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

Huishang She[1,2], Yan Hao[1], Gang Song[1], Xu Luo[3], Fumin Lei[1,2,4], Weiwei Zhai[1,2,4], Yanhua Qu[1,2]

[1] Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, Beijing, China

[2] College of Life Sciences, University of Chinese Academy of Sciences, Beijing, China

[3] Faculty of Biodiversity and Conservation, Southwest Forestry University, Kunming, China

[4] Center for Excellence in Animal Evolution and Genetics, Chinese Academy of Sciences, Kunming 650223, China

Abstract

Phenotypic plasticity facilitates organismal invasion of novel environments, and the resultant phenotypic change may later be modified by genetic change, so called "plasticity followed by genetic change". 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 gene expression plasticity that largely correlates with decrease hypoxia tolerance. Conversely, gene expression change 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.

Key words: Gene expression, plasticity, acclimation, genetic variation, plasticity reinforcement, plasticity reversion

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 et al. 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 "phenotype plasticity following by genetic change" hypothesis (*i.e.*, 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 the plasticity changes the phenotype in the same direction as the 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 act 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). 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 (Buchanan et al. 2010; 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). With a proper experimental design, it is possible to quantify plastic change and adaptive evolution of the gene expression (*i.e.*, Campbell-Staton et al., 2021). 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 2800 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; Nicole et al. 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 acclimate 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 Fig. 1, we quantify gene expression change stemming from hypoxia acclimation and highland colonization and define 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.

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,

121 such as the co-regulation of genes underpinning physiological functions, we analyzed the
122 resultant expression data for all individuals using weighted gene correlation network analysis
123 (WGCNA, Langfelder & Horvath 2008). Weighted correlation network is a system biology
124 method for identifying groups of highly co-expressed genes (modules), summarizing
125 module-level expression and identifying important genes within modules (hub genes).
126 Consequently, these co-expressed genes can capture expression-stage correlations due to
127 the hypoxia-dependent expression. We identified six and nine modules of co-expressed
128 genes with their expression changes that are significantly associated with the three stages
129 (*i.e.*, ancestral, plastic and colonized stages) for the flight and cardiac muscle, respectively
130 (Fig. 2a and Fig. S1). Across these modules, we identified 3220 and 682 hub genes based on
131 the gene significance function (GS>1st quartile and P<0.05) for the cardiac and flight muscles,
132 respectively (Fig. 2b-c).
133
134 Using these candidate genes we examined expression-stage relationship. We compared the
135 gene expression levels between the birds at the ancestral stage and those at the plastic stage,
136 as well as between the birds at the ancestral stage and those at the colonized stage. We
137 attributed genes showing the same direction of change to the reinforcement group and
138 those showing opposite direction of change to the reversion group as described in Ho and
139 Zhang (2018). Using a threshold of <100% and >50% increase/reduction of gene expression
140 between two comparisons, we attributed 22 and 6 genes to the reinforcement group and 69
141 and 33 genes to the reversion group for flight and cardiac muscle, respectively. Interestingly,
142 we found fractions of genes with reversion plasticity are larger than those of genes with
143 reinforcement plasticity. In order to explore whether this result is robust with different
144 thresholds, we used a range of thresholds of <150% and >100%, <200% and >150%,
145 and >200% to attribute gene to reinforcement and reversion groups, and found an excess of
146 genes with reversion plasticity in four out of six comparisons (two-tailed binomial test,
147 P<0.05, Fig. 2d-e). These results thus suggest that high-elevation colonization generally
148 reverses gene expression plasticity.
149

150 If the reinforcement and/or reversion gene expression plasticity in hypoxic tolerance are the
151 target of selection in the high-elevation environment, we would expect to see an increase in
152 the genetic divergence between the ancestral and colonized populations. To evaluate this we
153 searched for genomic signatures of selection using resequencing data from 12 lowland and
154 11 highland tree sparrows generated from a previous study (Qu et al. 2020). Significantly
155 elevated genetic divergence (*i.e.*, F_{ST}) within the candidate genes beyond the genic
156 background would provide evidence for selection acting on this group of co-expressed genes
157 (*i.e.*, polygenic adaptation). We thus calculated average F_{ST} for the genes with reinforcement
158 or reversion plasticity using all SNPs included in the genic region, 2kb up-stream and
159 down-stream regions, respectively. We considered the up-stream and down-stream regions
160 because gene regulation may also refer to genetic variation in nearby non-coding regions. By
161 comparing to the F_{ST} distributions generated from 100 random samplings of the same
162 number of SNPs from the genic background (see Methods), we found that the empirical F_{ST}
163 values significantly increased in the 2kb up-stream and/or down-stream regions of the
164 candidate genes, but not in the genic regions ($P < 0.05$, Fig. 2d-e). In addition, we found that
165 the empirical F_{ST} values increased significantly only in the genes with the levels of
166 reinforcement/reversion plasticity reaching above 200%. This observation thus suggests that
167 selection driven genetic change is more targeted on the non-coding regions and depends on
168 the magnitude of reinforcement/reversion plasticity.
169

170 ***Gene expression and muscle phenotype analyses show reinforcement of adaptive plasticity***
171 ***and reversion of maladaptive plasticity***

172 The evolutionary outcome of the phenotypic plasticity for a population colonizing a novel
173 environment depends on the direction of plastic change with respect to the local optimum
174 of novel environment. If the plastic change is close to the local optimum, *i.e.*, adaptive
175 plasticity, natural selection likely reinforces adaptive plasticity. Conversely, if the plasticity
176 moves the phenotypic change away from the local optimum, *i.e.*, maladaptive plasticity,
177 natural selection should reduce or reverse the reaction norm and restore the phenotypic
178 change back to the original ancestral values (Campbell-Staton et al. 2021). In order to

179 investigate phenotypic plasticity and its evolutionary consequence, we analyzed two
180 datasets that included gene expression and muscle phenotype collected from the lowland
181 ancestral and highland colonized tree sparrows (Qu et al. 2020).
182
183 To categorize gene expression plasticity of a give gene as adaptive or maladaptive, we made
184 the assumption that great hypoxia tolerance is adaptive at hypoxic condition of
185 high-elevation environment, whereas low hypoxia tolerance is maladaptive and results in
186 reduced fitness. Based on the correlation coefficients from the correlation analysis between
187 gene expression and muscle phenotypic values of the ancestral and colonized individuals
188 (see Methods), we categorized the direction of expression-phenotype correlation as positive
189 or negative for these muscle phenotype-associated genes ($P < 0.05$, Fig. 3a and Fig. S2). We
190 then characterized the differences in expression observed for the ancestral-plastic groups
191 and the ancestral-colonized groups as congruent or incongruent with increased hypoxia
192 tolerance. Because the muscle phenotypic changes observed in the colonized tree sparrows
193 have been shown to enhance oxygen delivery and improve metabolic capacity in other
194 highland animals (Giordano 2005; Storz et al. 2010; Scott 2011; Scott et al. 2015), we thus
195 deemed that the gene expressions in which the direction of plastic change matched the
196 expectation for increase hypoxia tolerance as putatively adaptive, while those that opposed
197 this expectation were considered to be putatively maladaptive (categorization workflow see
198 Fig. 3a). We identified 2037 and 1866 muscle phenotype-associated genes in the flight and
199 cardiac muscle, respectively (Fig. 3b-c). Interestingly, we found that the tree sparrows
200 displayed more genes with maladaptive plasticity than genes with adaptive plasticity
201 (two-tailed binomial test, flight muscle, 1709 vs. 328, cardiac muscle 1023 vs. 843,
202 $P<0.2.2e-^{16}$). These results indicate that hypoxia-induced plasticity of gene expression may
203 be maladaptive for muscle physiological performance in highland environment.
204
205 The observed adaptive and maladaptive gene expressions in the tree sparrows suggest that
206 selection may have acted to reinforce and/or reverse gene expression plasticity. We then
207 quantified the direction of the correlation as the fold change of gene expression between

208 the ancestral and plastic groups (plasticity) and between the ancestral and colonized groups
209 (evolved divergence). Consistent with the observation that selection has acted to reduce the
210 magnitude of maladaptive plasticity, we found that gene expression plasticity and evolved
211 divergence displayed a strong negative correlation (cardiac muscle, R=-0.87, P<0.001; flight
212 muscle, R=-0.92, P<0.001, Fig. 3d-e). Conversely, we found a positive correlation between
213 gene expression plasticity and evolved divergence in the genes that showed adaptive
214 expression plasticity (cardiac muscle, R=0.92, P<0.05; flight muscle, R=0.84, P<0.001, Fig.
215 3d-e). Together, these results suggest that whether plastic changes in the gene expression
216 are reinforced or reversed depends primarily on if they are functionally adaptive or
217 maladaptive in the high-elevation environment.
218
219 We subsequently examined expression-stage relationship using the same thresholds as
220 above, *i.e.*, <100% and >50%, <150% and >100%, <200% and >150%, and >200% of
221 reversal/reinforcement plasticity. In six out of eight comparisons, we found that the fractions
222 of genes with reversion plasticity were larger than those of genes with reinforcement
223 plasticity (two tailed binomial test, P<0.01, Fig. 3f-g). We then quantified the genetic
224 divergence of these genes between ancestral and colonized tree sparrows by testing for
225 statistically significant increase of F_{ST} as compared to the F_{ST} distributions generated from
226 100 random samplings (see Methods). We found that the empirical F_{ST} values were
227 significantly larger in the genes within the <200% and >150% category (cardiac muscle,
228 P<0.05) and >200% category (flight and cardiac muscle, P<0.05), but not in those within
229 other categories with low levels of reinforcement and reversion plasticity. These results
230 suggest that the strength of selection may depend on the magnitude of reinforcement and
231 reversion plasticity. Interestingly, we found that the genetic divergence between the
232 ancestral and colonized tree sparrows tends to increase in the 2kb up-stream and
233 down-stream regions of these genes (P < 0.05, Fig. 3f-g). This observation suggests that
234 selection on gene expression plasticity related to muscle phenotype drives primarily on
235 genetic variation in regulatory regions.

236 **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 et al. 2017; Rivera et al. 2021). Using an integrative approach of field-based studies, experimentally implemented acclimation experiment, multiomics 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 great insights into the mechanism of plasticity followed by genetic change.

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 initial 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). 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 require 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

295 quantifying phenotype plasticity and how to relate this to genetic change. This is timely, as
296 the role of plasticity in enabling species to invade new habitats and survive environmental
297 change is only beginning to be understood (Campbell-Staton et al. 2017; 2018; Ho et al.
298 2020). Such an understanding is important, not least because numerous species are being
299 introduced to new regions of the globe through human intervention and spread as invasive
300 species (Visser 2008; Wingfield et al. 2011).

301 Materials and Methods

302 ***Sampling***

303 We generated transcriptomic data for hypoxia-acclimated lowland tree sparrows by exposing
304 five lowland tree sparrows to hypoxic condition for 30 days using a hypoxic chamber. The
305 oxygen content in the chamber was set to 14% of the oxygen content (simulating the oxygen
306 concentration at 3200 m a.s.l., 70% of ~20%, latter of which is the content of oxygen content
307 at sea level), which is similar to condition where we collected the highland tree sparrows
308 (Heimahe, Qinghai, 3200 m a.s.l.). We collected flight muscle from the four individuals and
309 cardiac muscle from three individuals. In addition, we extended the analyses of the
310 transcriptomic and muscle histological data for three sparrows from lowland (Beijing and
311 Hebei, 100 m a.s.l.) and highland (Heimahe, 3200 m a.s.l., Figure 1) collected by Qu et al.
312 (2020). In total, we used twelve samples for flight muscle and nine samples for cardiac
313 muscle (Supplementary Table 1).
314

315 ***Transcriptome sequencing***

316 RNA libraries were constructed for the flight and cardiac muscles and sequenced on an
317 Illumina HiSeq4000 platform. After filtering low-quality, adapter-contaminated, and N-rich
318 reads (>10%), a total of 134, 128 and 120 million reads of transcriptional data were
319 generated for the lowland, hypoxia-exposed lowland tree sparrows and highland tree
320 sparrows, respectively (Supplementary Table S1). We mapped cleaned reads against the tree
321 sparrow genome using STAR (Alexander et al. 2013) and calculated gene expression level and
322 intensity using RSEM (Li & Dewey 2011) and DESeq 1.22.1 (Anders & Huber 2010).

323

324 ***WGCNA analyses***

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

336 ***Identify gene regulation associated with muscle phenotype***

337 To identify the genes with expression levels that correlate to evolved differences in the
338 muscle phenotypes in the tree sparrows, we used the lowland and highland individuals from
339 Qu et al. (2020) for which RNAseq and muscle phenotypic data were available. We correlated
340 the levels of gene expression with muscle phenotypes in the lowland and highland
341 individuals. We used three phenotypes for the flight muscle (capillary per fiber, fiber area
342 and perimeter) and two for the cardiac muscle (capillary density and fiber diameter). We
343 used Pearson correlation to test the association between each of the phenotypic traits and
344 expression profile of each gene (TPM, transcripts per million). A threshold of P<0.05 was
345 used to detect muscle-associated genes.

346

347 We then followed Campbell-Staton et al. (2021) to categorize genes as positive (positively
348 correlated with muscle phenotypes) or negative regulators (negatively correlated with
349 muscle phenotypes). The positive regulators showing increased expression (up-regulation)
350 and negative regulators showing decreased expression (down-regulation) in the highland

351 tree sparrows were considered to indicate adaptive plasticity. Conversely, the positive
352 regulators showing decreased expression (down-regulation) and negative regulators showing
353 increased expression (up-regulation) in highland tree sparrows were deemed as indicating
354 maladaptive plasticity (Fig. 3a-c). We calculated the log-transformed change in gene
355 expression between the lowland ancestral and acclimated groups and between the lowland
356 ancestral and highland colonized groups, and then tested the correlation between the two
357 log-transformed change values using Spearman's rank correlation (Fig. 3d-e).
358
359 ***Reinforcement and reversion analyses***
360 To investigate the magnitude of reinforcement and reversion plasticity, we followed the
361 method described in Ho and Zhang (2018). Specifically, expression levels of each gene in the
362 three stages were treated as E_lowland (expression level at ancestral stage), E_hypoxia
363 (expression level at plastic stage) and E_highland (expression level at colonized stage). We
364 used a threshold of 50% of the ancestral gene expression level, *i.e.*, 50% * E_lowland, to
365 detect an excess of gene expression change. We identified genes with an excess of plastic
366 change if it satisfied the condition of |(E_hypoxia-E_lowland)|> 50% * E_lowland. Likewise,
367 we identified genes with an excess of evolutionary change if it met the condition of
368 |(E_highland-E_hypoxia)|> 50%* E_lowland. If the genes showed same direction in the
369 expression change at the plastic and colonized stages, we regarded these genes as
370 reinforcement expression. If the genes showed opposite direction in the expression change
371 at the plastic and colonized stages, we regarded these genes as reversion expression. We
372 categorized these genes to reinforcement and reversion groups and then tested if the
373 proportions of the genes in the two groups were significantly different from the expected
374 proportions using two-side binomial tests. To test the impact of the choice of thresholds on
375 the results, we analyzed the data using a range of thresholds, *i.e.*, <100% and >50%, <150%
376 and >100%, <200% and >150%, and >200%. This setting aimed to obtain independent
377 gene-sets for subsequently genetic divergence analyses, because a geneset selected by relax
378 threshold (*i.e.*, 50%) also included genesets obtained by the stringent thresholds (*i.e.*, 100%,
379 150% and 200%) thus the result of former is partially dependent on those of the latters.

380

381 ***Comparing genetic divergence for genes with reinforcement and reversion expression***
382 ***plasticity***

383 To calculate the genetic divergence between the lowland ancestral and highland colonized
384 tree sparrows, we used 23 individuals from Qu et al. (2020) for which re-sequencing data
385 were available. After mapping the raw reads to the tree sparrow genome using BWA v0.7.17
386 (Li & Durbin 2009), we obtained mean sequence coverage of 17x for each individual (Table
387 S2). We called variants with GATK v 3.7 (McKenna et al. 2010) and Samtools v1.2
388 (http://www.htskib.org/) and filtered single nucleotide polymorphism (SNP) using VCF tools
389 and GATK with minimum coverage = 138, root mean square mapping quality >= 20, distance
390 of adjacent SNPs >=5, distance to a gap >=5bp and read quality value >=30.

391

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

406

407 **Data accessibility**

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

410

Authors' contributions

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

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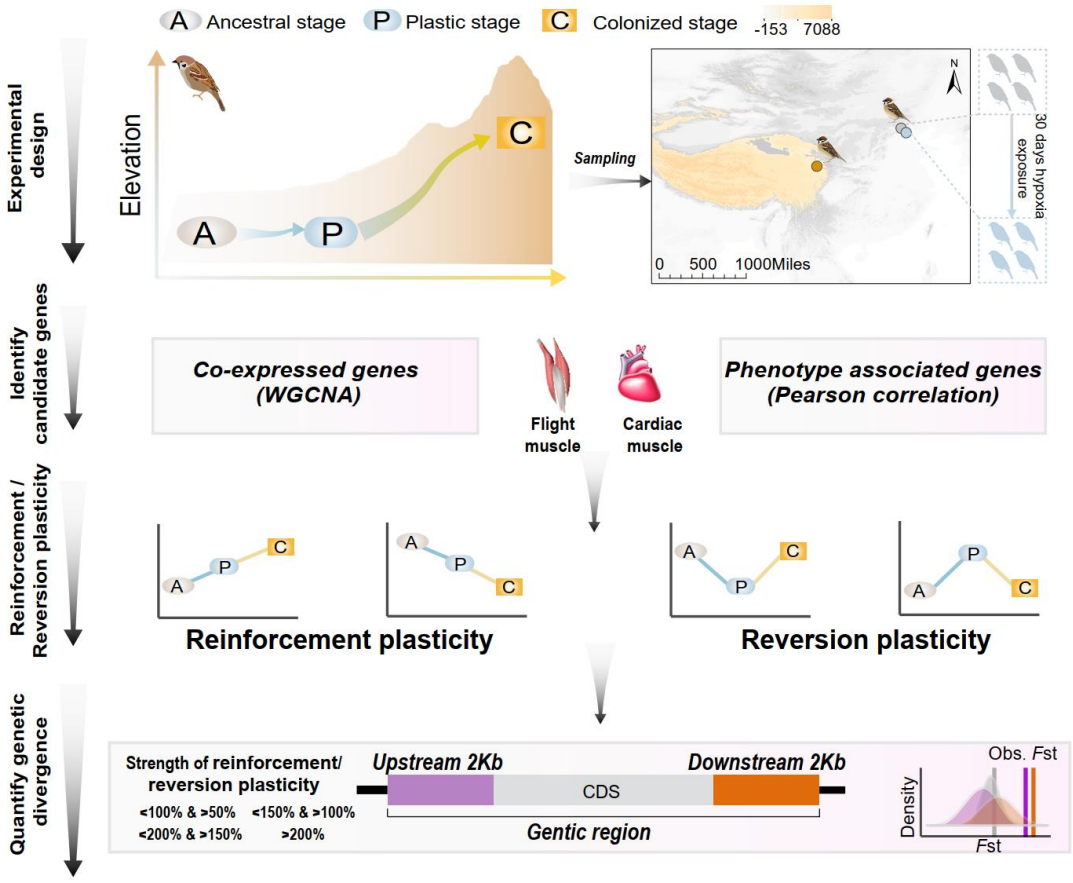

Figure 1. A schematic representation of quantifying gene expression plasticity and genetic change in relevant genes. **Experimental design**, transcriptomic data of the flight and cardiac muscle were collected from tree sparrows obtained in their original lowland habitat (ancestral stage), experimental 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 ancestral 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 were 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.

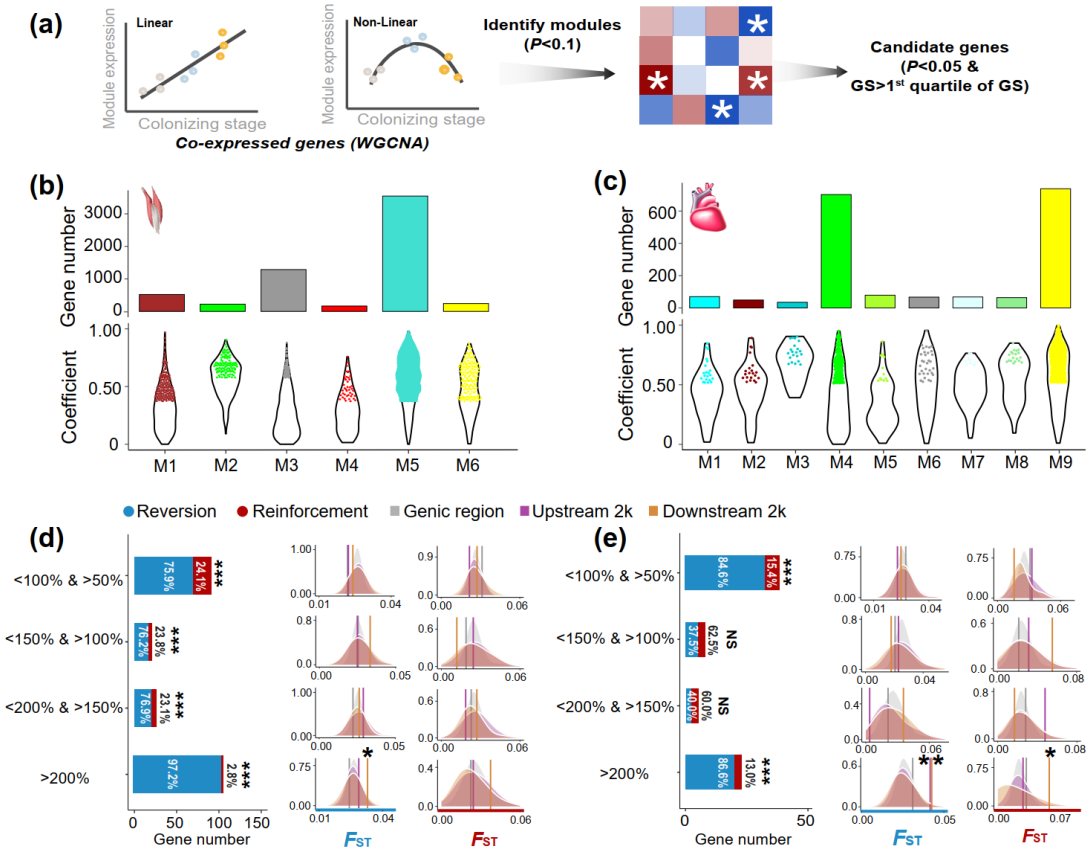

Figure 2. The WGCNA analysis identified co-expressed genes and reinforcement and reversion of expression plasticity. (a) Pearson linear correlation is used to identify genes with expression reinforcement and nonlinear regression to identify genes with expression reversion. Regulatory modules were identified 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 muscle (*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) and (e) Left, genes with expression plasticity being reinforced (red) or reversed (blue) at the colonized stage identified for the flight (d) and cardiac muscle (e), respectively. There are more genes showing reversion plasticity than those showing reinforcement plasticity in flight muscle (d, all comparisons, two-tailed binomial test, *P*<0.001) and cardiac muscle (e, two of four comparisons, two-tailed binomial test, *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; ***, *P* < 0.001.

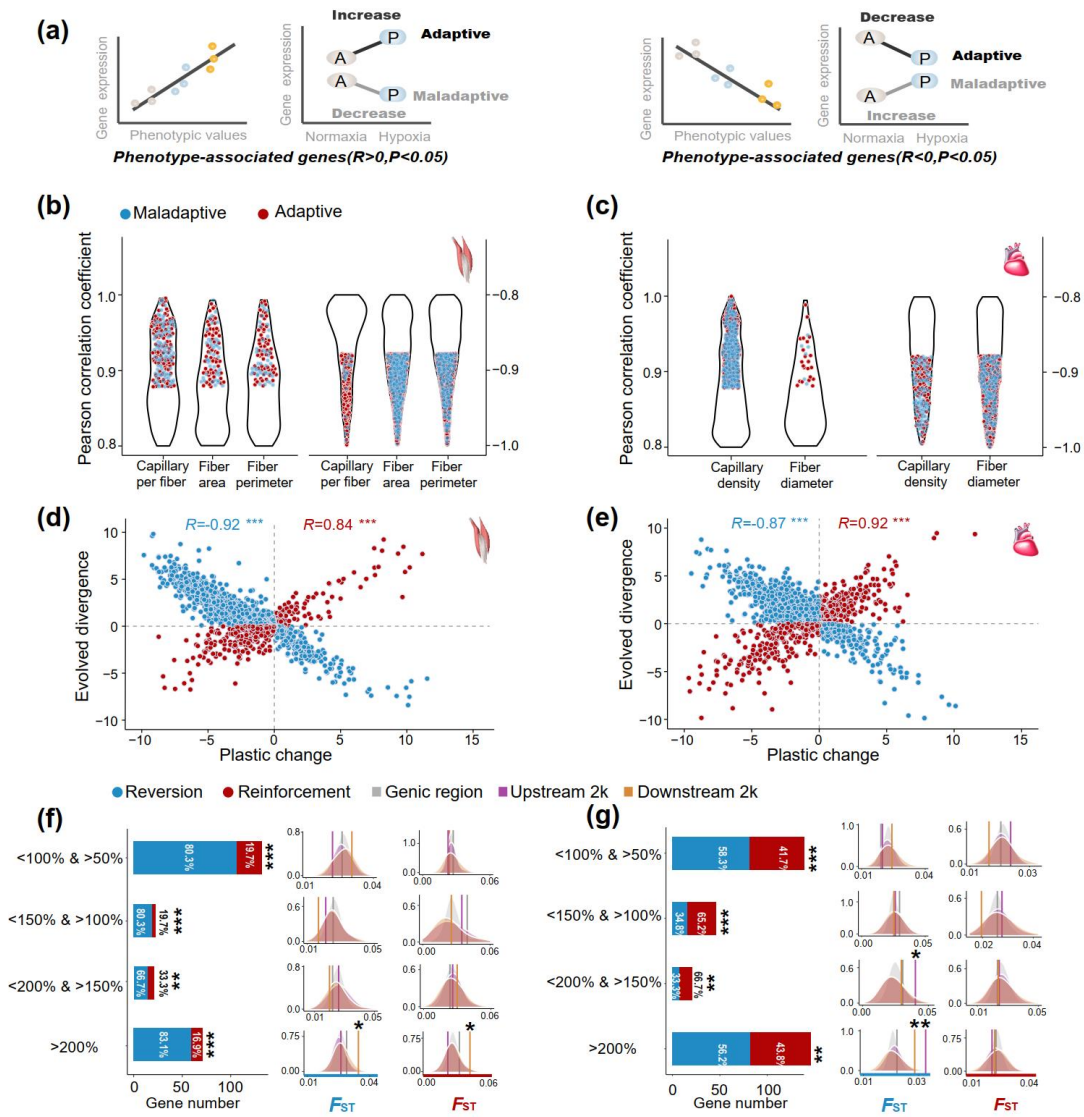

Figure 3. Genes with expression levels associated with muscle phenotypes and adaptive and maladaptive plasticity. (a) Gene expression profiles were correlated with muscle phenotypes. The genes with expression changes that correlate positively with muscle phenotypes were considered to be positive regulators, and those that correlate negatively with muscle phenotypes were deemed to be negative regulators. The positive regulators showing increased expression (up-regulation) and negative regulators showing decreased expression (down-regulation) in highland tree sparrows were considered to be adaptive plasticity. Conversely, the positive regulators showing decreased expression (down-regulation) and negative regulators showing increased expression (up-regulation) in highland tree sparrows were deemed as maladaptive plasticity. (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) and (e) The fold changes of gene expression between the ancestral and plastic stages (plastic change) and between the ancestral and colonized stages (evolved divergence) show positive correlation for the genes showing adaptive plasticity and negative correlation for genes showing maladaptive plasticity (Spearman's rank correlation, P<0.001) in the flight muscle (d) and cardiac

610 muscle (e). (f) and (g) Left, candidate genes were classified to four categories with different
611 magnitudes of reinforcement or reversion plasticity. More genes show reversion plasticity than those
612 show reinforcement plasticity (six out of eight comparisons, two-tailed binomial test, P<0.01). Right,
613 the empirical F_{ST} values increase significantly in the 2k up-stream and/or down-stream regions of the
614 genes having reinforcement/reversion plasticity <200% and >150% (cardiac muscle) and >200% (flight
615 and cardiac muscle). (f) flight muscle, (g) cardiac muscle. Vertical lines, the empirical F_{ST} values; shades,
616 permutated F_{ST} distributions generated from 100 times sampling. **, $P < 0.01$; ***, $P < 0.001$.