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Higher oxygen content and transport characterize high-altitude ethnic Tibetan women with the highest lifetime reproductive success

Shenghao Ye[a], Jiayang Sun[a], Sienna R. Craig[b], Anna Di Rienzo[c], David Witonsky[c], James J. Yu[d], Esteban A. Moya[d], Tatum S. Simonson[d], Frank L. Powell[d], Buddha Basnyat[e], Kingman P. Strohl[f], Brian D. Hoit[g], and Cynthia M. Beall[h,1]

Affiliations are included on p. 9.

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We chose the "natural laboratory" provided by high-altitude native ethnic Tibetan women who had completed childbearing to examine the hypothesis that multiple oxygen delivery traits were associated with lifetime reproductive success and had genomic associations. Four hundred seventeen (417) women aged 46 to 86 y residing at ≥3,500 m in Upper Mustang, Nepal, provided information on reproductive histories, sociocultural factors, physiological measurements, and DNA samples for this observational cohort study. Simultaneously assessing multiple traits identified combinations associated with lifetime reproductive success measured as the number of livebirths. Women with the most livebirths had distinctive hematological and cardiovascular traits. A hemoglobin concentration near the sample mode and a high percent of oxygen saturation of hemoglobin raised arterial oxygen concentration without risking elevated blood viscosity. We propose ongoing stabilizing selection on hemoglobin concentration because extreme values predicted fewer livebirths and directional selection favoring higher oxygen saturation because higher values had more predicted livebirths. *EPAS1,* an oxygen homeostasis locus with strong signals of positive natural selection and a high frequency of variants occurring only among populations indigenous to the Tibetan Plateau, associated with hemoglobin concentration. High blood flow into the lungs, wide left ventricles, and low hypoxic heart rate responses aided effective convective oxygen transport to tissues. Women with physiologies closer to unstressed, low altitude values had the highest lifetime reproductive success. This example of ethnic Tibetan women residing at high altitudes in Nepal links reproductive fitness with trait combinations increasing oxygen delivery under severe hypoxic stress and demonstrates ongoing natural selection.

Tibetan people | hypoxia | altitude | reproduction | genetic selection

Significance

We report a study designed to explore the extent to which variation in oxygen delivery physiology of ethnic Tibetan women aged 46 to 86 living ≥3,500 m altitude in Upper Mustang District, Nepal, related to the number of livebirths. Among women with long marriages and early first births, combinations of traits enhancing oxygen delivery to tissues characterized those with the highest lifetime reproductive success. Considering the collective contributions of sociocultural factors and the multiple biological traits contributing to the internal environment provided a fresh way to test hypotheses about ongoing natural selection under the stress of high-altitude hypoxia.

Testing the hypothesis of natural selection acting on adaptive human phenotypes requires integrating many data sources, including reproductive histories, sociocultural factors, physiology, and genotypes. Populations with a long history of exposure to severe stress and the opportunity for natural selection provide an ideal context to address these challenges. A few populations have thrived for thousands of years under high-altitude hypoxia and exhibit distinctive traits consistent with positive natural selection (1). Today's ethnic Tibetan population descends from ancestors with roughly 10,000 y of year-round high-altitude residence (2). Ethnic Tibetans have distinctive biology thought to reflect adaptations in the oxygen delivery system that counter environmental hypoxia (1, 3–5).

The Tibetan suite of traits encompasses the respiratory, hematological, and cardiovascular systems (6). For example, it includes high resting ventilation and brisk hypoxic ventilatory response (HVR), minimal elevation of hemoglobin concentration or pulmonary artery pressure above typical sea-level values, and high peripheral blood flow in many Tibetan individuals (7–10). This Tibetan pattern contrasts with hemoglobin concentration and pulmonary artery pressure elevation and a blunted HVR among many Andean highlanders and migrants to high altitudes (1, 3).

Women living at high altitudes (≥2,500 m) can encounter additional stress during pregnancy. High-altitude pregnancy increases the risk of preeclampsia or low birthweight (review in refs. 11 and 12), which raises the risk of maternal or infant death (13, 14). When comparing the pregnancy-related biology of Tibetan women with that of migrants to high altitudes, Tibetan women have lower hemoglobin concentration, higher oxygen saturation of hemoglobin and uterine artery blood flow, and heavier newborns (11, 15). Among Tibetan women who have completed childbearing, unelevated hemoglobin concentration, higher oxygen saturation, and a higher pulse rate correlate with higher lifetime

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[1]To whom correspondence may be addressed. Email: cmb2@case.edu.

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reproductive success (16). This pattern of human variation suggests the action of natural selection on oxygen delivery phenotypes.

Abundant evidence of genetic selection is also available. Dozens of loci show genomic signals of selection among Tibetans (17–27). Few of those studies included biological traits, and the reported associations remain to be replicated to rule out chance findings or unknown confounding factors (28). However, among the selection signals, two are highly replicated across studies: the *Egl-9 Family Hypoxia Inducible Factor 1* (*EGLN1*) and *Endothelial PAS Domain Protein 1* (*EPAS1*) loci, both of which play pivotal early roles in the response to hypoxia and maintaining oxygen homeostasis. The *EGLN1* locus encodes the oxygen sensor prolyl hydroxylase domain 2 (PHD2), which regulates the accumulation of hypoxia inducible factors (HIFs). *EPAS1* encodes the alpha subunit of hypoxia inducible factor 2 (HIF2). HIF2 regulates the expression of hundreds of genes (29). These two loci harbor alleles unique to indigenous Tibetan Plateau populations and have been repeatedly associated with the characteristic unelevated hemoglobin concentrations (27).

Moreover, the PHD2/HIF2 pathway is involved in the respiratory and cardiovascular response to hypoxia (30). A mouse line with the Tibetan Plateau *EGLN1* substitution (D4E/C127S) had a brisk ventilatory response to hypoxia (31). One active copy of the Tibetan Plateau haplotype of *EPAS1* protected a mouse line against elevated pulmonary artery pressure (31). Among Tibetans, SNP sites in *EPAS1* were associated with lower pulmonary artery pressure at altitude and upon exposure to acute hypoxia at low altitudes (4, 32). A brisk HVR and minimally elevated pulmonary artery pressures characterize the Tibetan response pattern (7, 10). These laboratory and natural experimental data support the hypothesis that natural selection has or is acting on these phenotypes and that genetic selection has acted on the genomes.

Consequently, this study examines the extent to which within-sample variation in respiratory, hematological, and cardiovascular traits associated with variation in lifetime reproductive success, measured as the number of livebirths. We assembled data on sociocultural information, biological characteristics, reproductive success, and previously collected genotypic data (27) of 417 ethnic Tibetan women 46 to 86 y of age residing at altitudes ranging from 3,500 to 4,100 m in Upper Mustang District, Nepal. We hypothesized that hemoglobin concentration, percent of oxygen saturation of hemoglobin, pulse, hypoxic heart rate response (HHRR), HVR, and cardiovascular anatomy and function would predict lifetime reproductive success and have genomic associations.

Materials and Methods

Study Population. The population of the Upper Mustang District of Nepal, located along the border of Nepal and the Tibet Autonomous Region, are Nepali citizens who self-identify as ethnically Tibetan: They speak Tibetan dialects and share economic, religious, and sociocultural practices common among highland Tibetans (16). Villages range from 3,500 to 4,100 m altitude (11,550 to 13,530′). Foreigners were forbidden in the area until 1992 when costly visitor permits became legal.

Study Sample. In 2012, we enrolled ethnic Tibetan women 39 y and older who had been married or pregnant and were lifelong native residents of Upper Mustang (16). Most women (62%) had joined their husband's family in a nearby village after marriage, while 15% remained in their natal households. 94% had no schooling. 43% had used contraception, which became available in the early 1990s, to stop pregnancies (33). We recruited 431 women of the 605 who participated in our 2012 study (86% of 504 still alive) (16, 27, 33, 34) to join a follow-up study in 2019. Power calculations based on the 2012 data estimated a sample of 400 would provide 80% power to detect effect sizes of 0.04 pregnancies or live births. The 417 women who provided written informed consent, interview, anthropometric, and biological data in 2012 and 2019 form the present sample. Their demographic and biological characteristics closely resemble those of the larger 2012 sample (*SI Appendix*, Table S3) and indicate that ascertainment bias is unlikely to confound our results.

Institutional review boards at Case Western Reserve University (IRB 2017-2082), Dartmouth College, and the Nepal Health Research Council (Reg. no. 256/2018 approved the protocol.

Data Collection. A research team of US-based researchers, ethnic Tibetan female nurses, and research assistants from Nepal collected data during June to August of 2019 in temporary laboratories in three villages, Lo Monthang (3,800 m), Tsarang (3,500 m), and Ghami (3,500 m). *SI Appendix* describes the ambient conditions and protocols for data collection, including anthropometry, echosonography, and hypoxic ventilatory and heart rate response tests.

Data Analyses. *SI Appendix* explains the analyses in detail (*SI Appendix*, Fig. S1). We conducted exploratory data analyses on 115 variables and reduced them to 50 potential predictors, including age, use of contraception, and altitude of residence (*SI Appendix*, Table S2). Since our sample contains a subgroup that did not have echosonography (echo) or HVR data, our comprehensive modeling of lifetime reproductive success used three datasets: 1) the *full sample* of 417 women, 2) the *subset of 364* women with completed with echocardiography (echo) and HVR data (the *complete sample*), and 3) the *subset of 53* women who did not have echo or HVR data. Table 1 shows the similar demographic and biological characteristics of the full and complete samples.

Parametric and nonparametric statistical analysis gave biologically meaningful models of the influential predictors of lifetime reproductive success. A hybrid, semiparametric model analysis used generalized additive modeling (GAM) of the outcomes with the 50 covariates; a Poisson regression analysis applied generalized linear modeling (GLM) on a combination of the original and transformed variables; and cross-validation evaluated the robustness of the models. Nonparametric analysis used tree-based models to efficiently incorporate missing information and nonlinear, complex relationships.

Two types of analyses deployed for evolutionary analyses include Lande-Arnold matrix approaches and Cox regressions. We did not use the Lande and Arnold selection gradient approach because our model fits the data better and is biologically interpretable. For instance, our data-dependent change point analysis shows that women's cumulative number of childbirths flattens on average after their reproductive period, which is sensible and consistent with human biology. Cox regressions were inappropriate here because the Cox regression model is designed for the time-to-first event response data. However, our interest was in the total number of events, measuring reproductive success.

Genomic analyses. We used previously published genotype data obtained at approximately 3.5M single nucleotide polymorphisms (SNPs) (27) (available at https://datadryad.org/stash/dataset/doi:10.5061/dryad.bp46m) to conduct a genome-wide association study (GWAS) and a candidate SNP analysis of the significant or influential variables included in our final models obtained as described above. DNA extracted from saliva samples (Oragene ®) provided the material for genotyping.

Accessibility of Information. Genomic data are available at the site described in the previous paragraph. Please contact the Cleveland Clinic BioRepository (CCBioR) https://my.clevelandclinic.org/departments/biorepository to enquire about the availability of DNA. Phenotypic data are available at https://doi.org/10.17605/OSF.IO/JUYCS (35). Protocols and related materials are available at https://tetragardenia-sl53.squarespace.com/config/. Codes are available in tables and cited publications.

Results

Four hundred and seventeen women 46 to 86 y of age reported 2,193 pregnancies, resulting in 2,076 livebirths, 71 miscarriages, and 46 stillbirths. They averaged 5.3 pregnancies and 5.2 livebirths with a range of variation from zero to 14 (Fig. 1 and Table 1). Table 1 describes all the women, a large subset with complete information, including echocardiograms and HVR tests, and a

Table 1. Characteristics (mean ± SD) of women in the full sample, the subset with complete data analyzed using parametric and nonparametric approaches, and the small subset of women without echo or HVR data*

	Full sample of 417	Complete subset of 364 with echocardiography and HVR	Subset of 53 without echocardiography or HVR
Continuous variables (*Bold letters are acronyms)	mean ± SD, range	mean ± SD, range	mean ± SD, range
Altitude of residence, km	3.8 ± 0.15, 3.5 to 4.1	3.8 ± 0.15, 3.5 to 4.1	3.7 ± 0.15, 3.5 to 4.0
Age, yrs	58.8 ± 9.26, 46 to 86	58.9 ± 8.94, 46 to 86	65.9 ± 9.22, 46 to 84
# pregnancies (n = 407)	5.3 ± 2.82, 0 to 15	5.3 ± 2.77, 0 to 14	5.3 ± 3.18, 0 to 15
# livebirths (n = 407)	5.2 ± 2.82, 0 to 14	5.2 ± 2.63, 0 to 14	5.3 ± 2.83, 1 to 12
Age at first pregnancy/birth, yrs (**b1mage**)	23.8 ± 4.22, 14 to 43	23.9 ± 4.33, 14 to 43	23.1 ± 3.20, 17 to 32
Length of marriage, yrs (**lmrge**)	25 ± 11.3, 2 to 53, n = 396	22 ± 12.4, 0 to 49	32 ± 10.5, 13 to 53, n = 48
BMI kg/m^2	21.1 ± 3.03, 13.7 to 31.6	21.3 ± 3.00, 15.4 to 31.6	20.0 ± 3.05, 13.7 to 30.1
BSA m^2	1.4 ± 1.27, 1.1 to 1.8, n = 396	1.4 ± 1.26, 1.1 to 1.8, n = 360	1.3 ± 0.13, 1.1 to 1.6, n = 53
Temp (Aural Temperature, ° F)	97.8 ± 0.46, 94 to 100, n = 411	97.8 ± 0.96, 94 to 100, n = 358	97.7 ± 0.95, 94 to 99, n = 53
Hemoglobin concentration, gm/dL (**HB**)	13.8 ± 1.30, 8.4 to 17.7	13.9 ± 1.23, 8.5 to 17.2	13.7 ± 1.71, 8.4 to 17.7
% oxygen saturation, finger plethysmography	86.2 ± 4.09, 65.0 to 94.3	86.6 ± 3.74, 68.0 to 94.3	83.5 ± 5.39, 65.0 to 92.0
% oxygen saturation, forehead reflectance (**SatRmAir**)	91.0 ± 3.897, 74.9 to 99.6 (n = 376)	91.1 ± 3.76, 76.5 to 99.6	87.5 ± 5.49, 75 to 93, n = 41
Pulse	73 ± 9.1, 48 to 105 (n = 414)	72 ± 8.5, 48 to 96	79 ± 10.8, 58 - 105 (n = 50)
Systolic blood pressure, mmHg	126.5 ± 22.68, 82.0 to 218.0	125.9 ± 22.20, 82.0 to 218.0	130.0 ± 25.73, 90.7 to 196.0
Diastolic blood pressure, mmHg	82.9 ± 12.10, 51.3 to 132.7	83.1 ± 11.71, 57.3 to 132.7	81.9 ± 14.60, 51.3 to 119.7
RVOTvti, cm	16.3 ± 3.81, 7.9 to 30.7, n = 395	16.3 ± 3.71, 7.9 to 30.7, n = 360	16.2 ± 4.75, 8.0 to 29.4, n = 35
LVOTDiamIndex, cm/m^2	1.5 ± 016, 1.0 to 2.1, n = 397	1.4 ± 0.16, 1.0 to 2.1, n = 361	1.5 ± 0.14, 1.2 to 1.8, n = 34
LADIndex, cm/m^2	2.2 ± 0.38, 1.5 to 3.6, n = 398	2.2 ± 0.36, 1.5 to 3.6, n = 361	2.4 ± 0.46, 1.8 to 3.4, n = 35
AODIndex, cm/m^2	2.5 ± 0.29, 1.77 to 3.80 (n = 397)	2.5 ± 0.28, 1.79 to 3.48 (n = 361)	2.5 ± 0.35, 1.77 to 3.80 (n = 35)
HHRR, bpm/Δ%sat	0.7 ± 0.32, 1.5 to 3.6, n = 397	0.7 ± 0.32, 0.1 to 2.4, n = 360	0.5 ± 0.28, 0.14 to 1.01, n = 12
Categorical variables	% of sample (#)	% of sample (#)	% of sample (#)
Marital status	N = 417	N = 364	N = 53
Currently married	59.7 (246)	59.0 (213)	58.5 (31)
• Divorced/Separated	4.3 (18)	4.7 (17)	1.9 (10)
• Never married	3.3 (14)	3.0 (11)	3.8 (2)
• Widowed	33.7 (141)	33.2 (120)	35.8 (18)
Use of contraception	N = 416	N = 363	N = 53
• Never	57.0 (237)	54.3 (197)	75.5 (40)
• Yes	43.0 (179)	45.7 (166)	24.5 (13)
# Miscarriages	N = 407	N = 357	N = 47
• 0	85.8 (351)	86.8 (307)	78.7 (37)
• 1	10.5 (43)	10.2 (36)	14.9 (7)
• 2	3.4 (14)	2.8 (10)	6.4 (3)
• 3	0.2 (1)	0.3 (1)	–
# Twin pregnancies	N = 407	N = 357	N = 53
0	94.5 (387)	94.7 (338)	98 (49)
1	4.7 (19)	5.0 (18)	2 (1)
2	0.2 (1)	0.3 (1)	0

*BMI—body mass index (kg/m^2), BSA—body surface area (0.007184 × (Height(cm)$^{0.725}$) × (Weight(kg)$^{0.425}$), Temp—aural temperature ° F, HBoxy = hemoglobin concentration × percent of oxygen saturation of hemoglobin by finger plethysmography, RVOTvti–right ventricular outflow tract velocity time integral, LVOTDiamIndex—left ventricular outflow tract diameter/BSA, LADIndex—left atrial diameter/BSA, AODIndex—aortic root diameter/BSA, HHRR—hypoxic heart rate response (ΔHR/ΔSpO2 by forehead reflectance).

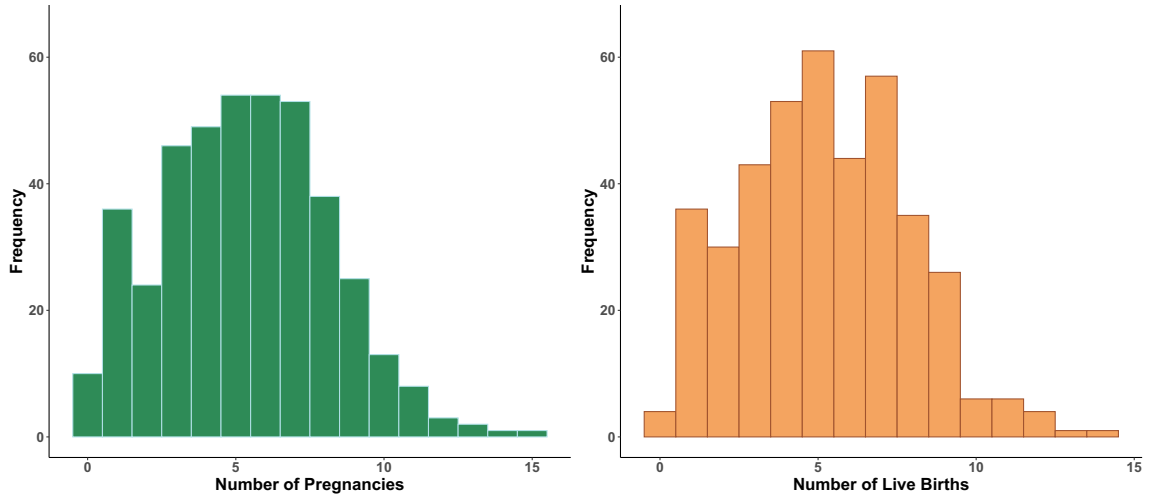

Fig. 1. The number of pregnancies and livebirths ranged from 0 to 15 and 0 to 14 respectively, with a mode of five for both traits (n = 417 women who had been pregnant or married).

small subset without echocardiograms or HVR tests. Our analyses focus on livebirths because women may not have recognized early, lost pregnancies; parallel analyses of pregnancies appear in Supporting Information. The two measures of lifetime reproductive success (number of pregnancies and number of livebirths) correlate highly (r = 0.971, $P < 0.001$).

We first considered natural selection on oxygen transport phenotypes. 364 women provided complete information on all variables. The best parametric analysis had a mixture of linear and nonlinear components and showed that women 63 y and older had the most livebirths (Table 2); those women had their children before family planning technology became accessible. An earlier age at first birth and a longer marriage predicted more livebirths, reflecting a longer exposure to the possibility of pregnancy. A high percent of oxygen saturation of hemoglobin, an intermediate hemoglobin concentration of approximately 13.4 gm/dL, and a low HHRR predicted more livebirths. Wide left ventricular outflow tract relative to body size (LVOTDiamIndex) or having twins interacted with a later age at first birth to predict more livebirths. We speculate that lower HHRRs allow a longer time to fill the ventricle with oxygenated blood. The hemoglobin and oxygen saturation results align with previous reports on Tibetan women (16, 36, 37). Our findings link high oxygen content (hemoglobin concentration and oxygen saturation) and oxygen transport (HHRR and LVOTDiamIndex) to more livebirths.

Fig. 2, *Left* shows the relationship between the predicted number of livebirths and hemoglobin concentration and the distribution of hemoglobin concentration values. The predicted livebirths increase sharply from the lowest hemoglobin concentrations to the mode, decrease gradually at higher values, and suggest stabilizing selection. Fig. 2, *Right* shows that ever-higher oxygen saturation predicts more livebirths and suggests directional selection.

Next, a nonparametric, data-driven, and machine-based approach [classification and regression tree analysis (38)] identified homogenous groups of women based on specific combinations of

Table 2. General linear model (GLM) analysis of the number of livebirths among the 364 women with complete response information (Quasi R^2 = 0.49)

variable	b1mage	lmrge	age2	LVOT-DiamIndex	Temp	Hb2	b1mage*LVOT-DiamIndex	b1mage*twins	b1moag-e*Hb2	lmrge*age2	age2*HHRR	HHR-R*Temp
P-value	0.031	0.0017	2.7*10^−5	0.005	0.045	0.039	0.01	0.019	0.028	0.004	0.043	0.038

$$\begin{aligned}\log(\#livebirth) \sim\ & 30.86 - 1.15 * b1mage + 0.12 * lmerge + 2.92 * age2 + 0.047 * SatRmAir \\ & - 9.86 * HHRR + 2.80 * LVOTDiam - 0.35 * Temp - 1.17 * twins - 4.41 * Hb2 \\ & - 0.11 * b1mage * LVOTDiam + 0.01 * b1mage * Temp + 0.06 * b1mage * twins \cdot \\ & + 0.21 * b1mage + Hb2 - 0.058 * lmrge * age2 - 1.22 * age2 * HHRR \\ & - 0.048 * SatRmAir * HHRR + 0.17 * HHRR * Temp\end{aligned}$$

Here, the variables b1mage, lmrge, SatRmAir, HHRR, and LVOTDiam=LVOT correspond to the acronyms in Table 1. In addition, the nonlinear relationships of y = #livebirth with age, BMI, and Hb were determined using additional analyses presented below:
For predicting the number of livebirths (y), the nonlinear relationships are

$$g_{hb}(x) = \begin{cases} -0.389 + 0.161x, & x \le 13.4 \\ 1.942 - 0.012x & x >> 13.4 \end{cases} \quad [1]$$

$$g_{BMI}(x) = (x - 21.95)^2,$$

$$g_{age}(x) = \begin{cases} 0.050 + 0.0294x, & x \le 63 \\ 1.901, & x > 63 \end{cases}$$

Here, $age2 = g_{age}(age)$, $hbavg2 = g_{hb}(hb)$ in Eq. **1**, and the *P*-values for the significant variables are given in the table.

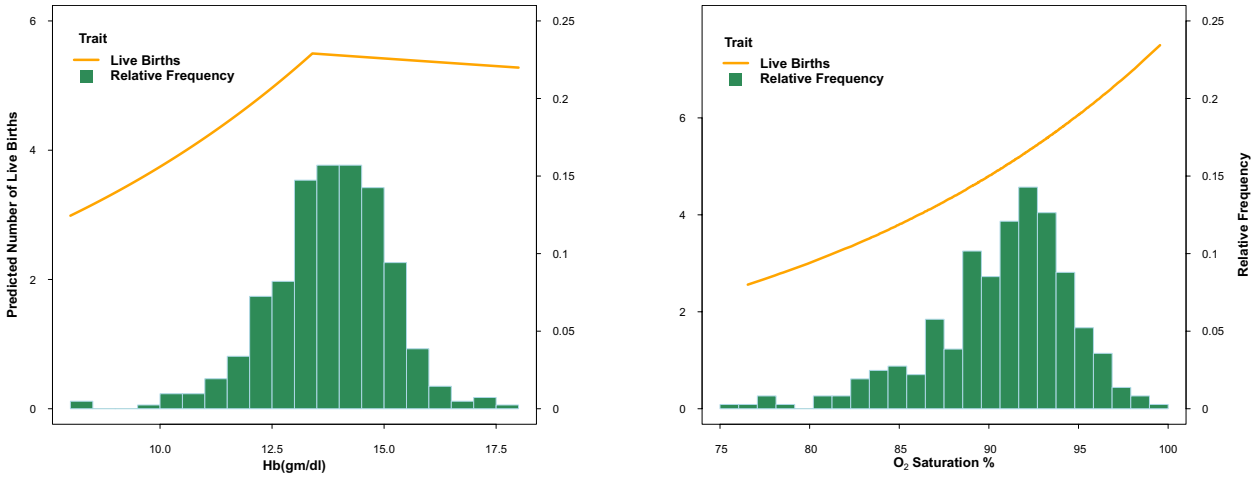

Fig. 2. *Left*: Predicted numbers of livebirths (yellow line, calculated using the GLM model equation in Table 2 allowing only the transformed hemoglobin concentration values to vary) across the range of variation of hemoglobin concentration (rust-colored histogram). The calculated optimal hemoglobin concentration is 13.4 gm/dL. *Right*: Predicted numbers of livebirths (yellow line, calculated using the GLM model equation in Table 2 allowing only the percent of oxygen saturation (forehead reflectance values) to vary across the range of variation of oxygen saturation (green histogram).

traits and values most associated with a particular number of livebirths. Fig. 3 shows the resulting tree. The variable in the box at the top labeled "length of the first marriage, yrs" links a sequence of nodes/boxes to an oval at the bottom. That oval shows the average number of livebirths of a maximally homogeneous group of women with the traits described. The tree in Fig. 3 shows the length of the first marriage as the primary factor distinguishing women with few or many livebirths, followed by the age at first pregnancy/birth. At the bottom left, women with 2.4 livebirths were married for fewer than 24 y and had a first pregnancy at 27 or later. At the bottom right, five traits identified two groups of women with eight or more livebirths among those with longer marriage, earlier first birth, and a right ventricle stroke distance (RVOTvti, the distance blood travels toward the lungs per heartbeat) above the sixth percentile (>11 cm). Women averaging 9.4 livebirths also had left ventricle outflow tract diameter normalized for body surface area (LVOTDiamIndex) above the 65th percentile (>1.5 cm/m^2). Women with 8.5 livebirths had an LVOTDiamIndex below the 65th percentile, a pulse below the 5th percentile (68 f/min), and a percent oxygen saturation above the 32nd percentile. One successful combination of traits included oxygen transport traits reflecting deoxygenated blood flow into the lungs and oxygenated

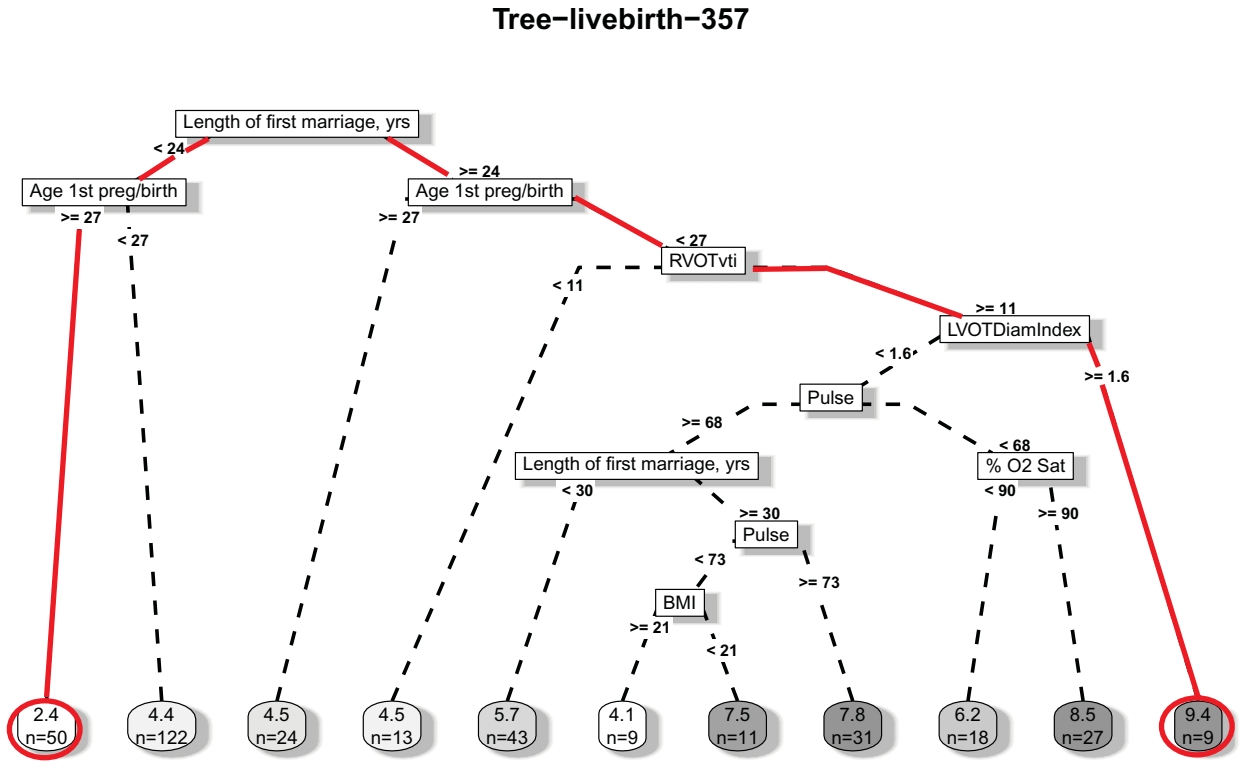

Fig. 3. Tree-based model of the number of livebirths in the complete sample of 364; 357 had a livebirth. The boxes represent nodes of algorithm-identified influential variables. The numbers on the descending branches denote the decision rule dividing a node in two maximally homogeneous groups. Successive nodes and branches apply decision rules that result in combinations of traits indicating homogeneous groups of women and their livebirths and sample size in the ovals. The red lines show trait combinations characterizing women with the fewest (*Bottom Left*) and most (*Bottom Right*) livebirths.

blood out of the left ventricle of the heart into the aorta and systemic circulation. A second successful combination of traits reflected a combination of high blood flow into the lungs and a smaller left ventricle coupled with a low pulse and high oxygen saturation.

A good parametric analysis provides the best-fitted model within an assumed form, such as linear, quadratic, or specific nonlinear models, and is efficient and easy to interpret if the model form is a reasonable approximation to the data. Nonparametric analysis, which does not assume a specific form, has minimal assumptions and offers robustness and flexibility in real-data analysis when the model form is unknown. If parametric and nonparametric fits yield similar results, the assumed parametric form is justified. Hence, both analyses are crucial for responsible data science.

Indeed, the parametric and nonparametric analyses reinforce and refine one another. Both identified the mother's age at her first pregnancy/birth and the length of her first marriage as the most influential variables. The parametric analysis showed a linear correlation between higher saturation and more births; the tree-based analysis located the favorable range above 90%–the average was 91% (Table 1). Both analyses identified lower heart rates as beneficial, whether under added hypoxia or in room air. Both linked a large left ventricle outflow diameter, particularly above 1.5 cm/m^2, to many livebirths. Two lines of evidence suggest the larger diameter supported reproduction rather than resulted from it. A larger ventricle outflow diameter can offset somewhat the effects of a late first birth (Table 2) and a smaller one can support many births when combined with high saturation (Fig. 3).

Sample without Echocardiography or HVR Tests. Women whose echocardiography exams excluded them from the HVR test comprise most of this subset of 53 women (*SI Appendix*, Table S1). The tree in Fig. 4 displays nodes different from those for the complete sample. Diastolic blood pressure topped the livebirths tree. Unexpectedly, women with the fewest livebirths (three) had healthy or mildly hypertensive diastolic blood pressure, while those with the most (eight) had diastolic hypertension (95 mmHg or more, above the 89th percentile).

Entire Sample. Among the *entire sample* of 417 women, the tree-based model (*SI Appendix*, Fig. S4) confirms two groups of women defined by relatively large or small left ventricles. A group with an average of nine livebirths had the larger 35% of LVOTDiamIndex values. A group with an average of 9.6 livebirths had the smaller 65% of LVOTDiamIndex values, were 71 or older, and had a BMI above the 56th percentile (20.4 kg/m^2). The full range of LVOTDiamIndex values can support many livebirths, although with different trait combinations at low or high values.

Parallel analyses of the number of pregnancies presented in *SI Appendix* reveal the same relationships and add left atrial diameter and the number of miscarriages as significant and influential variables (*SI Appendix*, Figs. S5–S7 and Table S4).

Genome-Wide and Candidate Gene Associations. Our genomics analyses evaluated the extent of genetic contribution to variation in the biological variables used in the analyses just described. Percent of oxygen saturation (forehead reflectance) associated with 45 tightly linked SNPs and HHRR associated with 12 tightly linked SNPs (Table 3) at a significance level of ($P \leq 5 \times 10^{-7}$), slightly below the conventional cutoff for genome-wide significance (Fig. 5 *B* and *C*). Nevertheless, we report these findings because these two traits correlated with the number of livebirths (Table 2). The average minor allele effect size was −2.8% for oxygen saturation (forehead reflectance). HHRR-associated SNPs included eight with a minor allele effect size of −0.16 and four with an effect size of +0.13 beats per minute/Δ%sat; the weighted average effect size was −0.76 beats per minute/Δ%sat. These results add to the genomic evidence relating to oxygen saturation (36, 39–42) and provide evidence relating to HHRR.

Candidate SNP analysis at the *EPAS1* SNP (rs76242811) detected an association with hemoglobin concentration (P = 0.00032) (Fig. 5*E*). rs76242811 is a high-frequency intronic variant with a strong signal of selection found among populations indigenous to the Tibetan Plateau. Homozygotes for the minor allele at this site in the gene encoding the transcription factor subunit HIF2A had the highest hemoglobin concentration (Table 3), consistent with previous analyses (review in ref. 27). Apart from this *EPAS1* SNP rs76242811, none of the SNPs in Table 3 and Fig. 5 lies near a known gene.

Discussion

We hypothesized that pulmonary, respiratory, hematological, and cardiovascular elements of a suite of traits involved in oxygen delivery and the response to high-altitude hypoxia would predict lifetime reproductive success and show genomic associations. We found influential and significant hematological and cardiovascular predictors. High-altitude native resident Tibetan women with higher oxygen content (modal hemoglobin concentration and progressively higher oxygen saturation) and oxygen transport (lower HHRR and wider left ventricles) tend to have more livebirths. Generally, these

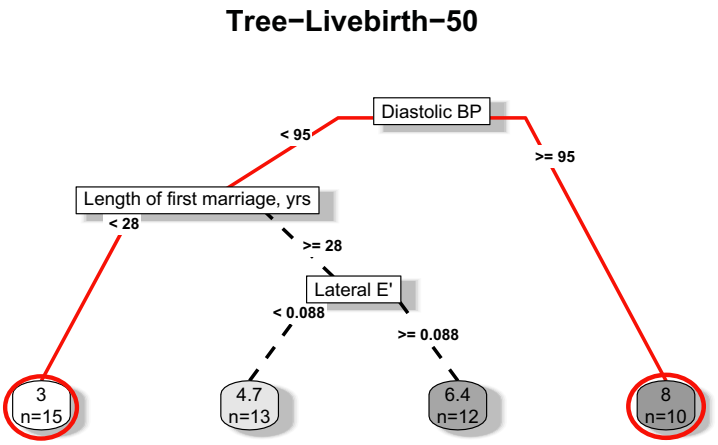

Fig. 4. Tree-based model of the number of livebirths in the sample of 53 women without echocardiograms or HVR tests; 50 had a livebirth.

Table 3. Genotype–Phenotype analyses. Genome-wide significant ($P < 5 \times 10^{-8}$) or near significant ($P \leq 5 \times 10^{-7}$) traits correlated with lifetime reproductive success measured as the number of livebirths (number of pregnancies in the case of LADIndex, cm/m^2)

Trait	CHR	Number of SNPS	Rs ID of top (rows 1 to 4) or candidate (row 5) SNP	minor allele frequency	beta value	SE of beta	p_LRT
1. BMI, kg/m^2	9	6	rs9299270	0.31	1.36	0.313	2.19E-08
2. Oxygen saturation (forehead reflectance), %	1	45	rs6660557	0.054	−2.77349	0.562799	1.02E-07
3. HHRR, bpm/Δ%sat	13	15	rs3011468	0.365	−0.16799	0.034637	2.55E-07
4. LADIndex, cm/m^2	12	4	rs7314052	0.11	0.201	0.0401	2.41E-07
5. Hb, gm/dL	2	1 (*EPAS1*)	rs76242811	0.211	0.410	0.107	3.32E-04

The top SNP (rows 1 to 4) is that with the most significant effect/noneffect allele. The Tibetan Plateau haplotype defining SNP (row 5) is the *EPAS1* SNP with the lowest *P*-value. The beta value is the effect size of the minor allele. p_LRT is the *P* value of the likelihood ratio test implemented in GEMMA.

results matched expectations. However, respiratory and pulmonary traits did not predict the number of livebirths. Previous work with younger samples of Tibetans across the altitude range of 3,658 to 4,200 m reported distinctly high resting ventilation, HVR (1, 3), and minimal elevation of pulmonary artery pressure (7, 10). Therefore, we hypothesized that these traits would improve reproductive success. This study did not support that hypothesis, perhaps because of the older age range, different measurement protocols, or sensitivity to other environmental factors.

Our results refined the understanding of hemoglobin concentration values by showing that the optimum hemoglobin concentration of 13.4 gm/dL for livebirths is close to the mode of 13.5 gm/dL. Sharply lower values below the optimum show fewer and fewer predicted livebirths, likely related to arterial oxygen content too low to support pregnancy or fetal development (Fig. 2). The slow decline in predicted livebirths above the optimum may reflect a change from past selection against very high hemoglobin concentrations that raise blood viscosity. High blood viscosity burdens the cardiovascular system; ventricles work harder, cardiac output falls, impaired blood flow, and oxygen diffusion ensues. Resulting costs to the individual include poorer exercise capacity and a higher risk of Chronic Mountain Sickness (44, 45). None of the women in this study had excessively high hemoglobin concentrations, consistent with past directional selection favoring lower values. These findings are consistent with studies and meta-analyses showing that intermediate maternal hemoglobin concentrations before and during pregnancy are optimal for pregnancy outcomes in many populations (46–51). Finding this association among Andean women living as high as 4,000 m seems paradoxical; however, robust expansion of plasma volume during pregnancy may be particularly relevant in the context of the generally high hemoglobin concentrations among Andean highlanders (52). The available evidence on blood volume during high-altitude pregnancy is equivocal. Comparing women of Andean and European descent at 3,600 m, Vargas and colleagues reported that absolute blood volume was lower among Andean women, although the two samples maintained the same volume per kilogram body weight through pregnancy (53). Available evidence finds that the

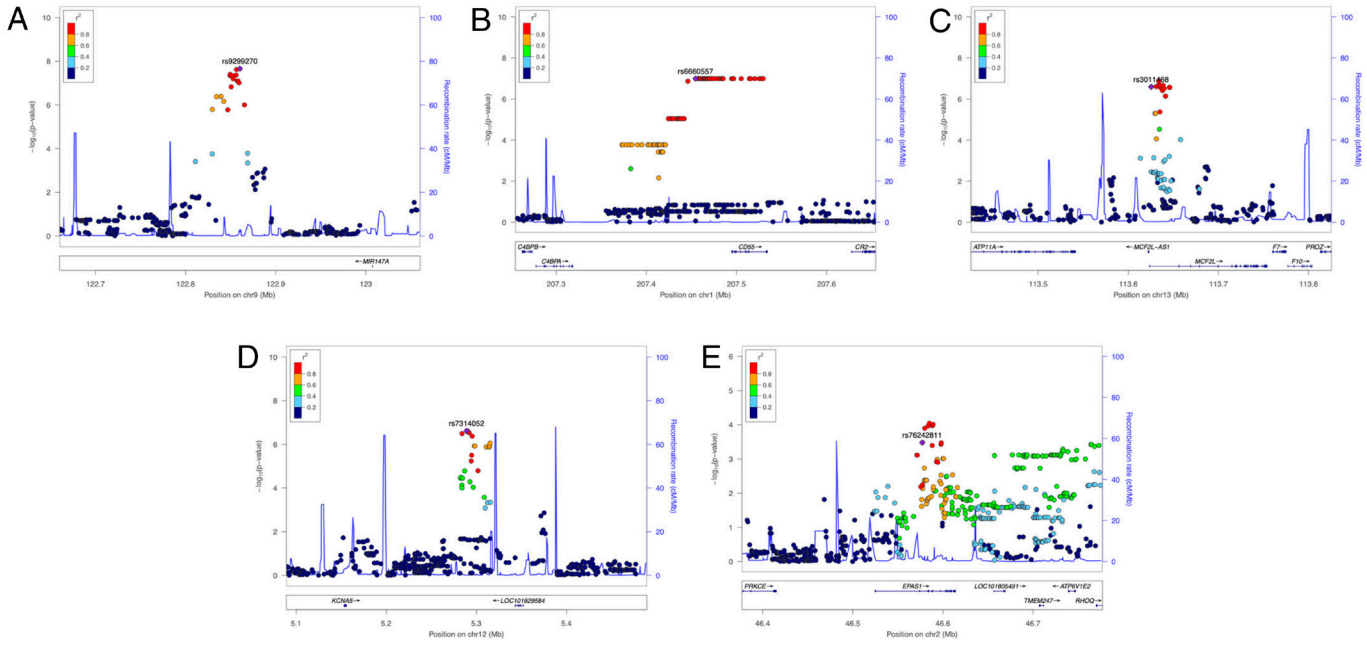

Fig. 5. LocusZoom plots (43) show the extent of the association signals listed in Table 3. The Y-axes show the −log10 scale values and the x-axes indicate chromosomal position. (*A*) BMI at chr 9, (*B*) Oxygen saturation at chr 1, (*C*) HHRR at chr 13, (*D*) LADindex at chr 12, and (*E*) Hb at *EPAS1* (chr 2).

EGLN1/*EPAS1* oxygen homeostasis pathway does not exert a large influence on hemoglobin concentration in the Andean populations as it does in the Tibetan (54–57).

An expanded plasma volume (not a lower hemoglobin mass) contributes to Tibetans' unelevated hemoglobin concentrations (52). An expanded baseline plasma volume, further expansion during pregnancy, and cardiovascular responses to hypoxia (58) could tax the cardiovascular system. This study provides evidence of cardiovascular associations with lifetime reproductive success. The tree-based analyses showed that the full range of LVOTDiamIndex values could support high reproductive success, depending on the values of other traits. Relatively large left ventricles (LVOTDiamIndex) pumping oxygenated blood to the systemic circulation predicted more livebirths if blood flow to the lungs was above a low minimum (6 to 8th percentile of RVOTvti). Interestingly, a relatively small left ventricle also supported many births if combined with an oxygen saturation at or above 90% (the mean was 91%). A second combination of traits supporting many pregnancies was the rapid flow of deoxygenated blood to the lungs (RVOTvti) and significant volumes of oxygenated blood from the heart (LVOTDiamIndex and LADIndex) to the systemic circulation. These combinations imply large caliber arteries or effective flow-mediated vasodilation to accept large blood volumes from the heart without raising blood pressure. Some women do not accomplish this. The tree-based analyses of the 53 women without echocardiograms or HVR tests show high blood pressure among some women.

The values of the traits involved in natural selection, correlating with or influencing lifetime reproductive success in this sample, lie close to or within the usual sea-level range, such as unelevated hemoglobin concentration and higher oxygen saturation. This is consistent with the hypothesis "in which …directional selection… offsets environmentally induced changes." (6 page 4125).

Regarding genetic selection, we conducted hypothesis-free GWAS and hypothesis-driven candidate gene analyses. Genome-wide analyses did not detect genome-wide significant associations with the candidate variables influencing reproductive success; none showed selection signals, similar to a recent study of new Tibetan mothers and neonates at high altitudes (37). This report of a genetic association with oxygen saturation remains to be replicated, as do other reports (39–42, 58). Candidate gene analyses replicated previous reports of an association with hemoglobin concentration. This study identified a set of SNP sites just below the cutoff for genome-wide significance whose most frequent alleles increased saturation by 2.8%. This result supports our earlier report that ethnic Tibetan women with higher estimated oxygen saturation genotypes had higher reproductive success (36). Furthermore, we reported a genome-wide significant association of *EPAS1* with low deoxygenated hemoglobin (Hemoglobin concentration × (100-percent of oxygen saturation)/100) and polygenes lowering deoxygenated hemoglobin (27). Selection for modal hemoglobin concentration and against deoxygenated hemoglobin likely reinforces selection for higher oxygen saturation and may represent another instance of the pleiotropic effects of *EPAS1* (59).

This study design combines retrospective reproductive history and current physiology and reasons that traits measured after the reproductive period inform about maternal biology during the childbearing years (for example, maintaining a relatively high percent of oxygen saturation or an optimal hemoglobin concentration). However, traits measured after reproduction may also reflect selective survival, biological aging, social and cultural factors, or the costs of reproduction. We consider these potential confounders next.

Selective Survival. Fifty-six women in the original sample died between 2012 and 2019. Survivors were 11 y younger and had a two percent higher oxygen saturation (fingertip measurement). Hemoglobin concentration measured in 2012 did not differ between survivors and nonsurvivors to 2019 (*SI Appendix*, Table S4). The 45 women who had moved away from the study area did not differ in hemoglobin concentration or oxygen saturation from the remaining residents. Selective survival or retention related to these oxygen delivery traits seems unlikely to have influenced the distribution of these traits in the present sample. Combined with evidence that the larger sample from which this sample was drawn did not have ascertainment bias (16), and the close demographic and biological resemblance in the 2012 and 2019 samples (*SI Appendix*, Table S5) we infer that the analyzed sample is representative of the population.

Age, Period, and Cohort Effects. A woman's current phenotype or place in the range of variation may or may not correspond to that during her childbearing years. We do not have that information for most traits. We have shown stable hemoglobin concentrations from 2012 to 2019, apart from a 0.5 gm/dL increase if menopause occurred in that interval. The average percent of oxygen saturation (fingertip measurement) decreased by less than one percent over 7 y (60). For traits assessed only for this study, left heart anatomy measures correlated weakly (LVOTDiamIndex) or moderately (LADIndex) and directly with age (*SI Appendix*, Fig. S3). Right heart function measured as RVOTvti did not correlate with age. Thus, we infer that biological aging had minor effects on some current phenotypes.

Women of different ages may also differ due to events in particular years (period effects) or having different characteristics (cohort effects). Disentangling aging, social, and cultural changes is notoriously difficult (61) without lifelong, longitudinal data. Contraception first became available in Upper Mustang during the early 1990s, and its use became more common in the late 1990s. Just 18% of those 60 and older had used contraception compared with 67% of the younger women. The older women began using contraception after a median of six pregnancies, and younger women began doing so after a median of four pregnancies (33). The exploratory data analysis excluded residential altitude and education as potential predictors of lifetime reproductive success. (Only six percent of the sample had received formal education, generally just a few years of primary school; the number of pregnancies or livebirths did not vary with education.) Our analyses considered age, period, and cohort by including age and contraception use in the final set of potential predictors and, in effect, controlled for them.

Costs of Reproduction. Adjustments to pregnancy may permanently alter a woman's physiology, accumulate over her pregnancies (62), and introduce uncertainty as to whether the current phenotype supported or resulted from reproduction. Two large studies of more than 43,000 white, black, Hispanic, and Asian US women with an average age of 62 reported that left ventricular mass and volume increased with each livebirth, particularly among women with five or more livebirths yet remained within typical healthy values (63–65). Because the Tibetan women in this sample averaged 62 y of age and had approximately five livebirths, the direct association of LVOTDiamIndex with the number of livebirths (Fig. 3, Table 2, and *SI Appendix*, Fig. S4) could arise in part from pregnancy-induced cardiac remodeling (66).

The subset of 53 women who did not have echocardiograms or HVR tests may show marked reproductive costs reflected by the high diastolic pressures among those with the most pregnancies and livebirths. Diastolic blood pressure generally drops during pregnancy, and women with diastolic hypertension before or

during pregnancy have poor pregnancy outcomes (67–70). Therefore, we suggest these women likely did not have diastolic hypertension during their numerous pregnancies.

Limitations and Strengths

This study focused on the primary candidate physiological systems for adaptations to high-altitude hypoxia: respiratory, hematological, and cardiovascular systems comprising the distinctive Tibetan biology. Future work could address whether the expanded plasma volume reported for male Tibetans (52) occurs among women and the consequences of further expansion during pregnancy (71). Pairing measurements of cardiopulmonary with peripheral vascular traits could be informative, as would serum traits reflecting hemoglobin synthesis and iron homeostasis (e.g., erythropoietin or hepcidin). We do not have serum measurements of relevant biomarkers because community engagement before mounting the fieldwork revealed that the women emphatically did not want to provide blood samples. We respected that wish.

The modest sample size, by GWAS standards, afforded statistical power sufficient to detect only large phenotypic effects. Simultaneously, the large sample by physiology standards gave necessary statistical power to identify traits relevant to reproductive success. Balancing the need for careful measures of physiology with the need for sufficient sample sizes for GWAS will continue to challenge field studies.

Strengths include managing these limitations using several statistical approaches to increase confidence in our findings. Multiple analyses converged on a few outcome variables and increased our confidence in their associations with lifetime reproductive success, although we cannot infer causality. Nonlinear transformation of age, BMI, and hemoglobin concentration reflected the data and biologically realistic relationships. Tree-based analyses of these complex data provided flexible modeling with minimal assumptions. Cross-validation analyses confirmed the robustness of these models and the likelihood of similar performance in a replication sample. A follow-up study with a larger sample size is desirable. Good to excellent intraclass correlations and small differences between test–retest measurement of the physiological traits reflect our effort to reduce measurement noise (*SI Appendix*, Table S2C). Formally considering sociocultural factors resulting in prolonged exposure to the possibility of pregnancy enabled the detection of influential oxygen delivery traits. The large sample of women who had completed their families enabled assessing lifetime reproductive success in the context of their social and physical environments, further strengthening this study.

The two measurements of the percent of oxygen saturation of hemoglobin strengthened the study. Fingertip measurements obtained by transmittance plethysmography enabled longitudinal analysis of variables collected by reflectance plethysmography of the fingertip. Forehead measurements obtained by reflectance plethysmography correlated with reproductive success and had genomic associations (16, 36).

Linking multiple oxygen delivery traits with reproductive success and genomes in a sample exposed to lifelong hypoxic stress and the added physiologic stress of pregnancy advances the understanding of human evolution and adaptation. Lifetime reproductive success is a complex trait. Including sociocultural information, linear and nonlinear analyses provide a fresh approach to studying this essential outcome measure of natural selection. These findings show that overlapping physiological homeostatic systems support a favorable internal environment for reproductive success in the extreme external environment of high-altitude hypoxia.

Summary

In summary, we detected traits relating to high lifetime reproductive success in a sample of more than 400 ethnic Tibetan women, lifetime residents at ≥3,500 m. Stabilizing selection favoring a modal hemoglobin concentration and directional selection favoring high oxygen saturation of hemoglobin together increased oxygen content and links to high lifetime reproductive success. Combining optimal levels of hemoglobin concentration near the sample mode with oxygen saturation ranging up to 99% raised oxygenated hemoglobin concentration without increasing blood viscosity and correlated with more livebirths. Indicators of blood flow into the lung and out of the heart related nonlinearly to high lifetime reproductive success. Two beneficial combinations defend convective oxygen transport from the heart into the systemic circulation. High flow of deoxygenated blood to the lungs and oxygenated blood into the systemic circulation benefited reproductive success as did relatively low flow to the lungs combined with oxygen saturation. These findings suggest multiple ways to maintain homeostasis and achieve many livebirths.

Linkages among hematological and cardiovascular phenotypes, genomics, and reproductive success among ethnic Tibetan women residing at 3,500 m or higher in Nepal demonstrate natural selection favoring oxygen delivery under the severe stress of high-altitude hypoxia.

Data, Materials, and Software Availability. Anonymized csv file data have been deposited in OSF (DOI: https://doi.org/10.17605/OSF.IO/JUYCS) (35).

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Author affiliations: [a]Statistics Department, George Mason University, Fairfax, VA 22030; [b]Anthropology Department, Dartmouth College, Hanover, NH 03755; [c]Human Genetics Department, University of Chicago, Chicago, IL 60637; [d]Division of Pulmonary, Critical Care, Sleep Medicine and Physiology, Department of Medicine, University of California San Diego, La Jolla, CA 92023; [e]Oxford University Clinical Research Unit-Nepal, Kathmandu 44600, Nepal; [f]School of Medicine, Division of Pulmonary, Critical Care and Sleep Medicine, Case Western Reserve University, Cleveland, OH 44106; [g]Harrington Heart and Vascular Institute, University Hospitals Cleveland Medical Center, Departments of Medicine and Physiology and Biophysics, School of Medicine, Case Western Reserve University, Cleveland, OH 44106; and [h]Anthropology Department, Case Western Reserve University, Cleveland, OH 44106

Author contributions: J.S., S.R.C., A.D.R., T.S.S., F.L.P., B.B., and C.M.B. designed research; S.R.C., J.J.Y., E.A.M., B.B., and C.M.B. performed research; S.Y., J.S., S.R.C., A.D.R., D.W., J.J.Y., E.A.M., T.S.S., F.L.P., B.D.H., and C.M.B. analyzed data; T.S.S. and F.L.P. designed hypoxic ventilatory response/hypoxic heart rate response protocol and interpretation; B.B. supported data collection in the field; and J.S., S.R.C., A.D.R., K.P.S., and C.M.B. wrote the paper.

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