lake, 3200 m a.s.l.). We collected flight muscle from the four individuals and cardiac muscle from three individuals. In addition, we extended the analyses of the transcriptomic and muscle histological data from tree sparrows that were collected from lowland (Beijing and Hebei, 100 m a.s.l.) and highland (Qinghai Lake, 3200 m a.s.l., Figure 1) collected in previous study (Qu et al. 2020). In total, we used twelve samples for flight muscle and nine samples for cardiac muscle (Supplementary Table 1). The

ancestral lowland and hypoxia-exposed lowland tree sparrows were collected from the same locality (Beijing) and the same season (pre-breeding). Only adult birds with similar body weight (approximately 18g) were used.

Transcriptome sequencing

RNA libraries were constructed for the flight and cardiac muscles and sequenced on an Illumina HiSeq4000 platform. After filtering low-quality, adapter-contaminated, and N-rich reads (>10%), a total of 134, 128 and 120 million reads of transcriptional data were generated for the lowland, hypoxia-exposed lowland tree sparrows and highland tree sparrows, respectively (Supplementary Table S1). We mapped cleaned reads against the tree sparrow genome using STAR (Dobin et al. 2013). We compared log-transformed transcript per million (TPM) across all genes and determined the most conserved genes (*i.e.*, coefficient of variance ≤ 0.3 and average TPM ≥ 1 for each sample) for the flight and cardiac muscles, respectively (Hao et al. 2023). We then compared the median expression levels of these conserved genes and found no difference among the lowland, hypoxia-exposed lowland, and highland tree sparrows (Wilcoxon signed-rank test, $P<0.05$, Supplementary Figure S5), suggesting that batch effect had little influence on the transcriptomic data. We then used TPM values to calculate gene expression level and intensity using RSEM (Li & Dewey 2011).

WGCNA analyses

We used WGCNA v. 1.41-1 (Langfelder and Horvath, 2008) to identify regulatory architecture for the flight muscle and cardiac muscle transcriptomes. Specifically, we used a principal component analysis (PCA) to summarize modules of genes expression with blockwiseModules function, and then used module eigengene values of the first principal component (PC) to test correlation between module expression and stages. We used Pearson linear correlation (cor function) to identify genes with expression reinforcement and nonlinear regression (polynomial regression model: Model = lm(y ~ poly(x, 5, raw = TRUE), data=data) to identify genes with expression reversion. Regulatory modules were identified as branches of the resulting cluster tree via the dynamic tree-cutting method and highly correlated modules ($P<0.1$) were merged. We used "GS > 1st quartile of GS" and "$P<0.05$" to identify important genes within modules (hub genes).

Identify gene regulation associated with muscle phenotype

To identify the genes with expression levels that correlated to evolved differences in the muscle phenotypes in the tree sparrows, we used the lowland and highland individuals from Qu et al. (2020) for which RNAseq and muscle phenotypic data were available. We correlated the levels of gene expression with muscle phenotypes in the lowland and highland individuals. We used three phenotypes for the flight muscle (capillary per fiber, fiber area and perimeter) and two for the cardiac muscle (capillary density and fiber diameter). We used Pearson correlation to test the association between each of the phenotypic traits and expression profile of each gene (TPM). A threshold of $P<0.05$ was used to detect muscle-associated genes. We then followed Campbell-Staton et al. (2021) to categorize genes as positive (positively correlated with muscle phenotypes) or negative regulators (negatively correlated with muscle phenotypes).

Subsequently, we compared the direction of gene expression plasticity at the plastic stage with those of the positive and negative regulators. Briefly, the direction of gene expression plasticity at the plastic stage matched the expectation for positive regulator (increased

expression) or negative regulator (decreased expression) in the colonized tree sparrows was considered to indicate adaptive plasticity. Conversely, those that opposed this expectation were considered to be maladaptive, *i.e.*, the direction of gene expression plasticity at the plastic stage showed decreased expression for the positive regulators and increased expression for negative regulators (Figure 3a).

Reinforcement and reversion plasticity analyses

To investigate the magnitude of reinforcement and reversion plasticity, we followed the method described in Ho and Zhang (2018). Specifically, expression levels of each gene in the three stages were treated as E_lowland (expression level at ancestral stage), E_hypoxia (expression level at plastic stage) and E_highland (expression level at colonized stage). We used a threshold of 50% of the ancestral gene expression level, *i.e.*, 50% * E_lowland, to detect an excess of gene expression change. We identified genes with an excess of plastic change if it satisfied the condition of |(E_hypoxia-E_lowland)|> 50% * E_lowland. Likewise, we identified genes with an excess of evolutionary change if it met the condition of |(E_highland-E_hypoxia)|> 50%* E_lowland. If the genes showed same direction in the expression change at the plastic and colonized stages, we regarded these genes as reinforcement expression. If the genes showed opposite direction in the expression change at the plastic and colonized stages, we regarded these genes as reversion expression. We categorized these genes to reinforcement and reversion groups and then tested if the proportions of the genes in the two groups were significantly different from the expected proportions using two-tailed binomial tests.

To explore whether an excess of genes with reversion plasticity was subject to random sampling errors (Mallard et al. 2018), we used a parametric bootstrap method as described in Ho and Zhang (2019). Specifically, we simulated the mean expression level of a gene at each stage following a Gaussian distribution within the mean equal to the observed mean expression of this gene at this stage and the standard deviation equal to the estimated standard error. We then draw a random variable from the above Gaussian distribution to represent an observation of the mean expression level of this gene at this stage. We drew random variables representing the mean expression level of gene at each of the three stages (*i.e.*, ancestral, plastic and colonized stages), and then computed gene expression changes at plastic stage and colonized stage and determined the reinforcement or reversion plasticity. This process is repeated 1000 times for each gene. If this gene exhibited reinforcement or reversion plasticity at least 950 repeats (P<0.05), we considered this gene showing reinforcement or reversion plasticity. The Gaussian distribution and random sampling were generated using rnorm function of R.

To test the impact of the choice of thresholds on the results, we analyzed the data using a range of thresholds, *i.e.*, <100% and >50%, <150% and >100%, <200% and >150%, and >200%. This setting aimed to obtain independent genesets for subsequently genetic divergence analyses, because a geneset selected by relax threshold (*i.e.*, 50%) also included genesets obtained by the stringent threshold (*i.e.*, 100%, 150% and 200%). In addition, we also implemented other categorization scheme to check the robustness of the results.

Specifically, we used three different bin settings (*i.e.*, 20%, 40% and 60% bin settings along the spectrum of the reinforcement/reversion plasticity), to group genes according their magnitude of plasticity. For each category in the three bin settings, we compared whether the proportion of the genes with reversion plasticity differed from that with reinforcement plasticity using two-tailed binomial tests.

Comparing genetic divergence for genes with reinforcement and reversion expression plasticity

To calculate the genetic divergence between the lowland ancestral and highland colonized tree sparrows, we used 12 lowland and 11 highland individuals for which re-sequencing data were available (Qu et al. 2020). After mapping the raw reads to the tree sparrow genome using BWA v0.7.17 (Li & Durbin 2009), we obtained mean sequence coverage of 17× for each individual (Table S2). We called variants with GATK v 3.7 (McKenna et al. 2010) and Samtools v1.2 (http://www.htskib.org/) and filtered single nucleotide polymorphism (SNP) using VCF tools and GATK with minimum coverage = 138, root mean square mapping quality >= 20, distance of adjacent SNPs >=5, distance to a gap >=5bp and read quality value >=30.

We calculated average F_{ST} using Vcftools (Danecek et al. 2011) with SNPs included in the genic region, 2kb up-stream and down-stream regions of the genes within each of the four categories, *i.e.*, <100% and >50%, <150% and >100%, <200% and >150%, and >200%. The up-stream and down-stream regions were considered because gene regulation may refer to genetic variation in the nearby genic regions. We permuted F_{ST} distributions for the genes within each category by random sampling the same number of SNPs, allowing for a fluctuation of 5% of total SNPs. We compared the empirical F_{ST} values to permutated F_{ST} distributions generated from 100 samplings and considered a threshold of $P<0.05$ to be statistically significant (empirical F_{ST}>95% percentile of permutated F_{ST} distribution). We calculated empirical F_{ST} values and permutated F_{ST} distributions for the genic region, 2kb up-stream and down-stream regions separately. To avoid the sampling bias (*i.e.*, sampling SNPs from neutral regions), our SNP sampling was constrained to genic region, 2k up-stream and down-stream regions of all genes (*i.e.*, 16925 genes). All analyses were conducted with the R statistical software package (R Foundation for Statistical Computing 2017).

Data accessibility

DNA sequencing reads used in this study have been deposited in Short Read Archive under the project number PRJNA417520.

Authors' contributions

Q.Y. designed research; S.H., Y.H., X.L., S.G. and Q.Y. performed research; F.L. provided highland samples; Q.Y. and S.H. wrote the paper.

Acknowledgements

We acknowledge Ying Xiong for logistic work in the hypoxia-exposed experiment. This research was funded by the Third Xinjiang Scientific Expedition and Research (XIKK) (2022xjkk0205), Second Tibetan Plateau Scientific Expedition and Research (2019QZKK0501) and the National Natural Science Foundation of China (NSFC32020103005).

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Reviewer #1 (Public Review):

She et al studied the evolution of gene expression reaction norms when individuals colonise a new environment that exposes them to physiologically challenging conditions. Their objective was to test the "plasticity first" hypothesis, which suggest that traits that are already plastic (their value changes when facing a new environment compared to the original environment) facilitates the colonisation of novel environments, which, if true, would be predicted to result in the evolution of gene expression values that are similar in the population that colonised the new environment and evolved under these particular selection pressures. To test this prediction, they studied gene expression in cardiac and muscle tissues in individuals originating from three conditions: lowland individuals in their natural environment (ancestral state), lowland individuals exposed to hypoxia (the plastic response state), and a highland population facing hypoxia for several generations (the coloniser state). They classified gene expression patterns as maladaptive or adaptive in lowland individuals responding to short term hypoxia by classifying gene expression patterns using genes that differed between the ancestral state (lowland) and colonised state (highland). Genes expressed in the same direction in lowland individuals facing hypoxia (the plastic state) as what is found in the colonised state are defined as adaptative, while genes with the opposite expression pattern were labelled as maladaptive, using the assumption that the colonised state must represent the result of natural selection. Furthermore, genes could be classified as representing reversion plasticity when the expression pattern differed between the plasticity and colonised states and as reinforcement when they were in the same direction (for example more expressed in the plastic state and the colonised state than in the ancestral state). They found that more genes had a plastic expression pattern that was labelled as maladaptive than adaptive. Therefore, some of the genes have an expression pattern in accordance with what would be predicted based on the plasticity-first hypothesis, while others do not.

As pointed out by the authors themselves, the fact that temperature was not included as a variable, which would make the experimental design much more complex, misses the opportunity to more accurately reflect the environmental conditions that the colonizer individuals face at high altitude. Also pointed out by the authors, the acclimation experiment in hypoxia lasted 4 weeks. It is possible that longer term effects would be identifiable in gene expression in the lowland individuals facing hypoxia on a longer time scale. Furthermore, a sample size of 3 or 4 individuals per group depending on the tissue for wild individuals may miss some of the natural variation present in these populations. Stating that they have a n=7 for the plastic stage and n= 14 for the ancestral and colonized stages refers to the total number of tissue samples and not the number of individuals, according to supplementary table 1.

Impact of the work:

There has been work showing that populations adapted to high altitude environments show changes in their hypoxia response that differs from the short-term acclimation response of lowland population of the same species. For example, in humans, see Erzurum et al. 2007 and Peng et al. 2017, where they show that the hypoxia response cascade, which starts with the gene HIF (Hypoxia-Inducible Factor) and includes the EPO gene, which codes for erythropoietin, which in turns activates the production of red blood cell, is LESS activated in high altitude individuals compared to the activation level in lowland individuals (which gives it its name). The present work adds to this body of knowledge showing that the short-term response to hypoxia and the long term one can affect different pathways and that acclimation/plasticity does not always predict what physiological traits will evolve in

populations that colonize these environments over many generations and additional selection pressure (UV exposure, temperature, nutriment availability).

Altogether, this work provides new information on the evolution of reaction norms of genes associated with the physiological response to one of the main environmental variables that affects almost all animals, oxygen availability. It also provides an interesting model system to study this type of question further in a natural population of homeotherms.

Erzurum, S. C., S. Ghosh, A. J. Janocha, W. Xu, S. Bauer, N. S. Bryan, J. Tejero et al. "Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans." Proceedings of the National Academy of Sciences 104, no. 45 (2007): 17593-17598.

Peng, Y., C. Cui, Y. He, Ouzhuluobu, H. Zhang, D. Yang, Q. Zhang, Bianbazhuoma, L. Yang, Y. He, et al. 2017. Down-regulation of EPAS1 transcription and genetic adaptation of Tibetans to high-altitude hypoxia. Molecular biology and evolution 34:818-830.

Reviewer #2 (Public Review):

This is a well-written paper using gene expression in tree sparrow as model traits to distinguish between genetic effects that either reinforce or reverse initial plastic response to environmental changes. Tree sparrow tissues (cardiac and flight muscle) collected in lowland and highland populations subject to hypoxia treatment were profiled for gene expression and compared in 1) highland birds; 2) lowland birds under normal conditions to test for differences in directions of changes between initial plastic response and subsequent colonized response.

The authors clarified several points and made revisions according to my comments. It is good to know that the highland and lowland samples were collected and processed at the same time and the previous publication reported part of the data. My concerns regarding the conclusions about reversal versus reinforcement remain even after the additional analyses. Further studies are needed to confirm these results.

Author Response:

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

She et al studied the evolution of gene expression reaction norms when individuals colonise a new environment that exposes them to physiologically challenging conditions. Their objective was to test the "plasticity first" hypothesis, which suggest that traits that are already plastic (their value changes when facing a new environment compared to the original environment) facilitates the colonisation of novel environments, which, if true, would be predicted to result in the evolution of gene expression values that are similar in the population that colonised the new environment and evolved under these particular selection pressures. To test this prediction, they studied gene expression in cardiac and muscle tissues in individuals originating from three conditions: lowland individuals in their natural environment (ancestral state), lowland individuals exposed to hypoxia (the plastic response state), and a highland population facing hypoxia for several generations (the coloniser state). They classified gene expression patterns as maladaptive or adaptive in lowland individuals responding to short term hypoxia by classifying gene expression patterns using genes that differed between the ancestral state (lowland) and colonised state (highland). Genes expressed in the same direction in lowland individuals facing hypoxia (the plastic state) as what is found in the colonised state are defined as adaptative, while

> *genes with the opposite expression pattern were labelled as maladaptive, using the assumption that the colonised state must represent the result of natural selection. Furthermore, genes could be classified as representing reversion plasticity when the expression pattern differed between the plasticity and colonised states and as reinforcement when they were in the same direction (for example more expressed in the plastic state and the colonised state than in the ancestral state). They found that more genes had a plastic expression pattern that was labelled as maladaptive than adaptive. Therefore, some of the genes have an expression pattern in accordance with what would be predicted based on the plasticity-first hypothesis, while others do not.*

Thank you for a precise summary of our work. We appreciate the very encouraging comments recognizing the value of our work. We have addressed concerns from the reviewer in greater detail below.

> *Q1. As pointed out by the authors themselves, the fact that temperature was not included as a variable, which would make the experimental design much more complex, misses the opportunity to more accurately reflect the environmental conditions that the colonizer individuals face at high altitude. Also pointed out by the authors, the acclimation experiment in hypoxia lasted 4 weeks. It is possible that longer term effects would be identifiable in gene expression in the lowland individuals facing hypoxia on a longer time scale. Furthermore, a sample size of 3 or 4 individuals per group depending on the tissue for wild individuals may miss some of the natural variation present in these populations. Stating that they have a n=7 for the plastic stage and n= 14 for the ancestral and colonized stages refers to the total number of tissue samples and not the number of individuals, according to supplementary table 1.*

We shared the same concerns as the reviewer. This is partly because it is quite challenging to bring wild birds into captivity to conduct the hypoxia acclimation experiments. We had to work hard to perform acclimation experiments by taking lowland sparrows in a hypoxic condition for a month. We indeed have recognized the similar set of limitations as the review pointed out and have discussed the limitations in the study, i.e., considering hypoxic condition alone, short time acclimation period, etc. Regarding sample sizes, we have collected cardiac muscle from nine individuals (three individuals for each stage) and flight muscle from 12 individuals (four individuals for each stage). We have clarified this in Supplementary Table 1.

> *Q2. Finally, I could not find a statement indicating that the lowland individuals placed in hypoxia (plastic stage) were from the same population as the lowland individuals for which transcriptomic data was already available, used as the "ancestral state" group (which themselves seem to come from 3 populations Qinghuangdao, Beijing, and Tianjin, according to supplementary table 2) nor if they were sampled in the same time of year (pre reproduction, during breeding, after, or if they were juveniles, proportion of males or females, etc). These two aspects could affect both gene expression (through neutral or adaptive genetic variation among lowland populations that can affect gene expression, or environmental effects other than hypoxia that differ in these populations' environments or because of their sexes or age). This could potentially also affect the FST analysis done by the authors, which they use to claim that strong selective pressure acted on the expression level of some of the genes in the colonised group.*

The reviewer asked how individual tree sparrows used in the transcriptomic analyses were collected. The individuals used for the hypoxia acclimation experiment and represented the ancestral lowland population were collected from the same locality (Beijing) and at the same season (i.e., pre-breeding) of the year. They are all adults and weight approximately 18g. We have clarified this in the Supplementary Table S1 and Methods. We did not distinguish males from females (both sexes look similar) under the assumption that both sexes respond similarly to hypoxia acclimation in their cardiac and flight muscle gene expression.

The Supplementary Table 2 lists the individuals that were used for sequence analyses. These individuals were only used for sequence comparisons but not for the transcriptomic analyses. The population genetic structure analyzed in a previously published study showed that there is no clear genetic divergence within the lowland population (i.e., individuals collected from Beijing, Tianjing and Qinhuangdao) or the highland population (i.e., Gangcha and Qinghai Lake). In addition, there was no clear genetic divergence between the highland and lowland populations (Qu et al. 2020).

> *Q4. Impact of the work*
>
> *There has been work showing that populations adapted to high altitude environments show changes in their hypoxia response that differs from the short-term acclimation response of lowland population of the same species. For example, in humans, see Erzurum et al. 2007 and Peng et al. 2017, where they show that the hypoxia response cascade, which starts with the gene HIF (Hypoxia-Inducible Factor) and includes the EPO gene, which codes for erythropoietin, which in turns activates the production of red blood cell, is LESS activated in high altitude individuals compared to the activation level in lowland individuals (which gives it its name). The present work adds to this body of knowledge showing that the short-term response to hypoxia and the long term one can affect different pathways and that acclimation/plasticity does not always predict what physiological traits will evolve in populations that colonize these environments over many generations and additional selection pressure (UV exposure, temperature, nutrient availability). Altogether, this work provides new information on the evolution of reaction norms of genes associated with the physiological response to one of the main environmental variables that affects almost all animals, oxygen availability. It also provides an interesting model system to study this type of question further in a natural population of homeotherms.*
>
> *Erzurum, S. C., S. Ghosh, A. J. Janocha, W. Xu, S. Bauer, N. S. Bryan, J. Tejero et al. "Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans." Proceedings of the National Academy of Sciences 104, no. 45 (2007): 17593-17598.*
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> *Peng, Y., C. Cui, Y. He, Ouzhuluobu, H. Zhang, D. Yang, Q. Zhang, Bianbazhuoma, L. Yang, Y. He, et al. 2017. Down-regulation of EPAS1 transcription and genetic adaptation of Tibetans to high-altitude hypoxia. Molecular biology and evolution 34:818-830.*

Thank you for highlighting the potential novelty of our work in light of the big field. We found it very interesting to discuss our results (from a bird species) together with similar findings from humans. In the revised version of manuscript, we have discussed short-term acclimation response and long-term adaptive evolution to a high-elevation environment, as well as how our work provides understanding of the relative roles of short-term plasticity and long-term adaptation. We appreciate the two important work pointed out by the reviewer and we have also cited them in the revised version of manuscript.

> ***Reviewer #2 (Public Review):***
>
> *This is a well-written paper using gene expression in tree sparrow as model traits to distinguish between genetic effects that either reinforce or reverse initial plastic response to environmental changes. Tree sparrow tissues (cardiac and flight muscle) collected in lowland populations subject to hypoxia treatment were profiled for gene expression and compared with previously collected data in 1) highland birds; 2) lowland birds under normal condition to test for differences in directions of changes between initial plastic response and subsequent colonized response. The question is an important and interesting one but I have several major concerns on experimental design and interpretations.*

Thank you for a precise summary of our work and constructive comments to improve this study. We have addressed your concerns in greater detail below.

> *Q1. The datasets consist of two sources of data. The hypoxia treated birds collected from the current study and highland and lowland birds in their respective native environment from a previous study. This creates a complete confounding between the hypoxia treatment and experimental batches that it is impossible to draw any conclusions. The sample size is relatively small. Basically correlation among tens of thousands of genes was computed based on merely 12 or 9 samples.*

We appreciate the critical comments from the reviewer. The reviewer raised the concerns about the batch effect from birds collected from the previous study and this study. There is an important detail we didn't describe in the previous version. All tissues from hypoxia acclimated birds and highland and lowland birds have been collected at the same time (i.e., Qu et al. 2020). RNA library construction and sequencing of these samples were also conducted at the same time, although only the transcriptomic data of lowland and highland tree sparrows were included in Qu et al. (2020). The data from acclimated birds have not been published before.

In the revised version of manuscript, we also compared log-transformed transcript per million (TPM) across all genes and determined the most conserved genes (i.e., coefficient of variance ≤ 0.3 and average TPM ≥ 1 for each sample) for the flight and cardiac muscles, respectively (Hao et al. 2023). We compared the median expression levels of these conserved genes and found no difference among the lowland, hypoxia-exposed lowland, and highland tree sparrows (Wilcoxon signed-rank test, $P<0.05$). As these results suggested little batch effect on the transcriptomic data, we used TPM values to calculate gene expression level and intensity. This methodological detail has been further clarified in the Methods and we also provided a new supplementary Figure (Figure S5) to show the comparative results.

The reviewer also raised the issue of sample size. We certainly would have liked to have more individuals in the study, but this was not possible due to the logistical problem of keeping wild bird in a common garden experiment for a long time. We have acknowledged this in the manuscript. In order to mitigate this we have tested the hypothesis of plasticity following by genetic change using two different tissues (cardiac and flight muscles) and two different datasets (co-expressed gene-set and muscle-associated gene-set). As all these analyses show similar results, they indicate that the main conclusion drawn from this study is robust.

> *Q2. Genes are classified into two classes (reversion and reinforcement) based on arbitrarily chosen thresholds. More "reversion" genes are found and this was taken as evidence reversal is more prominent. However, a trivial explanation is that genes must be expressed within a certain range and those plastic changes simply have more space to reverse direction rather than having any biological reason to do so.*

Thank you for the critical comments. There are two questions raised we should like to address them separately. The first concern centered on the issue of arbitrarily chosen thresholds. In our manuscript, we used a range of thresholds, i.e., 50%, 100%, 150% and 200% of change in the gene expression levels of the ancestral lowland tree sparrow to detect genes with reinforcement and reversion plasticity. By this design we wanted to explore the magnitudes of gene expression plasticity (i.e., Ho & Zhang 2018), and whether strength of selection (i.e., genetic variation) changes with the magnitude of gene expression plasticity (i.e., Campbell-Staton et al. 2021).

As the reviewer pointed out, we have now realized that this threshold selection is arbitrarily. We have thus implemented two other categorization schemes to test the robustness of the observation of unequal proportions of genes with reinforcement and reversion plasticity. Specifically, we used a parametric bootstrap procedure as described in Ho & Zhang (2019), which aimed to identify genes resulting from genuine differences rather than random sampling errors. Bootstrap results suggested that genes exhibiting reversing plasticity

significantly outnumber those exhibiting reversing plasticity, suggesting that our inference of an excess of genes with reversion plasticity is robust to random sampling errors. We have added these analyses to the revised version of manuscript, and provided results in the Figure 2d and Figure 3d.

In addition, we adapted a bin scheme (i.e., 20%, 40% and 60% bin settings along the spectrum of the reinforcement/reversion plasticity). These analyses based on different categorization schemes revealed similar results, and suggested that our inference of an excess of genes with reversion plasticity is robust. We have provided these results in the Supplementary Figure S2 and S4.

The second issue that the reviewer raised is that the plastic changes simply have more space to reverse direction rather than having any biological reason to do so. While a causal reason why there are more genes with expression levels being reversed than those with expression levels being reinforced at the late stages is still contentious, increasingly many studies show that genes expression plasticity at the early stage may be functionally maladapted to novel environment that the species have recently colonized (i.e., lizard, Campbell-Staton et al. 2021; Escherichia coli, yeast, guppies, chickens and babblers, Ho and Zhang 2018; Ho et al. 2020; Kuo et al. 2023). Our comparisons based on the two genesets that are associated with muscle phenotypes corroborated with these previous studies and showed that initial gene expression plasticity may be nonadaptive to the novel environments (i.e., Ghalambor et al. 2015; Ho & Zhang 2018; Ho et al. 2020; Kuo et al. 2023; Campbell-Staton et al. 2021).

> *Q3. The correlation between plastic change and evolved divergence is an artifact due to the definitions of adaptive versus maladaptive changes. For example, the definition of adaptive changes requires that plastic change and evolved divergence are in the same direction (Figure 3a), so the positive correlation was a result of this selection (Figure 3d).*

The reviewer raised an issue that the correlation between plastic change and evolved divergence is an artifact because of the definition of adaptive versus maladaptive changes, for example, Figure 3d. We agree with the reviewer that the correlation analysis is circular because the definition of adaptive and maladaptive plasticity depends on the direction of plastic change matched or opposed that of the colonized tree sparrows. We have thus removed previous Figure 3d-e and related texts from the revised version of manuscript. Meanwhile, we have changed Figure 3a to further clarify the schematic framework.

Reviewer #1 (Recommendations For The Authors):

> *Q1. Here are private recommendations that I think could help improve the manuscript. West-Eberhard was a pioneer back in 2003 in explicating the hypothesis of "plasticity first". I think it is important to cite their main work in the first paragraph of introduction and to use the term "plasticity-first", which is widely known among evolutionary biologists studying phenotypic plasticity, instead of "plasticity followed by genetic change", since the three papers cited in paragraph 1 call it « plasticity first ».*

> *West-Eberhard, M.J. (2003) Developmental Plasticity and Evolution, Oxford University Press.*

Thank you for suggesting West-Eberhard (2003) and we have cited this important work. We have also changed “plasticity followed by genetic change” to “plasticity first”.

> *Q2. Introduction. Line 5, Change for « On the one hand, if plasticity changes … »*

We have modified as suggested.

> *Q3. Line 52, Change for « …same direction as adaptive evolution does …»*

We have modified as suggested.

> *Q4. Line 66,When presenting papers that address the plasticity and evolution of gene expression in response to environmental variables, paper by Morris et al is another example that could be useful to include (but this is only a suggestion in case the authors missed it).*

Thank you for suggesting this nice work. We have cited Morris et al. (2014).

> *Q5. Line 94, Change for "We acclimated"*

We have modified as suggested.

> *Q6. In Figure 3, the figure in panel A and B is labelled "normaxia", but I think that "normoxia" is usually the term used.*

Thank you for spot the typo. We have modified Figure 3a and we no longer used the term “normaxia”.

> *Material and methods*

> *It would be important to merge supplementary table 1 and 2 and only present the individuals that were used with their respective cardiac and muscle libraries (if they come from the same individual?). Also, the origin of the individuals used in the hypoxia experiment should be explained at the beginning of the methods section and explicated in the supplementary table. Information on sex or stage of development (juvenile? Adult? Male? female?) and time of year (in breeding stage? Pre-migration (if any), etc) would allow the reader to see that individuals from lowland differed only in their exposure to hypoxia or not, or if other variables may affect gene expression patterns. Similarly, if all individuals form the highland are males and the lowland hypoxia exposed individuals are females (or juveniles versus breeders, or different time of year, etc) this should be stated in the methods. Gene expression is labile so the reader should know if other variables influence the results presented or not.*

Thank you for suggestion. We have added detailed information (i.e., age, collecting time and season) to the supplementary Table 1. We have also added this information to the Methods. Because the birds used in transcriptomic analysis (Supplementary Table 1) were different individuals from those used in the sequence analyses (Supplementary Table 2), these two tables cannot be merged.

References:

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