A Multilevel Assessment of Plasticity in Response to High-Altitude Environment for Agama Lizards

Yin Qi[1†], Tao Zhang[2†], Yayong Wu[3], Zhongyi Yao[1], Xia Qiu[1], Peng Pu[2], Xiaolong Tang[2], Jinzhong Fu[1,4] and Weizhao Yang[1]*

[1] Chengdu Institute of Biology, Chinese Academy of Sciences, Chengdu, China, [2] Department of Animal and Biomedical Sciences, School of Life Science, Lanzhou University, Lanzhou, China, [3] College of Life Sciences and Food Engineering, Yibin University, Yibin, China, [4] Department of Integrative Biology, University of Guelph, Guelph, ON, Canada

Upslope range shifting has been documented in diverse species in response to global warming. Plasticity, which refers to the ability of organisms to alter their phenotypes in changing environments, is crucial for the survival of those that newly migrated to a high-altitude environment. The scope and mechanisms of plasticity across biological levels, however, have rarely been examined. We used two agama lizards (genus *Phrynocephalus*) as model systems and a transplant experiment to comprehensively assess their plasticity on multiple organization levels. Two low-altitude (934 m) agama species, *Phrynocephalus axillaris* (oviparous) and *P. forsythii* (viviparous), were transplanted to a high-altitude site (3,400 m). After acclimation for 6 weeks in seminatural enclosures, plasticity was measured from bite force, tail display behavior, gene expression, and metabolome. Both lizards were capable of acclimating to the high-altitude environment without sacrificing their performance in bite force, but they also showed high plasticity in tail display behavior by either decreasing the intensity of a specific display component (*P. forsythii*) or by the trade-off between display components (*P. axillaris*). Genes and metabolites associated with lipids, especially fatty acid metabolism, exhibited significant differentiation in expression, compared to individuals from their native habitats. Improved fatty acid storage and metabolism appeared to be a common response among animals at high altitudes. Despite distinct reproductive modes that may differ in response to physiological pressure, the two lizards demonstrated high concordance in plasticity when they faced a novel environment at high altitudes. Taken together, lizards likely acclimate to high-altitude environments by reducing behavioral activity and increasing energy efficiency after range shifting. Our results provide new insights into our understanding of phenotypic plasticity and its importance in today's changing climate.

Keywords: high altitude, lizards, behavior, performance, gene expression, metabolome, plasticity

INTRODUCTION

Upslope range shifting has been documented in diverse species as a response to global warming because cooler regions likely relieve species from overheating and facilitate their survival (Thomas and Lennon, 1999; Parmesan and Yohe, 2003; Root et al., 2003; Thomas et al., 2006). However, moving up along an elevational gradient comes with several other inevitable environmental

stressors, including hypobaric hypoxia and intense UV radiation, which may reduce the successful reproduction and individual survival (Scheinfeldt and Tishkoff, 2010; Kouyoumdjian et al., 2019). Endemic high-altitude species have evolved several specific mechanisms that enable them to tolerate these stressors, such as improved O_2 uptake and modified metabolism (Qu et al., 2013; Zhang et al., 2014; Li et al., 2018). How newly range shifted species survive in the high-altitude environments remains to be explored.

Phenotypic plasticity plays a crucial role in facilitating organisms to live at high altitudes (Wilson and Franklin, 2002; Seebacher, 2005; Scoville and Pfrender, 2010; Corl et al., 2018). Plasticity in metabolism is particularly important (Seebacher, 2005; Horscroft et al., 2017). Abundant evidence has demonstrated that the oxygen transport system is highly plastic in dealing with severe hypoxia (Storz, 2007; He et al., 2013; Lui et al., 2015), while plasticity in metabolic activities also assists animals in coping with cold and hypoxia (Hammond et al., 2001; Seebacher, 2005). The Australian freshwater turtle *Chelodina longicollis*, for example, responds to cold stress by increasing the activity of regulatory enzymes (Seebacher et al., 2004). Correspondingly, gene expression, which connects genotype to metabolism, is highly plastic and also shows large differences between individuals living at different altitudes. For example, deer mice from high altitudes show a large-scale upregulation of genes associated with oxygen and metabolic fuel utilization, compared to low elevation individuals (Cheviron et al., 2014; Scott et al., 2015). Plasticity in animal behavior and performance is well known for buffering environmental changes along altitude gradients, especially for ectothermic species (Kearney et al., 2009; Enriquez-Urzelai et al., 2019). In a new environment, individuals often seek proper retreat sites to avoid the overheating risk (Enriquez-Urzelai et al., 2019) or immediately adjust their behavior patterns to match the local environments (Refsnider et al., 2018).

Despite these advancements, a holistic look at how a newly arrived animal may survive in a natural high-altitude environment is lacking. Previous studies were often carried out in laboratory settings with only one stressor considered, typically temperature or oxygen (Seebacher et al., 2004; Seebacher, 2005), but natural high-altitude environments are complex with multiple stressors. Therefore, animal transplant experiments from low- to high-altitude environments with a natural setting are highly desirable. Furthermore, plastic changes in response to high altitudes likely take place at multiple organization levels, including gene expression, metabolism, and performance. Plasticity in metabolic functions is likely essential for organisms to survive at high altitudes. Different pathways, such as oxidative phosphorylation and tricarboxylic acid cycle, have different capacities in heat production and oxygen utility (Voet and Voet, 1995; Cheviron et al., 2012). Several studies have also shown that the fatty acid metabolic pathway is crucial in dealing with high-elevation environments (Cheviron et al., 2012; Qu et al., 2013; Tang et al., 2013; Lui et al., 2015). Other levels of phenotypic variations, such as morphology and behavior, may also contribute to or depend on metabolic functions.

Toad-headed agama lizards (genus *Phrynocephalus*) provide an excellent system to investigate the plasticity in response
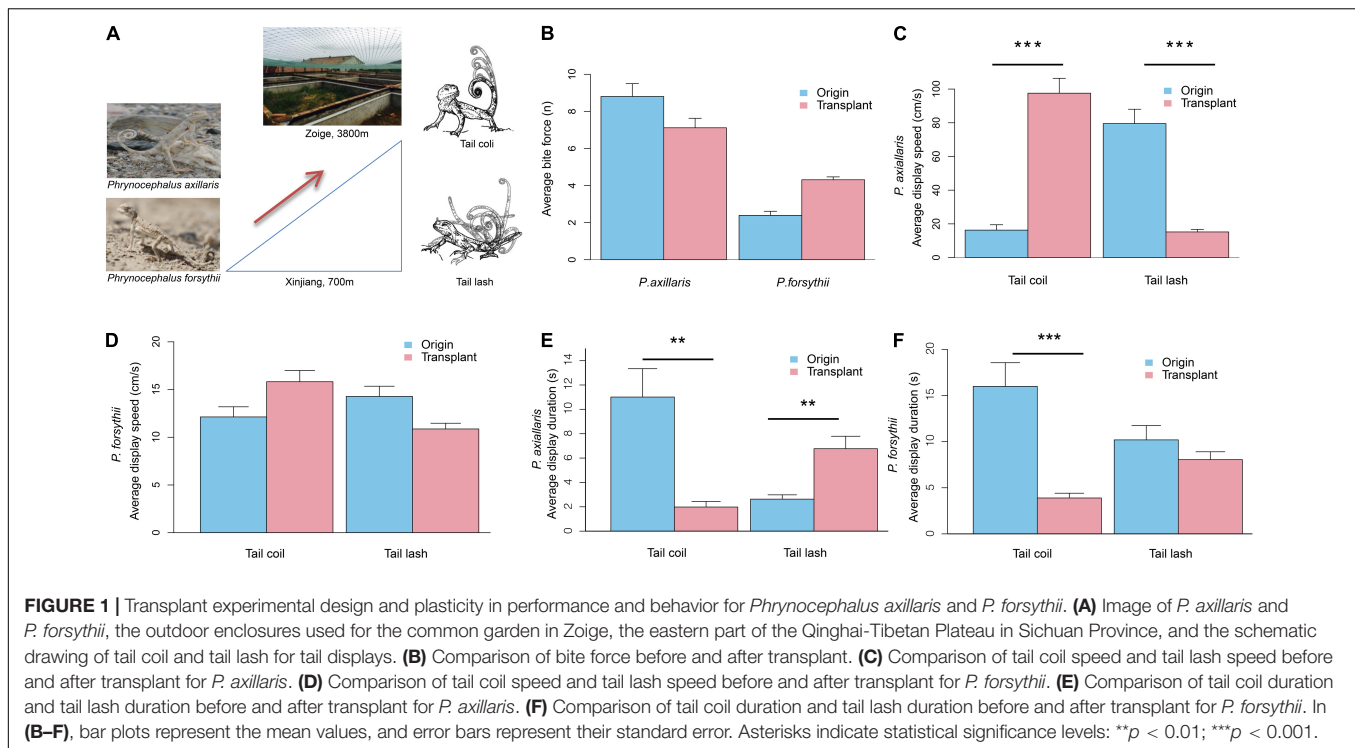
to high-altitude environments. As ectothermic species, agama lizards are sensitive to environmental changes. This genus is widely distributed in the Eurasian Arid Belt. Recent studies have shown that they have evolved a series of physiological and behavioral traits that are related to high-altitude habitats (Tang et al., 2013; Yang et al., 2014). In addition, these lizards use tail displays in their social communications, which are regulated by anaerobic metabolism (Zhu et al., 2021; Hu et al., 2022). Also, both oviparity (laying eggs) and viviparity (giving live birth) exist in this group of lizards, and viviparity has often been associated with high-altitude or high-latitude environments (Guo and Wang, 2007). The different reproductive modes may restrict or expand their plasticity in a new high-altitude environment. In this group of lizards, one oviparous species (*Phrynocephalus axillaris*) and one viviparous species (*P. forsythii*) are sympatric at low-altitude habitat, and *P. forsythii* has been assessed to face high extinction risk under global warming (Sinervo et al., 2018). Therefore, a comparison between *P. axillaris* and *P. forsythii* would provide an opportunity to reveal both common and specific patterns in response to high-altitude environments for agama lizards.

In this study, we aimed to make a multilevel assessment of the plasticity in response to high-altitude environments for agama lizards. Specifically, we examined (1) whether agama lizards were capable of acclimation from low- to high altitudes and (2) the patterns of plasticity on performance, behavior, gene expression, and metabolome. To achieve these objectives, we transplanted two sympatric agama species *P. axillaris* (oviparous) and *P. forsythii* (viviparous), from their original low-altitude habitat to high-altitude environments in the Qinghai-Tibetan Plateau. Their bite force was measured to assess their performance, and their gene expression profiles from heart, liver, and muscle, as well as blood metabolomes, were obtained to examine their patterns of plasticity.

MATERIALS AND METHODS

Experimental Design

We used a transplanting design to simulate lizards' upslope range shifting. Two *Phrynocephalus* species, *P. axillaris* and *P. forsythii* from Kuerle, Xinjiang, China (41.50386°N, 86.2290°E, elevation = 934 m a.s.l.), were sampled on June 18–July 2, 2017, and transplanted to a high-altitude environment on July 4, 2017, in Zoige, the eastern part of Qinghai-Tibetan Plateau in Sichuan Province (33.71389°N, 102.48543°E, elevation = 3,400 m a.s.l.). Although a control treatment in the original site would provide consistent conditions for comparison after transplantation, it was difficult to conduct experiments without a well-equipped field workstation in the original site. To minimize bias caused by microhabitat differences, we built six outdoor seminatural enclosures (length × width × height = 5 m × 5 m × 1.5 m, **Figure 1A**), and the settings resembled the original habitat as much as possible. We used sand from a field site of *P. vlangalii*, a sister species of *P. forsythii*, as substrate, and had a fishing net suspended above each enclosure to reduce the risk of bird predation. The lizards were kept in the enclosures for 6 weeks

FIGURE 1 | Transplant experimental design and plasticity in performance and behavior for *Phrynocephalus axillaris* and *P. forsythii*. **(A)** Image of *P. axillaris* and *P. forsythii*, the outdoor enclosures used for the common garden in Zoige, the eastern part of the Qinghai-Tibetan Plateau in Sichuan Province, and the schematic drawing of tail coil and tail lash for tail displays. **(B)** Comparison of bite force before and after transplant. **(C)** Comparison of tail coil speed and tail lash speed before and after transplant for *P. axillaris*. **(D)** Comparison of tail coil speed and tail lash speed before and after transplant for *P. forsythii*. **(E)** Comparison of tail coil duration and tail lash duration before and after transplant for *P. axillaris*. **(F)** Comparison of tail coil duration and tail lash duration before and after transplant for *P. forsythii*. In **(B–F)**, bar plots represent the mean values, and error bars represent their standard error. Asterisks indicate statistical significance levels: **$p < 0.01$; ***$p < 0.001$.

to acclimate to the new environments, and mealworms were provided every 3 days to offset food shortage.

We examined multiple levels of phenotypic plasticity by comparing lizard states before and after transplanting. The bite force was used to assess lizard body condition and performance in the new environments, while the tail display behavior, gene expression, and metabolism were used to assess the plasticity patterns.

Behavioral Data Collection

We collected data of tail display behavior from 25 males of *P. axillaris* and 26 males of *P. forsythii* from the original site (**Figure 1A**). The detailed protocols on display collection are described by Wu et al. (2018). First, we captured intruder males using noose from a different site 3 km away and measured their snout-vent length (SVL) to the nearest 0.01 mm using caliper. Then, the resident male was approached by a size-matched intruder using a 4-m-long fishing rod. Meanwhile, a video camera (HDR PJ670, Sony, Japan) was set up in front of the resident male and recorded the display response. The trial was terminated after the display stopped, or at a maximum of 5 min if no display was observed. At the conclusion of each trial, a ping-pong ball was placed at the location of the resident and recorded as a scale for subsequent analysis. The resident male was captured using a noose after filming. Immediately after capture, we measured the body temperature from the center of its dorsum with an IR thermometer (HT-866, HCJYET, China). The lizards were kept individually during the experiment. We also measured the SVL and bite force of each lizard. The SVL was measured using a caliper to the nearest 0.01 cm. The bite force was recorded

using a piezoelectric force transducer (Type 9203, Kistler Inc., Switzerland) that connected to a charge amplifier (Type 5995A, Kistler Inc., Switzerland) and fitted with two metal bite plates (Vanhooydonck et al., 2005). We encouraged the lizards to bite the plates by gently tapping the mouth using a blunt metal probe. To minimize bias due to motivation and physical condition, we repeated the measurement three times and used the maximum value as the final measurement (Vanhooydonck et al., 2005).

A total of 22 male *P. axillaris* and 23 male *P. forsythii* were transplanted from the original site to the high-altitude site. Individuals of each species were randomly divided into three groups (7–8 for each) and kept in seminatural enclosures. After acclimation for 6 weeks, we recorded the social display behavior and measured SVL and bite force in the same way. All raw data of the behavioral experiment are shown in **Supplementary Table 1**.

Bite Force Plasticity Analysis

We analyzed the correlation between bite force and transplant treatments using linear models in the *nlme* package (Pinheiro et al., 2018) in R version 4.0.5 (R Core Team, 2015). The bite force was log-transformed before modeling to meet the assumption of Gaussian error distribution. We established a linear model with the transplant treatment as the main effect while considering individual body temperature as co-variable.

Display Digitization and Plasticity Analysis

To quantify the tail display, we tracked the motion of tail tips following the methods outlined by Hedrick (2008) in MATLAB

2015b (MathWorks Inc., Natick, MA, United States). The x-y coordinates of tail tips were determined using the DLT dv5 software (Hedrick, 2008) with successive frames. We extracted two tail display variables, the average display speed and display duration. The average display speed was defined as the average distance moved by tail tip within a specific time. The display duration was defined as the time of each display bout. We quantified the two variables for both tail coil display and tail lash display to examine whether tail display plasticity differed between components. As the orientation of the lizard relative to the camera likely affected tail display quantification (Bian et al., 2016), we categorized each display as either facing toward or away from the camera or at the right angle to the camera. Finally, we transformed the grid-based display variables to Euclidean distance in MATLAB using the ping-pong ball as scale.
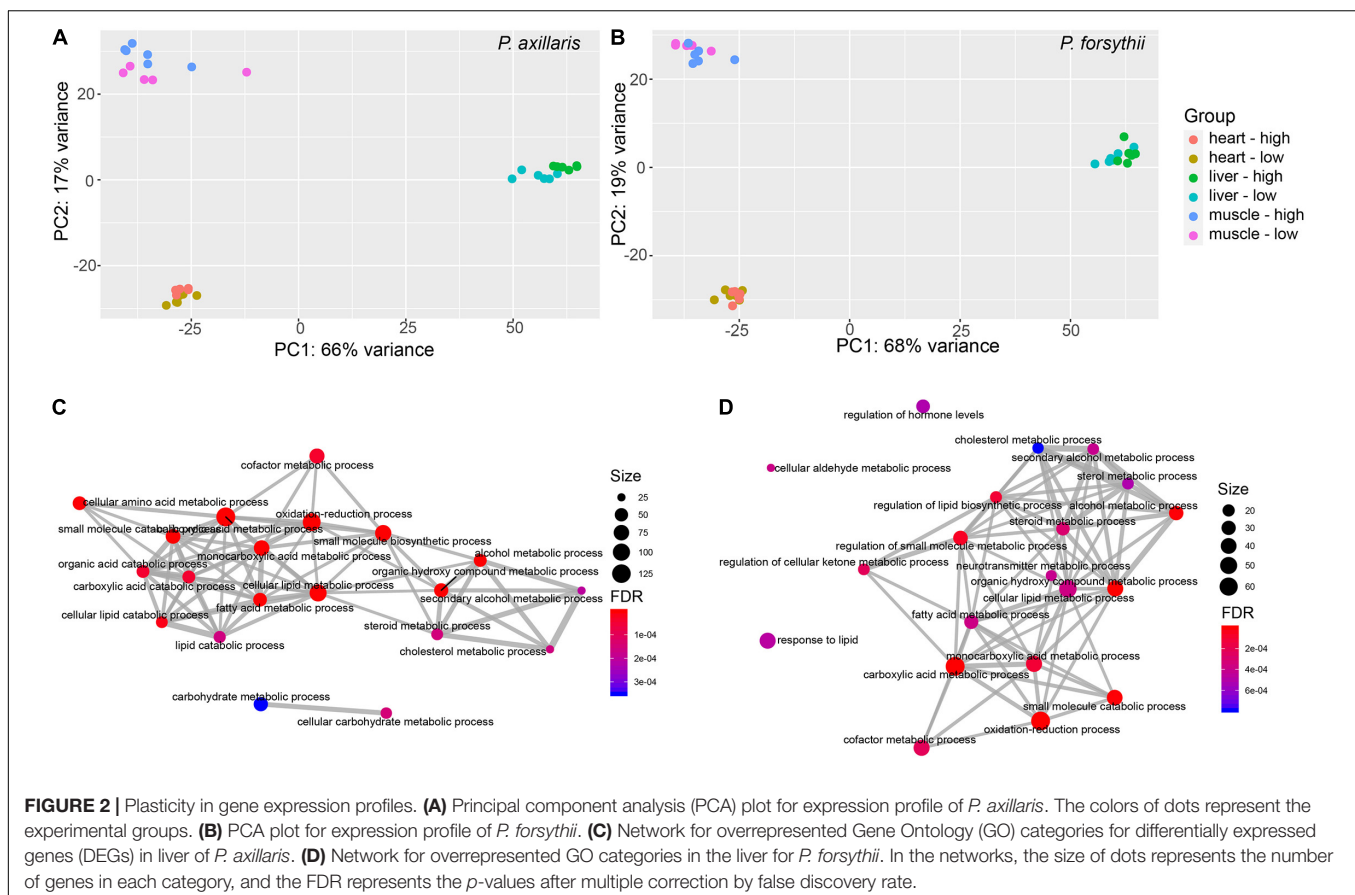
We analyzed the correlation between tail display and transplant treatments using linear mixed models in the *nlme* package (Pinheiro et al., 2018) in R version 4.0.5 (R Core Team, 2015). All independent variables were log-transformed to meet the assumptions of the linear mixed model. For average tail display speed, we considered transplant treatment and display orientation as fixed effects, with lizard identity as a random effect and assuming a Gaussian error distribution. For display duration, we considered treatment as fixed effects, with lizard identity as a random effect and assuming a Gaussian error distribution. We included the SVL of residents as covariates in both tail

display speed and duration models to account for the potential effect of body size.

Transcriptome Sequencing Analysis

For each species, 6 individuals from the original habitat and 6 transplanted individuals were subjected to part of the examination. Tissues of the heart, liver, and muscle were collected immediately after euthanization on the days of collection or immediately after the completion of the transplant experiments. The sample information is provided in **Supplementary Table 2**. Total RNA was extracted from each tissue sample according to the TRIzol protocols (Invitrogen, Carlsbad, California). Paired-end sequencing with a read length of 150 base pairs (bp) was carried out on the Illumina HiSeq2500 platform by Novogene (Beijing, China).

Raw sequencing reads were first cleaned by excluding the adapter sequences and low-quality base calls using a Novogene pipeline. Trimmomatic version 0.35 (Bolger et al., 2014) was used to trim the clean reads with LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15, and MINLEN:70. We checked the read quality before and after trimming using FastQC version 0.11.8 (Andrews, 2010). Subsequently, quality-filtered reads of three different tissue samples from one representative individual of each species (A2, G1, and M2 for *P. axillaris*; C1, I1, and O1 for *P. forsythii*) were used in *de novo* assembly *via* Trinity version 2.8.4 after *in silico* read normalization (Grabherr et al., 2011;

FIGURE 2 | Plasticity in gene expression profiles. **(A)** Principal component analysis (PCA) plot for expression profile of *P. axillaris*. The colors of dots represent the experimental groups. **(B)** PCA plot for expression profile of *P. forsythii*. **(C)** Network for overrepresented Gene Ontology (GO) categories for differentially expressed genes (DEGs) in liver of *P. axillaris*. **(D)** Network for overrepresented GO categories in the liver for *P. forsythii*. In the networks, the size of dots represents the number of genes in each category, and the FDR represents the *p*-values after multiple correction by false discovery rate.

Haas et al., 2013). Since Trinity usually generates a large number of assembled transcripts, among which many may have questionable biological significance, we used kallisto version 0.44.0 (Bray et al., 2016) to quantify the abundance of the assembled transcripts and build expression matrices. Transcripts with "transcripts per million transcripts" (TPM) less than three were removed to generate the final assembly for each species. To obtain orthologous sequences among the two species, a best reciprocal hit (BRH) method was applied for the final assemblies *via* BLAST + version 2.7.1 (Camacho et al., 2009).

The clean reads for each tissue sample were mapped to the transcriptome assemblies for corresponding species by STAR version 2.6 (Dobin et al., 2013). The quantity of reads that matched to the same transcripts was counted using the HTSeq-count tool with the "union" resolution mode (Anders et al., 2015). The expression similarity among samples was calculated by the Euclidean distance and visualized by clustering heatmap after regularized log-transformation (rlog) of normalized counts *via* DESeq2 version 1.20 (Love et al., 2014). Principal component analysis (PCA) was used to assess the relationship between samples. We compared the samples that belonged to the same species separately to examine the expression divergence among

different tissue types within one species. Differentially expressed genes (DEGs) were estimated using generalized linear models in edgeR package version 3.22.5 (Robinson et al., 2010; McCarthy et al., 2012). We used a strict thresol to characterize DEGs, with fold-change $\geq$ 2 and adjusted *p*-value < 0.05 [false discovery rate (FDR)].

Functional annotation was applied by aligning the transcripts to the UniProtKB/Swiss-Prot database (release "2018_08") with BLAST hits *E*-value cutoff greater than 10^{-5}. For transcripts with annotation information, overrepresentation test of DEGs was calculated using the clusterProfiler package (Yu et al., 2012) in R with annotation to the Gene Ontology (GO) category and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway databases. The minimum number of transcripts required for each test of a given category was 10.

Metabolomic Assay Analysis

For each species, 10 individuals from the original habitat and 10 transplanted individuals were subjected to this part of the examination. The sampling of blood plasma was conducted immediately after euthanization on the days of collection or the completion of the transplant experiment. In detail, each lizard

FIGURE 3 | Plasticity in main biochemical clusters of metabolites identified in metabolome assay. Red color denotes metabolites from positive ion mode, and blue color denotes metabolites from negative ion mode. The top panels represent the metabolome in *P. axillaris*, and the bottom panels represent that in *P. forsythii*.
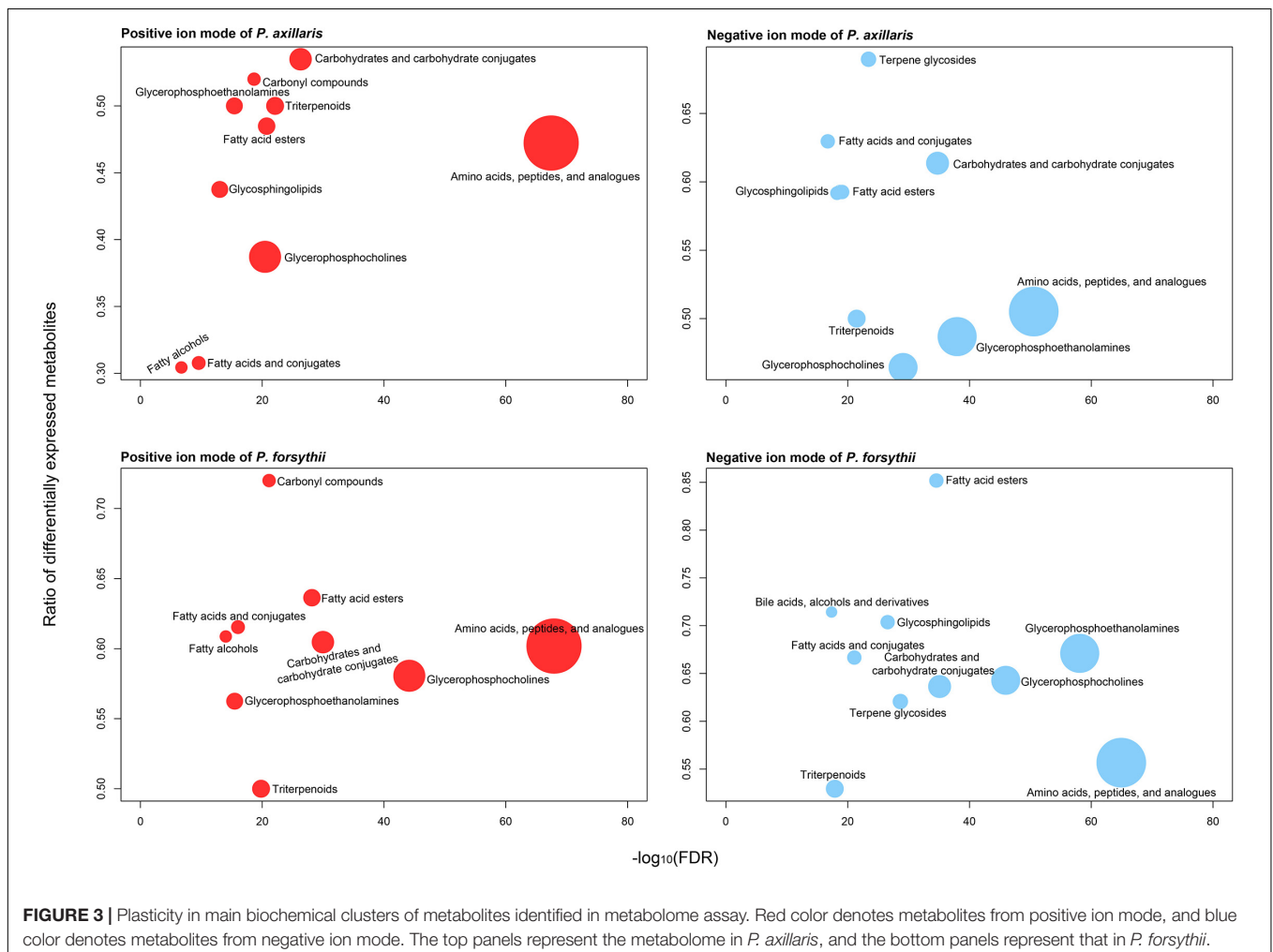
was fixed on a polyvinyl board. Then, we used ophthalmic scissors to remove the skin of the neck and make a cut on the Jugular vein. Notably, 40 μl blood plasma was extracted by a capillary (length: 100 mm; inner diameter: 1.55 mm) from the vein. Finally, the plasma was pushed into a 200-μl tube and then immediately stored in liquid nitrogen. The plasma samples were inputted for metabolome assay using the liquid chromatography-mass spectrometry (LC-MS) method (Brown et al., 2011; Dunn et al., 2011) and an ultra-performance liquid chromatography (UPLC)-TripleTOF 5600 system (AB SCIEX, Majorbio, Shanghai, China). Due to the different properties of metabolites, two modes (i.e., negative and positive ion modes) were used to detect metabolites. The raw data from LC-MS passed through a series of quality control phases, including baseline filtering, peak identification, integration, retention time correction, and peak alignment, and finally were normalized using the software XCMS (Tautenhahn et al., 2012). Final data matrices contained retention time, mass-to-charge ratio, and peak intensity. The identification of metabolites was carried out using substance-matching in

the METLIN and HMDB databases and an in-house standard library according to the retention time and m/z. The SIMCA-14.1 (Umetrics, Kinnelon, United States) was used to perform orthogonal partial least squares discriminant analysis (OPLS-DA) for obtaining variable influence on projection (VIP) values. Differentially expressed metabolites (DEMs) were defined using the following cutoff criteria: (1) Benjamini-Hochberg-adjusted p-values being less than 0.05; (2) fold-change (expressed as the ratio of average metabolite abundance between groups) being less than 0.5 for downregulation or being larger than 2 for upregulation; (3) VIP being larger than 1.

The Chemical Similarity Enrichment Analysis (ChemRICH; Barupal and Fiehn, 2017) was conducted to classify the metabolites into biochemical clusters from metabolomic database using Chemical Translation Service (CTS) and PubChem Identifier Exchange Service (Barupal et al., 2018). ClassyFire (Feunang et al., 2016) was used to automatedly annotate metabolite classification with default parameters. In addition, to further identify key metabolic processes for various metabolites,
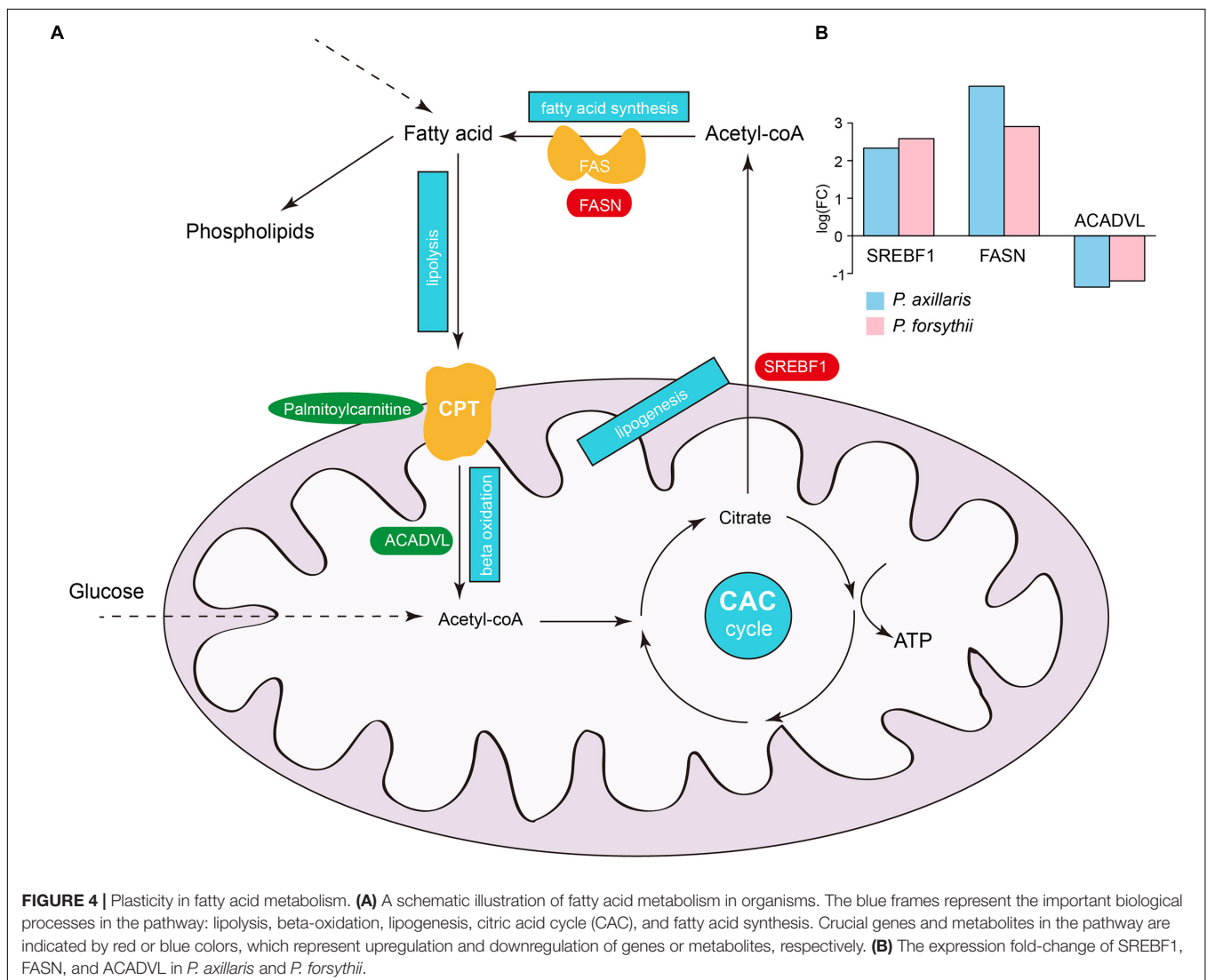

FIGURE 4 | Plasticity in fatty acid metabolism. **(A)** A schematic illustration of fatty acid metabolism in organisms. The blue frames represent the important biological processes in the pathway: lipolysis, beta-oxidation, lipogenesis, citric acid cycle (CAC), and fatty acid synthesis. Crucial genes and metabolites in the pathway are indicated by red or blue colors, which represent upregulation and downregulation of genes or metabolites, respectively. **(B)** The expression fold-change of SREBF1, FASN, and ACADVL in *P. axillaris* and *P. forsythii*.

null diffusion-based enrichment was conducted with the KEGG pathway database by *runDiffusion* function using the FELLA package (Picart-Armada et al., 2018).

RESULTS

Plasticity in Bite Force

We found no significant change in bite force in *P. axillaris* (from 8.81 ± 0.70 n, $n = 11$ to 7.12 ± 0.51 n, $n = 17$; $t = -0.95$, $p = 0.35$; **Figure 1B**) and in *P. forsythii* (from 2.40 ± 0.22 n, $n = 14$ to 4.31 ± 0.16 n, $n = 18$; $t = 0.74$, $p = 0.47$; **Figure 1B**) after being acclimated to high-altitude environments.

Plasticity in Tail Display Behavior

We detected clear plasticity in tail display behavior in both species. In *P. axillaris*, average tail coil speed was significantly increased (from 16.28 ± 3.19 cm/s, $n = 23$ to 97.55 ± 8.82 cm/s, $n = 20$; $t = 5.06$, $p = 0.0004$; **Figure 1C**), while average tail lash speed was significantly decreased (from 79.50 ± 8.52 cm/s, $n = 22$ to 15.19 ± 1.49 cm/s, $n = 22$; $t = -5.28$, $p = 0.0005$; **Figure 1C**) after being acclimated to high elevation area. Meanwhile, the average tail coil duration decreased significantly from 11.01 ± 2.33 s ($n = 23$) to 1.98 ± 0.46 s ($n = 20$; $t = -3.31$, $p = 0.007$), while average tail lash duration increased significantly from 2.63 ± 0.35 s ($n = 22$) to 6.77 ± 1.02 s ($n = 22$; $t = 4.00$, $p = 0.003$; **Figure 1E**). In *P. forsythii*, we found no significant change in average tail coil speed (from 12.13 ± 1.06 cm/s, $n = 13$ to 15.82 ± 1.18 cm/s, $n = 22$; $t = 1.63$, $p = 0.13$; **Figure 1D**), in average tail lash speed (from 14.28 ± 1.07 cm/s, $n = 20$ to 10.87 ± 0.60 cm/s, $n = 20$; $t = -0.97$, $p = 0.35$; **Figure 1D**), and in average tail lash duration (from 10.19 ± 1.55 s, $n = 20$ to 8.05 ± 0.85 s, $n = 20$; $t = -0.85$, $p = 0.41$; **Figure 1F**) after being acclimated to high elevation area, but the average tail coil duration decreased significantly from 15.99 ± 2.57 s ($n = 13$) to 3.89 ± 0.52 s ($n = 22$; $t = -4.73$, $p = 0.0004$; **Figure 1F**).

Plasticity in Gene Expression

A total of 44,000,068–123,811,250 and 42,179,214–91,710,426 raw reads were generated for *P. axillaris* and *P. forsythia* by Illumina sequencing, respectively. After filtering, 41,832,664–118,600,900 and 39,967,100–90,040,782 reads were retained, respectively (**Supplementary Table 3**). For *P. axillaris*, 27,894 transcripts were obtained with an N50 size of 2,372 bp and a mean length of 1,224 bp. For *P. forsythii*, 31,519 transcripts were obtained with an N50 size of 2,185 bp and a mean length of 1,205 bp. By the BRH method, 8,892 orthologous transcripts were identified among the two species.

The PCA plot showed that the tissue-specific expression was very consistent for all types of tissues within the two species, and the expression divergence for any type of tissues between low- and high altitudes was lower than any cross-tissue comparisons (**Figures 2A,B**). Generally, transcriptomes from heart and muscle were less diverged than from liver for both species. As for DEGs of *P. axillaris*, 1,612 were identified in heart between low- and high-altitude groups, with 690 upregulated and 922 downregulated, given low-altitude sample as a reference; 2,792 DEGs were identified in liver, with 1,485 upregulated and 1,307 downregulated; 2,993 DEGs were identified in muscle, with 1,365 upregulated and 1,628 downregulated. Similarly, for *P. forsythii*, 913 DEGs were identified in heart, with 405 upregulated and 408 downregulated; 1,301 DEGs were identified in liver, with 722 upregulated and 579 downregulated; 1,994 DEGs were identified in muscle, with 868 upregulated and 1,126 downregulated.

After functional annotation for DEGs of *P. axillaris*, a total of 32 GO categories and 1 KEGG pathways were identified as overrepresented in heart, most of which were associated with muscle function (e.g., GO: 0006936 muscle contraction, GO: 0003012 muscle system process) and small molecule metabolism (e.g., GO: 0019320 hexose catabolic process, GO:1901135 carbohydrate derivative metabolic process). In muscle, 158 GO categories and 7 KEGG pathways were overrepresented by DEGs, concentrating mostly on muscle function, including GO: 0003012 muscle system process, GO: 0006936 muscle contraction, and GO: 0006941 striated muscle contraction, and also associating with acid metabolism, such as GO: 0043436 oxoacid metabolic process. In liver, different from the other two tissues, 77 GO categories and 13 KEGG pathways were identified as overrepresented by DEGs, which were mostly associated with lipid metabolism, including GO: 0044255 cellular lipid metabolic process, GO: 0006631 fatty acid metabolic process, and GO: 0008202 steroid metabolic process. Similar patterns were also found in overrepresented KEGG pathways, such as map00071 fatty acid degradation and map01212 fatty acid metabolism (**Figure 2C** and **Supplementary Figure 1**).

For the DEGs of *P. forsythii*, four GO categories and one KEGG pathway were overrepresented in heart, which were all related to circadian regulation, including GO: 0032922 circadian regulation of gene expression, GO: 0009649 entrainment of the circadian clock, and pathway hsa04710: circadian rhythm. In muscle, 60 GO categories but no KEGG pathways were overrepresented by DEGs. Those categories were mostly related to cell migration and blood vessel development, including GO: 0030334 regulation of cell migration, GO: 0048514 blood vessel morphogenesis, and GO: 0010594 regulation of endothelial cell migration. In liver, 115 GO categories and 13 KEGG pathways were overrepresented. Similar to *P. axillaris*, most of those functional categories were also associated with lipid metabolism, including GO: 0008202 steroid metabolic process and GO: 0006631 fatty acid metabolic process, and pathways, such as map00071 fatty acid degradation (**Figure 2D** and **Supplementary Figure 1**).

Plasticity in Metabolism

A total of 4,523 features of metabolites were detected in the metabolome assay, among which 4,309 features were retained after filtering. After ChemRICH classification, 1,109 and 985 metabolites were identified with biochemical annotation for positive and negative ion mode of LC-MS, respectively. For *P. axillaris*, 421 DEMs were identified in positive ion mode with 83 upregulated and 338 downregulated; 482 DEMs were identified in negative ion mode with 80 upregulated and 412 downregulated. Similarly, for *P. forsythii*, 565 DEMs were identified in positive ion mode with 106 upregulated and 459

downregulated; 554 DEMs were identified in negative ion mode with 117 upregulated and 437 downregulated. Both positive and negative ion modes suggested that the patterns of metabolome for the two species were quite similar. For all biochemical clusters, amino acids, peptides, and analogs contained the most metabolites for both species, among which approximately 55% of the metabolites were differentially expressed. However, clusters associated with lipids were identified, showing a high ratio of DEMs, such as fatty acid esters, fatty acids and conjugates, and glycosphingolipids (**Figure 3**).

A total of 29 DEMs were mapped to KEGG pathways, among which 16 and 26 were from *P. axillaris* and *P. forsythii*, respectively. A high proportion (50.0% for *P. axillaris* and 53.8% for *P. forsythii*) of DEMs were associated with lipids, including those concordantly regulated in both species, such as the upregulation of 17α-Hydroxypregnenolone (cortisol synthesis and secretion) and sphingosine phosphocholine (sphingolipid metabolism) and the downregulation of cholesterol sulfate (steroid hormones biosynthesis) and L-palmitoylcarnitine (fatty acid degradation). In addition, lipid metabolism-related pathways were overrepresented by DEMs in both species, including fatty acid degradation, primary bile acid biosynthesis, and taurine and hypotaurine metabolism (**Supplementary Table 4**).

Fatty Acid Metabolism

Fatty acid metabolism appeared to be a major source of plasticity for both DEGs and DEMs, and therefore, we further examined detailed expression patterns for genes and metabolites associated with the fatty acid metabolic pathway (**Figure 4A**). We identified a total of 13 genes and 4 symbolic metabolites within the pathway that was differentially expressed in both species. Intriguingly, the patterns of up- or downregulation for those genes were identical in the two lizards, suggesting a common acclimation process in response to high-altitude environments for *Phrynocephalus* species. Among those genes, three play crucial roles in the fatty acid metabolic pathway: sterol regulatory element-binding transcription factor 1 (SREBF1), fatty acid synthase (FASN), and very long-chain-specific acyl-CoA dehydrogenase (ACADVL) (**Figure 4B**). SREBF1 acts as an inductor of lipogenesis in liver leading to increased storage of fatty acid as triglycerides for organisms. FASN encodes the enzyme FASN that catalyzes the synthesis of fatty acid from acetyl-CoA and is regulated by SREBF1 (Bhuiyan et al., 2009; Bouchard-Mercier et al., 2012). On the opposite side, ACADVL catalyzes the first step of mitochondrial fatty acid beta-oxidation, which digests fatty acid into acetyl-CoA before the citric acid cycle to produce energy, specifically for long-chain fatty acids (Miller et al., 2015). Among the metabolites within the pathway, L-palmitoylcarnitine is an important ester derivative of carnitine, which plays a core role in fatty acid metabolism by transporting long-chain fatty acids into mitochondria (Wood et al., 1984; Mutomba et al., 2000). In both *P. axillaris* and *P. forsythii*, SREBF1 and FASN showed significant upregulation, while ACADVL and the L-palmitoylcarnitine showed significant downregulation, which strongly suggested increased storage and decreased digestion of fatty acids.

DISCUSSION

The upslope range shifting is one of the primary responses for species under climatic changes (Pecl et al., 2017). Animals are predicted to colonize high-altitude regions following the shifting of suitable climatic conditions (Lenoir and Svenning, 2015). At high altitudes, key environmental factors, such as low oxygen availability, high levels of UV radiation, and their interactions, pose severe challenges to organisms, especially ectothermic species (Li et al., 2018; Sun et al., 2018). Our transplant experiments demonstrate that toad-headed lizards (*P. axillaris* and *P. forsythii*) from low altitudes are capable of acclimating to high-altitude environments without sacrificing their performance. *P. axillaris* showed high plasticity in tail display behavior, with increased tail coil speed and tail lash duration, as well as decreased tail coil duration and tail lash speed, while *P. forsythii* only showed reduced tail coil duration at high altitudes. Genes and metabolites associated with fatty acid metabolism were also identified with significant differentiation in expression when compared to individuals from low-altitude native habitats. Moreover, a large proportion of metabolites showed decreased expression for transplanted groups. Those consistent results imply that toad-headed lizards acclimate to high-altitude environments by reducing the behavioral intensity and increasing energy efficiency in multiple ways. Despite distinct reproductive models, the two lizards have highly concordant plasticity.

Plasticity in behavior plays a crucial role in dealing with the challenges of high-elevation environments from an energy cost perspective (Refsnider et al., 2018; Enriquez-Urzelai et al., 2020). As the direct connection between organisms and their environments, animal behavior is intimately correlated with energy metabolism (Ros et al., 2006; Mowles, 2014). We found that both species showed a trend of decreasing their tail display intensity, either by a specific component (e.g., tail coil in *P. forsythii*) or by a trade-off between different components (e.g., tail coil and tail lash in *P. axillaris*). Tail displays of *Phrynocephalus* lizards play important roles in social conflict alleviation and mate assessment (Wu et al., 2018), but they are energetically costly (Zhu et al., 2021). Consistent with our results, high-altitude *Phrynocephalus* lizards often constrain their activity intensity or reduce the display complexity (Hu et al., 2022). Behavioral traits are intrinsically plastic; lizards and many other ectotherms are well known for adjusting their behavior quickly in a new environment (Refsnider et al., 2018; Enriquez-Urzelai et al., 2020).

Plasticity in nutrient and energy metabolism can balance the requirements of life activities in high-altitude environments (Storz et al., 2010; Zhang et al., 2018). As the primary energy storage for animals, fatty acids play a key role in high-altitude plasticity and adaptation (Cheviron et al., 2012; Lui et al., 2015). Tang et al. (2013) first discovered that high-altitude *Phrynocephalus* species (*Phrynocephalus erythrurus*) had high-fat utility compared to its low-altitude counterpart (*Phrynocephalus przewalskii*). In this study, several core genes and metabolites associated with fatty acid metabolism show concerted patterns of differential expression, suggesting a common plastic response

for both *P. axillaris* and *P. forsythii*. The *SREBF1* gene in both species is also significantly upregulated at high altitudes, which functions to facilitate the process of lipogenesis by transforming other nutrients into fatty acids (Bhuiyan et al., 2009; Bouchard-Mercier et al., 2012). The upregulation of FASN is consistent with this process, which directly catalyzes fatty acid synthesis (Bhuiyan et al., 2009). In addition, ACADVL is downregulated at high altitudes, which suppresses the breaking down of long-chain fatty acids during beta-oxidation (Miller et al., 2015). Some endotherms have similar plastic responses at high altitudes. Yang et al. (2006) found upregulated leptin gene at high altitudes for the plateau pika; leptin facilitates the lipolysis process and is a key regulator for fatty acid metabolism (Pan et al., 2014). All these results suggest a common response of fatty acid metabolism, through lipogenesis, synthesis, and lipolysis, at high altitudes.

Global climatic change has increasingly been a leading cause for biodiversity loss (Sala et al., 2000; Thomas et al., 2004; Valtonen et al., 2017). Studying plasticity in response to high-altitude environments will provide useful insights into how organisms cope with new environments after upslope range shifting. In addition to directly buffering the environmental stress, plasticity may also provide direction for adaptive evolution toward high altitudes. The "plasticity-first evolution" model favors phenotypic plasticity that may lead to adaptation and is supported by an increasing number of cases (Moczek et al., 2011; Jones and Robinson, 2018). Environmental changes may first trigger phenotypic plasticity, and the selection on genotypes that influence the plastic expression of phenotypes will follow. Therefore, elucidating the nature of plasticity will help to predict directions of evolution and to manage conservation projects for organisms under the pressure of climatic change. Clearly, more studies are needed to unravel a comprehensive profile for phenotypic plasticity in behavior, performance, and metabolism for upslope range shifting species in high-altitude environments.

DATA AVAILABILITY STATEMENT

The behavioral data that were collected in this study are available in the **Supplementary Material** of this article. All the sequencing data are deposited in National Genomics Data Center with accession number PRJCA008159.

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ETHICS STATEMENT

AUTHOR CONTRIBUTIONS

YQ, JF, and WY conceived the ideas and designed the experiments. YW, ZY, PP, and XQ conducted the field collection and transplant experiment. YQ and YW collected the behavior, performance, and RNASeq data. TZ and XT measured the metabolome assay. YQ, TZ, JF, and WY analyzed the data and drafted the manuscript. All authors read, edited, and approved the final submitted version of the manuscript.

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