UNIVERSITY OF CALIFORNIA

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Challenges in nature: individual vulnerability and its consequences for fitness

A dissertation submitted in partial satisfaction of the requirements

for the degree Doctor of Philosophy in Biology

by

Gabriela Medeiros de Pinho

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ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Biology

University of California, Los Angeles, 2021

Professor Daniel T. Blumstein, Co-Chair

Professor Robert K. Wayne, Co-Chair

In nature, species are constantly challenged by biotic and abiotic factors. In order to thrive, individuals need mechanisms that regulate their responses according to the experienced conditions. I have studied aspects of regulatory responses to gain insights into how individuals are affected by environmental challenges. All chapters capitalized on long-term data collected at individual level. Chapter 1 introduce and summarize all chapters. Chapter 2 explores how the social environment affects maternal stress levels (glucocorticoid hormones) in a flexibly social mammal. I found that agonistic behavior challenges mothers, while affiliative social bonds can lower stress levels in a context-specific manner. Pups from highly stressed mothers are less likely to survive unless they come from small litters. Chapter 3 uses the estimated biological age (or epigenetic age) to investigate how hibernation, an adaptation to survive highly challenging periods, may affect individual aging patterns. I observed a much slower biological aging during hibernation, which suggests that biological processes involved in hibernation suppress aging.

While chapters 2 and 3 evaluate the influence of the surrounding environment on individual fitness, chapter 4 focus on how inbreeding, an intrinsic individual trait, is associated with a higher aging speed. Indeed, inbred individuals appear to exhibit a higher age acceleration, but only at older chronological ages, which may be explained by a higher vulnerability of inbred individuals to the cumulative effects of environmental and physiological stressors. Thus, individual traits influence the chances of successful responses to challenges. And, while it is generally assumed that challenges affect negatively individual fitness, chapter 2 and 3 uncover strategies that maximize individual fitness under duress, which highlights the role of selective pressure on plastic phenotypes. Another contribution from this dissertation is the use of physiological and molecular regulatory responses to estimate potential costs to individual fitness. Both glucocorticoid hormone levels and age acceleration are indirect measures of individual health and wellbeing that can be used to monitor wild populations. In particular, chapters 3 and 4 are among the first studies to use individual aging acceleration as a proxy of individual fitness in wildlife, a method that is becoming more accessible to wildlife managers.

The dissertation of Gabriela Medeiros de Pinho is approved.

Kirk Lohmueller

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2021

DEDICATION PAGE

I dedicate this dissertation to all the Brazilian scientists struggling due to a deeply anti-science and anti-environment government.

My dedication goes particularly to female scientists: the world many times makes us feel small, as if science is not a career option for us, but we certainly have the potential to excel at it.

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ACKNOWLEDGMENTS

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VITA

1. EDUCATION

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2010. IDEA WILD (equipment purchases, $1,444.94), and Uatumã Biological Reserve (logistical support and consumables).

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2013. Lecture: "Bioinformatics" at the IV Workshop of Genetics, Conservation and Evolutionary Biology, INPA, Brazil.

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2011. Lecture: Genetic Committee Report of the Tapir Specialist Group (IUCN). Fifth International Tapir Symposium, Kuala Lumpur, Malaysia.

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Larison*****, B.; **Pinho*, G.M.**; Haghani, A.; Zoller, J.A.; Li, C.Z.; Finno, C.J.; Farrell, C.; Kaelin, C.B.; Barsh, G.S.; Wooding, B.; Robeck, T.R.; Maddox, D.; Pellegrini, M. & Horvath, S. 2021. Epigenetic models predict age and aging in plains zebras and other equids. *BioRxiv* (doi.org/10.1101/2021.03.29.437607). *Accepted in Communications Biology* ***Both authors contributed equally**

Chock, R.Y.; Clucas, B.; Peterson, E.K.; Blackwell, B.F.; Blumstein, D.T.; Church, K.; Fernández Juricic, E.; Francescoli, G.; Greggor, A.L.; Kemp, P.; **Pinho, G.M.**; Sanzenbacher, P.M.; Schulte, B.A. & Toni, P. 2021. Evaluating potential effects of solar power facilities on wildlife from an animal behavior perspective. *Conservation Science and Practice*, 3: e319.

Heissenberger*****, S.; **Pinho*, G.M**.; Martin, J.G.A. & Blumstein, D.T. 2020. Age and location influence the costs of compensatory and accelerated growth in a hibernating mammal. *Behavioral Ecology*, araa013 ***These authors contributed equally to this work**

Pinho, G.M.; Ross, X.O.; Reese, A. & Blumstein, D.T. 2019. Correlates of maternal glucocorticoid levels in a socially flexible rodent. *Hormones and Behavior*, 116: 104577.

Lima, D.O.; **Pinho, G.M.** & Fernandez, F.A.S. 2015. Spatial patterns of the semi-aquatic rodent Nectomys squamipes in Atlantic forest streams. *Journal of Natural History*, 50(7-8): 1-15.

Pinho, G.M.; Fonseca, R.; Farias, I.P. 2014. An opportunity for testing multiple paternity in a wild Jaguar (*Panthera onca*). *Biota Neotropica*, 14(3): e20140055.

Pinho, G.M.; Gonçalves da Silva, A.; Hrbek, T.; Venticinque, E.M. & Farias, I.P. 2014. Kinship and social behavior of lowland tapirs (Tapirus terrestris) in a central Amazon landscape. PLoS ONE, 9(3): e92507.

Pinho, G.M.; Lima, D.O.; Costa, P.N. & Fernandez, F.A.S. 2009. Epicrates cenchria (Brazilian Rainbow Boa) diet. Herpetological Review, 40 (3): 354-355.

CHAPTER 1

General Introduction

In nature, species are constantly challenged by biotic and abiotic factors. Such challenges may be intrinsic to an organism, or may come from the surrounding environment. Challenges can also be periodic, as seen in seasonal environments, or continuous, observed in individual aging or the increasingly cooler climate in North America in the late Pliocene and Quaternary [1,2]. While the time scale of challenges can widely vary, my research has focused on challenges that occur within an individual's lifetime.

Individual-level studies provide detailed insight into population and species dynamics [3,4], which may help explain patterns such as species distribution, longevity or conservation status. When environmental changes occur throughout life, genotypes that allow for plasticity in phenotypic expression may be favored [5–7]. In order to thrive, individuals need mechanisms that regulate their responses according to the experienced conditions. I have studied aspects of regulatory responses to gain insights into how individuals are affected by environmental challenges.

Briefly, chapter 2 explores how maternal stress levels (measured through glucocorticoid hormones) are associated with different aspects of the social environment, and tests whether maternal stress have consequences for offspring fitness. In chapters 3 and 4, I use epigenetic markers to estimate individual biological age, an useful measure of individual well-being when compared to their chronological age: an individual biologically older than their chronological age is likely under challenging conditions and may have a shorter longevity. Chapter 3 uses the estimated biological age (or epigenetic age) to investigate how hibernation, an adaptation to

survive highly challenging periods of food scarcity and low temperatures, may affect individual aging patterns. While chapters 2 and 3 evaluate the influence of the surrounding environment on individual fitness, chapter 4 focus on how intrinsic individual traits may make individuals more sensitive to challenges. More specifically, chapter 4 asks whether inbred individuals age at faster rates, which would suggest a higher vulnerability to challenging conditions.

All chapters capitalized on long-term data collected at individual level. Chapters 2 and 3 were developed in a wild population of yellow-bellied marmots (*Marmota flaviventer*) in Colorado (US) and the fourth chapter in a captive population of plains zebras (*Equus quagga*), kept in a semi-wild state in Western Cape (South Africa). The main results of each chapter are explained in further detail below.

The social environment & Maternal stress levels (published in Hormones & Behavior in 2019)

The social environment—defined as the set of all interactions between an individual and its conspecifics—may challenge individuals but may also promote well-being and/or modify their ability to cope with adversity [8–10]. The consequences of the social environment on individuals are often studied by manipulating group composition or structure in captive, domesticated species [11]. Such studies may not be applicable to wild populations since artificially selected lineages may have altered social phenotypes [12–14], and behavioral and endocrine responses have evolved in contexts characterized by multiple stressors. From the *in situ* studies, most have been conducted on highly social mammals, while little is known about the effects of the social environment on individuals of less social species [15–17]. Another limitation of the available literature on social stress is the sex bias: physiological and behavioral responses to social stress have been mostly

studied in males [18–21]. Yet, sex differences in the perception of the social environment are expected due to sex-specific social roles [18,19,22,23].

In this context, I became interested in studying the females from a wild population of a flexibly social mammal. Wild female marmots can live alone or form kin groups [24]. While large group sizes are characterized by more agonistic interactions and reproductive suppression [24], group living may also have advantages since solitary females occupy suboptimal environments [25] and exhibit more vigilant behavior than females in groups [26]. Group living may be particularly important to mothers and their offspring [27] because pups are extremely vulnerable to predators [28]. In this chapter, I evaluated how both affiliative and agonistic aspects of the social environment are associated with maternal stress in yellow-bellied marmots.

Exposure to stressors activates pathways in the hypothalamic-pituitary-adrenal axis that culminate in the production of glucocorticoid (GC) hormones [11,29]. Due to GCs role in coping responses, I used fecal glucocorticoid metabolites (FGM) as a proxy of stress levels, as it is often done in ecological studies [30–32]. Assuming that GC levels vary according to the magnitude of environmental challenges, and that individuals facing greater challenges have lower fitness, the cort-fitness hypothesis predicts that baseline GC levels will be negatively associated with reproductive success [33,34]. However, maternal responses to sub-optimal conditions may be adaptive when it reliably prepares offspring for their future environments [31], which could potentially increase reproductive success. Female marmot stress has been associated with litter size, offspring sex ratio, dispersal behavior and personality [35,36], but it is not clear whether offspring fitness is affected. This chapter further investigates female reproductive success under sub-optimal conditions through measures of offspring survival.

To study the association between the social environment and maternal stress (FGM levels), as well as their impact on offspring survival, I analyzed 10 years (2005-2012, 2014, 2015) of behavioral and trapping data collected from free-living yellow-bellied marmots in Colorado (USA). The data was analyzed with a top-down model selection [37] on linear mixed effects models (LMMs) for the dependent variable maternal FGM concentrations and on generalized LMMs with binomial distribution for the dependent variable pup survival (measured as summer, winter and annual survival). All models had female identity and year as random effects. Fixed effects included: group size, maternal age, litter size, predator index (classified as high or low), social attributes (for the maternal stress models), maternal FGM levels after pup emergence from burrows (for the pup survival models) and interactions between the variables.

To test how the social context is associated with maternal FGM levels, we estimated 10 social attributes from two separate social networks, one for affiliative interactions and another for agonistic interactions. These social attributes capture various dimensions of marmot social behavior [28,38] and may be correlated, so we used a principal component analysis to reduce them into uncorrelated measures. We fitted separate LMMs for affiliative and agonistic interactions. The female-agonistic dataset included 32 unique females that represented 42 breeding events over 8 years. After controlling for FGM seasonal and daily variation, maternal FGM level was positively associated with PC1 (interpreted as initiated aggression), PC2 (aggression received), and litter size (p = 0.014, 0.007 and 0.004, respectively).

The female-affiliative social network analysis included 51 unique females and represented 87 breeding events over 10 years. After controlling for FGM seasonal and daily variation, litter size was positively associated with maternal FGM (p = 0.001), and the interaction between PC3

(group cohesiveness) and predator index significantly explained variation in maternal FGM levels ($p = 0.020$). In low predation pressure environments, females with high group cohesion had lower FGMs than females with low group cohesion. In contrast, there was no apparent association between group cohesion and FGMs in high predation environments.

Offspring survival was measured for 91 litters from 59 females across 10 years. In the final models, maternal FGM after emergence was negatively associated with measurements of winter and annual survival ($p < 0.001$ and $= 0.001$, respectively). For the model fitted to summer survival data, we found a negative effect of predator index ($p = 0.031$), where pups were more likely to survive in environments with low predator pressure, and a significant effect of the interaction between maternal FGM after emergence and litter size ($p = 0.011$). During the active season, large litters from females with low FGM levels had higher chance of survival than smaller litters, but this pattern was inverted if females had high FGM levels.

We conclude that both affiliative and agonistic interactions may influence wild reproductive females from this socially flexible mammal, where agonistic behavior clearly challenges mothers. Interestingly, maternal stress can be buffered by affiliative social bonds, however it seem to be context-specific. Under low predation pressure, group cohesiveness was weakly associated with reduced maternal FGM, but may potentially act as a social buffer by reducing effects of external stressors [9,39]. In more social species, positive outcomes of social bonds on female fitness are more pronounced [40–44]. Since advantages of sociality are context dependent [45,46], identifying which conditions differ among less and more social species may reveal the conditions that have selected for more complex social systems in ancestral species.

Although female marmots with high FGM levels wean larger litters [27], their pups are significantly less likely to survive. Mothers with high FGM levels produced offspring with lower winter and annual survival. In addition to having a low annual reproductive success, stressed mothers have a low overwinter survival themselves [32], which supports the cort-fitness hypothesis [33,34]. The summer, however, shows an interesting scenario: when mothers have high FGM levels, individuals born into small litters have higher survival likelihood than individuals born into larger litters. Thus, mothers could potentially increase offspring survival likelihood by programming litter sizes according to environmental conditions.

Yellow-bellied marmots are obligate hibernators and most of the social interactions happen during their active season. Even though the size of social groups may influence which individuals share the same hibernaculum, the social interactions *per se* could be considered seasonally strict. In the next chapter, I compare the effects of the active and hibernation seasons on individual fitness by studying their aging patterns.

Hibernation & Aging patterns (under review in *Nature Ecology & Evolution*)

Aging is a poorly understood natural phenomenon, characterized by an age-progressive decline in intrinsic physiological function [47,48]. The high variation in disease and functional impairment risk among same-age individuals shows that biological age is uncoupled from chronological age [49–51]. Currently, DNA methylation based age estimators are arguably the most accurate molecular estimators of age [49,52–55] and have been used in human and wildlife aging studies. Age-adjusted estimates of DNAm age (or epigenetic age acceleration) are associated with a host of age-related conditions and stress factors, such as cumulative lifetime stress [56], all-cause mortality [57–61], and

age-related conditions/diseases [59,62–64]. These associations suggest that epigenetic age is an indicator of biological age [51,65].

Measures of epigenetic aging rates are associated with longevity at the individual level as well as across mammalian species [66,67], where long-lived species age more slowly at an epigenetic level [65,68–71]. Some species have longer lifespans than expected based on their body size [72,73], and a common characteristic among them is the capacity to engage in torpor [74,75]—a response to adverse conditions characterized by a dramatic decrease in metabolic rate [76–84]. Some of the physiological stresses from the transition between deep torpor and euthermy are similar to the ones experienced by the aging body (e.g., oxidative stress), and may promote responses in cellular signaling pathways that are essential for both longevity and torpor survival [74,84]. Considering that hibernation consists in cycles between torpor bouts and short periods of euthermy [79,85], this widespread adaptation to harsh and highly seasonal environments [79,86] may in fact lengthen lifespan through aging suppression.

Thus, I used epigenetic aging models to investigate whether hibernation slows biological aging in yellow-bellied marmots. Yellow-bellied marmots are a great model to first approach this hypothesis because of their long hibernation periods (7-8 months per year [87]) and the available information about female chronological age. Males have not been studied because most disperse and cannot be further monitored.

The DNAm profiling from 149 blood samples was performed with the custom Illumina chip HorvathMammalMethylChip40 [88], from which 31,388 probes mapped uniquely in the yellow-bellied marmot assembly. These samples were collected from 73 females with chronological ages varying from 0.01 to 12.04 years. This methylation level data was analyzed with two model

approaches: the epigenetic clock [52,53] (EC) and the epigenetic pacemaker [89–92] (EPM). The EPM models each individual CpG site as a linear function of an underlying epigenetic state of an individual. The epigenetic state is calculated through iterations implemented in a fast conditional expectation maximization algorithm [93], and is an estimate of age that, given the methylation rates and initial methylation levels for each site, minimizes the differences between known and estimated methylation levels in a specific sample for all sites included in the model. This epigenetic state changes with time in a non-linear fashion, and can be used to identify periods with variable rates of methylation changes throughout lifespan. All CpG sites with a higher than 0.7 absolute Pearson correlation coefficients between chronological age and methylation levels per site [91,92] were used in this model (EPM v.0.0.3[92]), resulting in 309 sites. A 10-fold cross validation was used to estimate epigenetic states. Under the EC, a linear correlation with chronological age is determined by attempting to fit a single coefficient to each CpG site. We fitted a generalized linear model with elastic-net penalization [94] to the chronological-age and methylation level data sets with a 10-fold cross validation using the glmnet v.4.0-2 package in R [95]. This model performs an automatic selection of CpG sites for age prediction, which resulted in 360 sites. Because the EC uses a penalized regression, it is less likely to capture seasonal effects than the EPM.

The epigenetic aging models developed with the EC and the EPM approaches were both highly accurate, with high correlations between epigenetic and chronological age (r = 0.98 and 0.92, respectively). The EPM identified variable rates of methylation changes throughout the marmot lifespan, with a rapid change in epigenetic age until marmots reached 2-years old, their age of sexual maturity [96,97], and a more linear and slower epigenetic aging after reaching adulthood. The

pattern observed in marmot epigenetic aging is consistent with the notion that methylation remodeling is associated with key physiological milestones [71]. This logarithmic relationship between methylation change rate and chronological age may be a shared trait in mammals, as has been described for multiple human tissues [52,89,91] and some species, including dogs [71] and mice [98].

To test the hypothesis that aging is slower during hibernation, the epigenetic age estimated by each epigenetic model was used as dependent variable in a Generalized Additive Mixed Model (GAMM). For both GAMMs, fixed effects included a cubic spline function for chronological age, and a cyclic cubic spline function for day of year. We also tested for the interaction between these two variables (using tensor product interaction with a cubic spline). Individual identity was added as random effect. GAMMs were fitted and checked using the mgcv R package v.1.8 [99]. The GAMM fitted to the EC epigenetic age explained 96.6% of the variation (Adj. R^2). The age spline was significant ($F = 1225.76$, $p < 0.0001$) and the cyclic spline for day of year was not ($p = 0.78$). The tensor interaction smooths was also not significant ($p = 0.11$).

The GAMM fitted to the EPM epigenetic age explained 95.6% of the variation. Both smooth terms significantly influenced marmot epigenetic state ($p < 0.005$), but the interaction between them was not significant ($p = 0.44$). As it can be observed from Figure 1, this GAMM suggests that biological aging slows during hibernation. There is a clear delay in epigenetic-state changes during hibernation. However, this delay is not observed in individuals in their first and second years of life (Figure 1A), despite the fact that we observed a non-significant interaction between chronological age and day of year. A weaker effect of slowed aging during hibernation in younger animals could be explained by their later hibernation start date [100–102] in addition to an overall higher metabolic rate during hibernation [87].

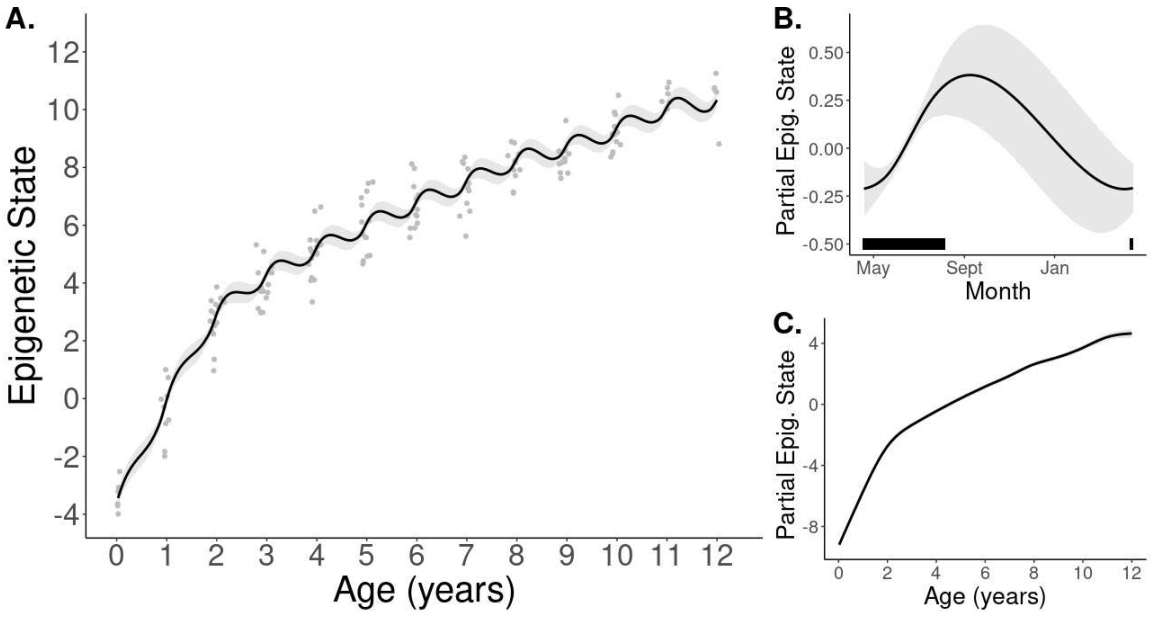

Figure 1. Visualization of the generalized additive mixed model with epigenetic states generated from the epigenetic pacemaker model using CpG sites highly correlated to chronological age (absolute $r > 0.7$). A) Changes in the epigenetic state (or epigenetic age) as individuals age. Points are actual data, while lines are the predictions from the model. B) Predictions generated with the partial effect of date of year (cyclic cubic smoother spline) on epigenetic state. The black horizontal bar represents when samples were collected and most of the marmot active season. C) Predictions generated with the partial effect of chronological age (cubic smoother spline) on epigenetic state. Buffers illustrate the 95% confidence intervals.

As expected, the EPM was better equipped to detect the seasonal pattern than the EC. To further increase confidence in our GAMMs using EPM epigenetic ages and test the impact of the limitations of our sampling in our results, we performed simulations to estimate the type 1 error and our power to detect the seasonal effect. The limitations of our sampling can be observed in Figure 1: blood samples could only be collected during the active season, instead of throughout the year. In this regard, we simulated two different traits: (1) a trait that increases linearly with age independently of the season; and (2) a trait that increases during the summer but not during the winter. The daily rate of increase for the first and second traits, and the among-individual and residual variance were set to similar ranges of the observed EPM data. The length of the active

season, day of the year, chronological age, and number of samples, were set using field data. We generated 1000 data sets for each trait and fit the GAMM. The proportion of simulations on trait 1 (no seasonal effect) that were significant for day of the year (cyclic cubic spline) indicated our type 1 error. The proportion of simulations on trait 2 (seasonal effect) that were significant was an indication of the power to detect this effect. As results, 76.5% of the simulations found a significant effect of seasons on the data simulated with a seasonal effect, indicating high power to detect a seasonal effect. From the 1000 GAMMs fitted to data simulated with a constant linear age effect, 7.3% had a significant season effect, which is a slightly higher type 1 error than expected (5%). Based on this result, we calculated a new significance critical value by estimating the 0.05 quantile of the p-value distribution from a null model. The 0.05 quantile was 0.0344, which is larger than in our data (p=0.003). From this, we concluded that our results were not driven by our sampling nor by statistical artifacts.

In sum, our main finding was the little to no change in epigenetic aging during hibernation. While hibernation may increase longevity by protecting individuals from predators and diseases [75], we suggest that the biological processes involved in hibernation are important contributors to the long lifespan seen in hibernators. Longevity is a key component of individual fitness, thus a better understanding about the interplay between aging and hibernation/torpor have multiple potential applications.

Until this point I have learned how individuals are affected by aspects of their surrounding environment. My last chapter moves the focus of my study to the individual characteristics that may affect individual vulnerability to challenges. I study whether inbreeding may accelerate individual aging.

Inbreeding & Aging patterns (accepted in *Communications Biology*)

Once an epigenetic clock (EC) model has been developed for a species, it can be applied on samples from individuals without age information in order to accurately predict their chronological age. Thus, ECs are a promising development for the aging of wild animals and have the potential to make significant contributions to wildlife conservation [103,104]. Information about individual chronological age in wild populations is key to estimate demographics trends and effectively manage threatened species [105]. A critical limitation to developing epigenetic aging models for wildlife is that these models need to be trained on samples from individuals of known age, therefore populations of non-model organisms with known-age individuals are of extreme importance [104,106]. In this regard, the first motivation for my last chapter was to develop epigenetic aging models for the genus *Equus*—comprised by a closely related group of 6 extant species, 5 of which range from near threatened to critically endangered [107,108]. To achieve this goal, we studied a captive population of plains zebras (*E. quagga*) maintained in a semi-wild state and tested whether an epigenetic clock trained with 76 blood samples from this population of known-age zebras can be used to estimate chronological age in 3 sister species: the non-threatened domestic horses (*E. caballus,* 188 samples), the endangered Grevy's zebras (*E. grevyi,* 5 blood samples), and the critically endangered Somali asses (*E. africanus somaliensis,* 7 blood samples).

All DNA methylation data were generated using a custom Illumina methylation array (HorvathMammalMethylChip40 [88]). The array contains 36 thousand probes, 31,836 of which mapped uniquely to the horse genome. We developed ECs for plains zebras by fitting a generalized linear model with elastic-net penalization using leave-one-out cross-validation in

glmnet v.4.0-2 in R [95,109]. To improve EC fit we square root transformed chronological age prior to fitting the models. As results, the EC for plains zebras had a high accuracy to estimate chronological age within and across species. The median absolute error of chronological age prediction was 0.56 years in plains zebras, 1.82 years in horses, 1.08 years in Grevy's zebras, and 1.15 years in Somali wild asses. Even though we had limited sample sizes for some species, the high accuracy of our EC model was expected because caballine and non-caballine equids are somewhat closely related (4-4.5 MYA [110,111]). Non-caballine species of equids are more closely related to plains zebras (1.28-1.75 MYA) than domestic horses, which may explain the lower errors for age estimation in Grevy's zebras and Somali asses and suggests that this model can be successfully used in the other species of the genus.

The plains zebras captive population studied here was founded with 19 wild zebras and has undergone artificial selection to reproduce the phenotype of the extinct quagga subspecies. Because this population has a complex pedigree, and there is an general expectation that inbreed individuals are more sensitive to environmental stress than outbred individuals [112], we asked whether inbred zebras age faster than non-inbred zebras. To answer this question, we developed an epigenetic pacemaker (EPM) model for the plains zebras. We chose to approach this question with the EPM model because it has previously been useful for investigating how environmental and life-history factors influence aging [91]. We only inputted sites in which methylation levels were highly correlated with individual chronological age (absolute Pearson correlation higher than 0.75) and epigenetic states were estimated using a leave-one-out cross validation with EpigeneticPacemaker 0.0.3 [92] in Python 3.7.4 [113]. Epigenetic state was strongly correlated with chronological age (r = 0.97) and showed a logarithmic epigenetic aging across zebra lifespan. As

has been found in humans and other species (Chapter 3 and Snir et al., 2019), young zebras undergo faster epigenetic changes than adult zebras.

To access individual inbreeding level, we used genotypes derived from two sources: RADseq data (42 individuals) and whole genome low coverage sequence data (28 individuals). For the second data set, we inputted the genotypes for the same RADseq sites from the first data set using GLIMPSE [114]. We used 313,645 autosomal SNPs to estimate the inbreeding coefficient F and to detect runs of homozygosity (ROH) in PLINK [115]. F was estimated using methods of moments, and ROH with the default settings with the exception that we increased the stringency to require detection of runs in 150 rather than 100 bp windows, and final runs had to be at least 1.5 MB long. We converted ROH to the inbreeding coefficient F_{ROH} [116] by dividing the total length of ROH for each individual by the length of the genome over which we screened for ROH [117].

After estimating inbreeding levels and developing the EPM model for plains zebras, we used multiple linear regressions to assess whether inbreeding is associated with age acceleration (epigenetic age – chronological age). We fitted two linear models in lmtest v.0.9-38 [118] package in R v.4.1.0 [109], running separate analyses for the two estimates of inbreeding (F and F_{ROH}). EPM age acceleration was the dependent variable and the independent variables were sex, chronological age, inbreeding, and the interaction between chronological age and inbreeding. The EPM age acceleration not associated with sex and was associated with an interaction between chronological age and both F and F_{ROH} ($p= 0.02$ and 0.004, respectively), indicating that the impacts of inbreeding on biological age increase with chronological age (Figure 2).

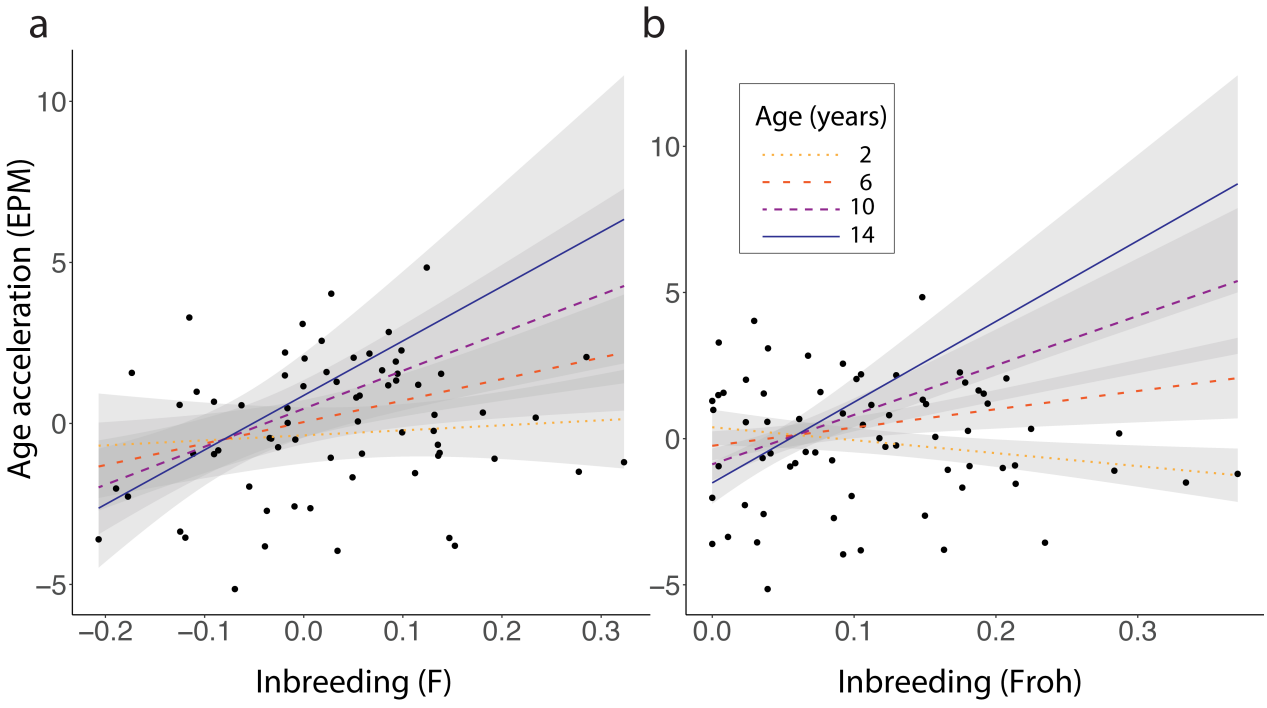

Figure 2. Relationship between EPM age acceleration and inbreeding in plains zebras. Lines represent the predicted age acceleration for individuals with different chronological ages. Gray areas show 95% confidence intervals. Black dots represent the individual plains zebra data.

In sum, this chapter is divided in two main parts. First, we generated valuable epigenetic aging models that can be used to predict chronological age in *Equus*. And second, we measured the effects of inbreeding on epigenetic aging, wherein inbred individuals appear to exhibit a higher age acceleration at older chronological ages. In general, a higher variation in age acceleration is expected in older individuals because they have been exposed to more environmental and physiological stresses than young individuals [52,119]. This trend has been observed in humans and other species (Chapter 3 and in Snir et al., 2019), including plains zebras. The fact that the inbreeding effect on age acceleration could be detected only at older ages may be explained by the cumulative effects of environmental and physiological challenges, where inbred individuals

are more strongly affected than outbred ones. Thus, our results support the idea that inbreeding may increase sensitiveness to challenges experienced throughout the life of a semi-wild animal.

General Conclusions

In this dissertation, I gained insights into how individuals are affected by social, abiotic and intrinsic challenges. While it is generally assumed that challenges affect negatively individual fitness, chapter 2 and 3 uncover strategies that maximize individual fitness under duress, which highlights the role of selective pressures on plastic phenotypes. Even though pup survival likelihood decreases with an increase in maternal stress in chapter 2, pups have enhanced chances of survival if they come from a small litter. Another study on the same yellow-bellied marmot population [35] found that older females under stressful conditions are more likely to produce smaller litters, which may suggest that older females are able to increase offspring survival likelihood by the manipulation of litter sizes. Experienced mothers have increased reproductive success compared to inexperienced females in other species [120–129], and, although not tested in this dissertation, females that are able to lean from experience may have a higher fitness output. Chapter 3 approaches a very different type of stress, but also offers a silver lining into potential positive outcomes associated with responses to duress. Even though a hibernating species have less chances to increase their reproductive success within a year, the lengthen of their lifespan due to the physiological plasticity associated with hibernation allows adults to have more mating opportunities in other years. Thus, not all challenges have purely negative effects on individuals. However, some individual characteristics may lower the chances of successful responses to challenges. In chapter 4 I have shown that inbreeding is one of such traits because it

may make individuals more susceptible to the cumulative negative effects of challenges experienced throughout life.

Another important aspect of this dissertation is the use of physiological and molecular regulatory responses to estimate potential costs to individual fitness. Glucocorticoid hormones have been used as a proxy of individual stress levels in many ecological and psychosocial studies [30–32], and can be considered a more traditional approach. But the exploration of epigenetic markers (DNA methylation levels) to estimate biological aging is a more recent development. In fact, chapters 3 and 4 are among the first studies to use individual aging acceleration as a proxy of individual fitness in wildlife. Age acceleration is measured by comparing an individual biological age (measured through epigenetic markers) to their chronological age. An individual biologically older than their chronological age are likely under suboptimal conditions. Both glucocorticoid hormone levels and age acceleration are indirect measures of individual health and wellbeing that can be used to monitor wild populations. Since glucocorticoid levels have conflicting results in different taxa [29,130–133], I expect that epigenetic aging models will be valuable for more ecological studies and population management in wild species. Given the small number of CpG sites required, an aging project in a wild population could be done relatively inexpensively using bisulfite sequencing or pyrosequencing [134–136], and the costs of the mammalian array (used in chapters 3 and 4) are decreasing. Thus, epigenetic aging models will likely become more accessible to wildlife managers, and I expect to be using it in future endeavors.

References

1. Nikol'skii, a. a. & Rumiantsev, V. Y. Center of species diversity of Eurasian marmots (Marmota, Rodentia) in an epi-platformal orogeny area. *Dokl. Biol. Sci.* **445**, 261–264 (2012).

2. Polly, P. D., Cardini, A., Davis, E. B., Steppan, S. J. & Quaternary, T. Marmot evolution and global change in the past 10 million years. in *Evolution of rodents: advances in phylogeny, palaeontology, and functional morphology.* (eds. Cox, P. G. & Hautier, L.) 246–276 (Cambridge Univ. Press, 2015).

3. Maltby, L. Studying stress: The importance of organism-level responses. *Ecol. Appl.* **9**, 431–440 (1999).

4. Ward, T. D. *et al.* Understanding the individual to implement the ecosystem approach to fisheries management. *Conserv. Physiol.* **4**, 1–18 (2016).

5. Gabriel, W. How stress selects for reversible phenotypic plasticity. *J. Evol. Biol.* **18**, 873–883 (2005).

6. Garland, T. & Kelly, S. A. Phenotypic plasticity and experimental evolution. *J. Exp. Biol.* **209**, 2344–2361 (2006).

7. Lafuente, E., Duneau, D. & Belade, P. Genetic basis of thermal plasticity variation in Drosophila melanogaster body size. *bioRxiv* **25**, (2018).

8. Beery, A. K. & Kaufer, D. Stress, social behavior, and resilience: Insights from rodents. *Neurobiol. Stress* **1**, 116–127 (2015).

9. Cohen, S. Social relationships and health. *Am. Psychol.* **59**, 676–684 (2004).

10. Holt-Lunstad, J., Smith, T. B. & Layton, J. B. Social relationships and mortality risk: A meta-analytic review. *PLoS Med.* **7**, e1000316 (2010).

11. Cruces, J., Venero, C., Pereda-pérez, I. & Fuente, M. De. The effect of psychological stress and social isolation on neuroimmunoendocrine communication. *Curr. Pharm. Des.* **20**, 4608–4628 (2014).

12. Chalfin, L. *et al.* Mapping ecologically relevant social behaviours by gene knockout in wild mice. *Nat. Commun.* **5**, 1–10 (2014).

13. Künzl, C., Kaiser, S., Meier, E. & Sachser, N. Is a wild mammal kept and reared in captivity still a wild animal? *Horm. Behav.* **43**, 187–196 (2003).

14. Künzl, C. & Sachser, N. The behavioral endocrinology of domestication: A comparison between the domestic guinea pig (*Cavia aperea f. porcellus*) and its wild ancestor, the cavy (*Cavia aperea*). *Horm. Behav.* **35**, 28–37 (1999).

15. Ferron, J. Social behavior of the golden-mantled ground squirrel (*Spermophilus lateralis*). *Can. J. Zool.* **63**, 2529–2533 (1985).

16. Maher, C. R. Effects of relatedness on social interaction rates in a solitary marmot. *Anim. Behav.* **78**, 925–933 (2009).

17. Yoerg, S. I. Solitary is not asocial: Effects of social contact in kangaroo rats (heteromyidae: Dipodomys heermanni). *Ethology* **105**, 317–333 (1999).

18. Palanza, P., Gioiosa, L. & Parmigiani, S. Social stress in mice: Gender differences and effects of estrous cycle and social dominance. *Physiol. Behav.* **73**, 411–420 (2001).

19. Palanza, P. & Parmigiani, S. How does sex matter? Behavior, stress and animal models of neurobehavioral disorders. *Neurosci. Biobehav. Rev.* **76**, 134–143 (2017).

20. Pittet, F., Babb, J. A., Carini, L. & Nephew, B. C. Chronic social instability in adult female rats alters social behavior, maternal aggression and offspring development. *Dev. Psychobiol.* **59**, 291–302 (2017).

21. Taylor, S. E. *et al.* Biobehavioral responses to stress in females: Tend-and-befriend, not fight-or-flight. *Psychol. Rev.* **107**, 411–429 (2000).

22. Kudielka, B. M. & Kirschbaum, C. Sex differences in HPA axis responses to stress: A review. *Biol. Psychol.* **69**, 113–132 (2005).

23. Stroud, L. R., Salovey, P. & Epel, E. S. Sex differences in stress responses: Social rejection versus achievement stress. *Biol. Psychiatry* **52**, 318–327 (2002).

24. Armitage, K. B. & Schwartz, O. A. Social enhancement of fitness in yellow-bellied marmots. *Proc. Natl. Acad. Sci.* **97**, 12149–12152 (2000).

25. Armitage, K. B. & Downhower, J. F. Demography of yellow-bellied marmot populations. *Ecology* **55**, 1233–1245 (1974).

26. Mady, R. P. & Blumstein, D. T. Social security: Are socially connected individuals less vigilant? *Anim. Behav.* **134**, 79–85 (2017).

27. Blumstein, D. T., Keeley, K. N. & Smith, J. E. Fitness and hormonal correlates of social and ecological stressors of female yellow-bellied marmots. *Anim. Behav.* **112**, 1–11 (2016).

28. Wey, T. W. & Blumstein, D. T. Social attributes and associated performance measures in marmots: Bigger male bullies and weakly affiliating females have higher annual reproductive success. *Behav. Ecol. Sociobiol.* **66**, 1075–1085 (2012).

29. Sapolsky, R. M., Romero, L. M. & Munck, A. U. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr. Rev.* **21**, 55–89 (2000).

30. Sheriff, M. J., Krebs, C. J. & Boonstra, R. Assessing stress in animal populations: Do fecal and plasma glucocorticoids tell the same story? *Gen. Comp. Endocrinol.* **166**, 614–619 (2010).

31. Sheriff, M. J. & Love, O. P. Determining the adaptive potential of maternal stress. *Ecol. Lett.* **16**, 271–280 (2013).

32. Wey, T. W., Lin, L., Patton, M. L. & Blumstein, D. T. Stress hormone metabolites predict overwinter survival in yellow-bellied marmots. *Acta Ethol.* **18**, 181–185 (2015).

33. Bonier, F., Martin, P. R., Moore, I. T. & Wingfield, J. C. Do baseline glucocorticoids predict fitness? *Trends Ecol Evol* **24**, 634–642 (2009).

34. Bonier, F., Martin, P. R., Moore, I. T. & Wingfield, J. C. Clarifying the Cort-Fitness Hypothesis: A response to Dingemanse et al. *Trends Ecol. Evol.* **25**, 262–263 (2010).

35. Monclús, R., Tiulim, J. & Blumstein, D. T. Older mothers follow conservative strategies under predator pressure: The adaptive role of maternal glucocorticoids in yellow-bellied marmots. *Horm. Behav.* **60**, 660–665 (2011).

36. Petelle, M. B., Dang, B. N. & Blumstein, D. T. The effect of maternal glucocorticoid levels on juvenile docility in yellow-bellied marmots. *Horm. Behav.* **89**, 86–91 (2017).

37. Zuur, A. F., Ieno, E. N., Walker, N. J., Saveliev, A. A. & Smith, G. M. *Models and Extensions in Ecology with R*. (Springer, 2009).

38. Fuong, H., Maldonado-Chaparro, A. & Blumstein, D. T. Are social attributes associated with alarm calling propensity? *Behav. Ecol.* **26**, 587–592 (2015).

39. Kiyokawa, Y. & Hennessy, M. B. Comparative studies of social buffering: A consideration of approaches, terminology, and pitfalls. *Neurosci. Biobehav. Rev.* **86**, 131–141 (2018).

40. Cameron, E. Z., Setsaas, T. H. & Linklater, W. L. Social bonds between unrelated females increase reproductive success in feral horses. *Proc. Natl. Acad. Sci.* **106**, 13850–13853 (2009).

41. Silk, J. B. *et al.* Strong and consistent social bonds enhance the longevity of female baboons. *Curr. Biol.* **20**, 1359–1361 (2010).

42. Silk, J. B. *et al.* The benefits of social capital: Close social bonds among female baboons enhance offspring survival. *Proc. R. Soc. B* **276**, 3099–3104 (2009).

43. Silk, J. B., Alberts, S. C. & Altmann, J. Social bonds of female baboons. *Science* **302**, 1231–1235 (2003).

44. Vander Wal, E., Festa-Bianchet, M., Réale, D., Coltman, D. W. & Pelletier, F. Sex-based differences in the adaptive value of social behavior contrasted against morphology and environment. *Ecology* **96**, 631–641 (2015).

45. Lahvis, G. P., Panksepp, J. B., Kennedy, B. C., Wilson, C. R. & Merriman, D. K. Social conditioned place preference in the captive ground squirrel (*Ictidomys Tridecemlineatus*): Social reward as a natural phenotype. *J. Comp. Psychol.* **129**, 291–303 (2015).

46. Silk, J. B. The adaptive value of sociality in mammalian groups. *Philos. Trans. R. Soc. B Biol. Sci.* **362**, 539–559 (2007).

47. Flatt, T. A new definition of aging? *Front. Genet.* **3**, 148 (2012).

48. Berdasco, M. & Esteller, M. Hot topics in epigenetic mechanisms of aging: 2011. *Aging Cell* **11**, 181–186 (2012).

49. Jylhävä, J., Pedersen, N. L. & Hägg, S. Biological Age Predictors. *EBioMedicine* **21**, 29–36 (2017).

50. Wagner, K. H., Cameron-Smith, D., Wessner, B. & Franzke, B. Biomarkers of aging: From function to molecular biology. *Nutrients* **8**, 338 (2016).

51. Field, A. E. *et al.* DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Mol. Cell* **71**, 882–895 (2018).

52. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol.* **14**, R115 (2013).

53. Hannum, G. *et al.* Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. *Mol Cell* **49**, 359–367 (2013).

54. Unnikrishnan, A. *et al.* The role of DNA methylation in epigenetics of aging. *Pharmacol. Ther.* **195**, 172–185 (2019).

55. Bocklandt, S. *et al.* Epigenetic Predictor of Age. *PLoS One* **6**, e14821 (2011).

56. Zannas, A. S. *et al.* Lifetime stress accelerates epigenetic aging in an urban, African American cohort: Relevance of glucocorticoid signaling. *Genome Biol.* **16**, 266 (2015).

57. Marioni, R. E. *et al.* The epigenetic clock and telomere length are independently associated with chronological age and mortality. *Int. J. Epidemiol.* **45**, 424–432 (2016).

58. Marioni, R. E. *et al.* DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol.* **16**, 25 (2015).

59. Perna, L. *et al.* Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clin. Epigenetics* **8**, 64 (2016).

60. Chen, B. H. *et al.* DNA methylation based measures of biological age: meta analysis predicting time to death. *Aging* **8**, 1844–1859 (2016).

61. Christiansen, L. *et al.* DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell* **15**, 149–154 (2016).

62. Horvath, S. & Levine, A. J. HIV-1 infection accelerates age according to the epigenetic clock. *J. Infect. Dis.* **212**, 1563–1573 (2015).

63. Horvath, S. *et al.* Accelerated epigenetic aging in Down syndrome. *Aging Cell* **14**, 491–495 (2015).

64. Horvath, S. & Raj, K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nat. Rev. Genet.* **19**, 371–384 (2018).

65. Parrott, B. B. & Bertucci, E. M. Epigenetic Aging Clocks in Ecology and Evolution. *Trends Ecol. Evol.* **34**, 767–770 (2019).

66. Horvath, S. *et al.* Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging* **7**, 1159–1170 (2015).

67. Wang, T. *et al.* Epigenetic aging signatures in mice livers are slowed by dwarfism, calorie restriction and rapamycin treatment. *Genome Biol.* **18**, 57 (2017).

68. Thompson, M. J., von Holdt, B., Horvath, S. & Pellegrini, M. An epigenetic aging clock for dogs and wolves. *Aging* **9**, 1055–1068 (2017).

69. Wagner, W. Epigenetic aging clocks in mice and men. *Genome Biol.* **18**, 107 (2017).

70. Lowe, R. *et al.* Ageing-associated DNA methylation dynamics are a molecular readout of lifespan variation among mammalian species. *Genome Biol.* **19**, 22 (2018).

71. Wang, T. *et al.* Quantitative Translation of Dog-to-Human Aging by Conserved Remodeling of the DNA Methylome. *Cell Syst.* **11**, 1–10 (2020).

72. Wilkinson, G. S. & Adams, D. M. Recurrent evolution of extreme longevity in bats. *Biol. Lett.* **15**, 20180860 (2019).

73. Austad, S. N. Comparative biology of aging. *J. Gerontol. A Biol. Sci. Med. Sci.* **64**, 199–201 (2009).

74. Wu, C. W. & Storey, K. B. Life in the cold: Links between mammalian hibernation and longevity. *BioMol. Concepts* **7**, 41–52 (2016).

75. Turbill, C., Bieber, C. & Ruf, T. Hibernation is associated with increased survival and the evolution of slow life histories among mammals. *Proc. R. Soc. B* **278**, 3355–3363 (2011).

76. Chen, Y. *et al.* Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. *Biochemistry* **40**, 11565–11570 (2001).

77. Knight, J. E. *et al.* mRNA stability and polysome loss in hibernating Arctic ground squirrels (*Spermophilus parryii*). *Mol. Cell. Biol.* **20**, 6374–6379 (2000).

78. Yan, J., Barnes, B. M., Kohl, F. & Marr, T. G. Modulation of gene expression in hibernating arctic ground squirrels. *Physiol. Genomics* **32**, 170–181 (2008).

79. Van Breukelen, F. & Martin, S. L. Molecular adaptations in mammalian hibernators: unique adaptations or generalized responses? *J. Appl. Physiol.* **92**, 2640–2647 (2002).

80. Morin, P. & Storey, K. B. Evidence for a reduced transcriptional state during hibernation in ground squirrels. *Cryobiology* **53**, 310–318 (2006).

81. van Breukelen, F. & Martin, S. L. Reversible depression of transcription during hibernation. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **172**, 355–361 (2002).

82. Azzu, V. & Valencak, T. G. Energy Metabolism and Ageing in the Mouse: A Mini-Review. *Gerontology* **63**, 327–336 (2017).

83. Schrack, J. A., Knuth, N. D., Simonsick, E. M. & Ferrucci, L. 'IDEAL' aging is associated with lower resting metabolic rate: The baltimore longitudinal study of aging. *J. Am. Geriatr. Soc.* **62**, 667–672 (2014).

84. Al-attar, R. & Storey, K. B. Suspended in time: Molecular responses to hibernation also promote longevity. *Exp. Gerontol.* **134**, 110889 (2020).

85. Carey, H. V., Andrews, M. T. & Martin, S. L. Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. *Physiol. Rev.* **83**, 1153–1181 (2003).

86. Armitage, K. B. *Marmot Biology: Sociality, Individual Fitness, and Population Dynamics*. (Cambridge University Press, 2014).

87. Armitage, K. B., Blumstein, D. T. & Woods, B. C. Energetics of hibernating yellow-bellied marmots (*Marmota flaviventris*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **134**, 101–114 (2003).

88. Arneson, A. *et al.* A mammalian methylation array for profiling methylation levels at conserved sequences. *bioRxiv* doi: 10.1101/2021.01.07.425637 (2021).

89. Snir, S., VonHoldt, B. M. & Pellegrini, M. A Statistical Framework to Identify Deviation from Time Linearity in Epigenetic Aging. *PLoS Comput. Biol.* **12**, e1005183 (2016).

90. Snir, S., Wolf, Y. I. & Koonin, E. V. Universal Pacemaker of Genome Evolution. *PLoS Comput. Biol.* **8**, e1002785 (2012).

91. Snir, S., Farrell, C. & Pellegrini, M. Human epigenetic ageing is logarithmic with time across the entire lifespan. *Epigenetics* **14**, 912–926 (2019).

92. Farrell, C., Snir, S. & Pellegrini, M. The Epigenetic Pacemaker: modeling epigenetic states under an evolutionary framework. *Bioinformatics* **36**, 4662–4663 (2020).

93. Snir, S. & Pellegrini, M. An epigenetic pacemaker is detected via a fast conditional expectation maximization algorithm. *Epigenomics* **10**, 695–706 (2018).

94. Zou, H. & Hastie, T. Regularization and variable selection via the elastic net. *J. R. Stat. Soc. B* **67**, 301–320 (2005).

95. Friedman, J., Hastie, T. & Tibshirani, R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *J. Stat. Softw.* **33**, 1–22 (2010).

96. Armitage, K. B. Reproductive strategies of yellow-bellied marmots: energy conservation and differences between the sexes. *J. Mammal.* **79**, 385–393 (1998).

97. Armitage, K. B. Reproductive competition in female yellow-bellied marmots. in *Adaptive strategies and diversity in marmots* (eds. Ramousse, R., Allainé, D. & Le Berre, M.) 133–142 (International Marmot Network, 2003).

98. Petkovich, D. A. *et al.* Using DNA Methylation Profiling to Evaluate Biological Age and Longevity Interventions. *Cell Metab.* **25**, 954–960 (2017).

99. Wood, S. N. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *J. R. Statist. Soc. B.* **73**, 3–36 (2011).

100. Kilgore, D. L. & Armitage, K. B. Energetics of Yellow-Bellied Marmot Populations. *Ecology* **59**, 78–88 (1978).

101. Armitage, K. B. Social and population dynamics of yellow-bellied marmots: results from long-term research. *Annu. Rev. Ecol. Syst.* **22**, 379–407 (1991).

102. Webb, D. R. Environmental harshness, heat stress, and *Marmota flaviventris*. *Oecologia* **44**, 390–395 (1980).

103. Jarman, S. N. *et al.* Molecular biomarkers for chronological age in animal ecology. *Mol. Ecol.* **24**, 4826–4847 (2015).

104. De Paoli-Iseppi, R. *et al.* Measuring animal age with DNA methylation: From humans to wild animals. *Front. Genet.* **8**, 106 (2017).

105. Beissinger, S. R. & Westphal, M. I. On the use of demographic models of population viability in endangered species management. *J. Wildl. Manage.* **62**, 821–841 (1998).

106. Nussey, D. H., Froy, H., Lemaitre, J. F., Gaillard, J. M. & Austad, S. N. Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. *Ageing Res. Rev.* **12**, 214–225 (2013).

107. Moehlman, P. D. *Equids: Zebras, Asses and Horses Status Survey and Conservation Action Plan*. (IUCN-the World Conservation Union, 2002).

108. Moehlman, P. D. K. & Sarah, R. B. *IUCN SSC Equid Specialist Group 2019 Report*. (2019).

109. R Core Team. *R: A language and environment for statistical computing*. *R Foundation for Statistical Computing* (2020).

110. Jónsson, H. *et al.* Speciation with gene flow in equids despite extensive chromosomal plasticity. *Proc. Natl. Acad. Sci. USA* **111**, 18655–18660 (2014).

111. Vilstrup, J. T. *et al.* Mitochondrial Phylogenomics of Modern and Ancient Equids. *PLoS One* **8**, e55950 (2013).

112. Fox, C. W. & Reed, D. H. Inbreeding depression increases with environmental stress: An experimental study and meta-analysis. *Evolution (N. Y).* **65**, 246–258 (2010).

113. Van Rossum, G. & Drake, F. L. *Python 3 Reference Manual*. (CreateSpace, 2009).

114. Rubinacci, S., Ribeiro, D. M., Hofmeister, R. J. & Delaneau, O. Efficient phasing and imputation of low-coverage sequencing data using large reference panels. *Nat. Genet.* **53**, 120–126 (2021).

115. Purcell, S. *et al.* PLINK: A tool set for whole-genome association and population-based linkage analyses. *Am. J. Hum. Genet.* **81**, 559–575 (2007).

116. McQuillan, R. *et al.* Runs of Homozygosity in European Populations. *Am. J. Hum. Genet.* **83**, 359–372 (2008).

117. Meyermans, R., Gorssen, W., Buys, N. & Janssens, S. How to study runs of homozygosity using PLINK? a guide for analyzing medium density SNP data in livestock and pet species. *BMC Genomics* **21**, 94 (2020).

118. Zeileis, A. & Hothorn, T. Diagnostic Checking in Regression Relationships. *R News* **2**, 7–10 (2002).

119. Bell, C. G. *et al.* DNA methylation aging clocks: Challenges and recommendations. *Genome Biol.* **20**, 249 (2019).

120. Broussard, D. R., Dobson, F. S. & Murie, J. O. Previous experience and reproductive investment of female Columbian ground squirrels. *J. Mammal.* **89**, 145–152 (2008).

121. Colas, S. Evidence for sex-biased behavioral maternal investment in the gray mouse lemur (*Microcebus murinus*). *Int. J. Primatol.* **20**, 911–926 (1999).

122. Dwyer, C. M. & Lawrence, A. B. Maternal behaviour in domestic sheep (*Ovis aries*): Constancy and change with maternal experience. *Behaviour* **137**, 1391–1413 (2000).

123. Künkele, J. & Kenagy, G. J. Inefficiency of lactation in primiparous rats: The costs of first reproduction. *Physiol. Zool.* **70**, 571–577 (1997).

124. Lunn, N. J., Boyd, I. L. & Croxall, J. P. Reproductive performance of female Antarctic fur seals: The influence of age, breeding experience, environmental variation and individual quality. *J. Anim. Ecol.* **63**, 827–840 (1994).

125. Schino, G. & Troisi, A. Neonatal abandonment in Japanese macaques. *Am. J. Phys. Anthropol.* **126**, 447–452 (2005).

126. Snyder, R. J., Perdue, B. M., Zhang, Z., Maple, T. L. & Charlton, B. D. Giant panda maternal care: A test of the experience constraint hypothesis. *Sci. Rep.* **6**, 27509 (2016).

127. Sunderland, N., Heffernan, S., Thomson, S. & Hennessy, A. Maternal parity affects neonatal survival rate in a colony of captive bred baboons (*Papio hamadryas*). *J. Med. Primatol.* **37**, 223–228 (2008).

128. Wang, Z. & Novak, M. A. Parental care and litter development in primiparous and multiparous prairie voles (*Microtus ochrogaster*). *J. Mammal.* **75**, 18–23 (1994).

129. Zedrosser, A., Dahle, B. & Swenson, J. E. The effects of primiparity on reproductive performance in the brown bear. *Oecologia* **160**, 847–854 (2009).

130. Bonier, F., Moore, I. T., Martin, P. R. & Robertson, R. J. The relationship between fitness and baseline glucocorticoids in a passerine bird. *Gen. Comp. Endocrinol.* **163**, 208–213 (2009).

131. Dipietro, J. A., Novak, M. F. S. X., Costigan, K. A., Atella, L. D. & Reusing, S. P. Maternal psychological distress during pregnancy in relation to child development at age two. *Child Dev.* **77**, 573–587 (2006).

132. Maguire, J. & Mody, I. Behavioral deficits in juveniles mediated by maternal stress hormones in mice. *Neural Plast.* **25016**, 2762518 (2016).

133. Whirledge, S. & Cidlowski, J. A. Glucocorticoids, stress, and fertility. *Minerva Endocrinol.* **35**, 109–125 (2010).

134. Crary-Dooley, F. K. *et al.* A comparison of existing global DNA methylation assays to low-coverage whole-genome bisulfite sequencing for epidemiological studies. *Epigenetics* **12**, 206–214 (2017).

135. Reed, K., Poulin, M. L., Yan, L. & Parissenti, A. M. Comparison of bisulfite sequencing PCR with pyrosequencing for measuring differences in DNA methylation. *Anal. Biochem.* **397**, 96–106 (2010).

136. Tost, J., Dunker, J. & Gut, I. G. Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing™. *Biotechniques* **35**, 152–156 (2003).

CHAPTER 2

Correlates of maternal glucocorticoid levels in a socially flexible rodent

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Correlates of maternal glucocorticoid levels in a socially flexible rodent

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ABSTRACT

While it is generally accepted that social isolation has detrimental effects on social species, little is known about the importance of social interactions in less social species–particularly for wild reproductive females. We studied socially-flexible yellow-bellied marmots (*Marmota flaviventer*) and asked whether features of the social environment are associated with maternal fecal glucocorticoid metabolite (FGM) concentrations. Since changes in maternal baseline glucocorticoids may have positive or negative consequences for offspring fitness, we were also interested in estimating their relationship with measures of reproductive success. We fitted generalized linear mixed effects models to a dataset including maternal FGM measurements, social network metrics, maternal/alloparental care, and pup FGM and survival. Agonistic interactions were positively associated with maternal FGM levels, while mothers that engaged in relatively more affiliative interactions had reduced FGM levels when living in environments with low predator pressure. Pups associated with mothers exhibiting high FGM levels had low annual survival rates, received less maternal/alloparental care and had higher FGM levels. Interestingly, offspring from mothers with high FGM levels were more likely to survive the summer when born in small litters. In sum, social interactions likely influence and are influenced by glucocorticoid levels of facultatively social females. Potential benefits of social bonds may be context-specific, and agonistic interactions may be tightly correlated with fitness. Female marmots exhibiting high FGM levels had overall low reproductive success, which is predicted by the cort-fitness hypothesis. However, under adverse conditions, offspring summer survival can be maximized if pups are born in small litters.

1. Introduction

The social environment—defined as the set of all interactions between an individual and its conspecifics—may challenge individuals but may also promote well-being and/or modify their ability to cope with adversity (Beery and Kaufer, 2015; Cohen, 2004; Holt-Lunstad et al., 2010). In nature, ecological and demographic factors modify social environments (Armitage and Downhower, 1974; Barash, 1974; Blumstein, 2013; Iossa et al., 2008; Klein et al., 2017; Negrin et al., 2016; Ondrasek et al., 2015; Pasinelli and Walters, 2002), and the degree to which social changes impact individuals depends on a variety of contextual factors, including group size, type of interactions, species social organization, sex and age (Anisman and Merali, 1999; Cohen, 2004; Hall, 1998; Palanza et al., 2001).

The consequences of the social environment on individuals are often studied by manipulating group composition or structure in captive, domesticated species (Cruces et al., 2014). Such studies may not be applicable to wild populations since artificially selected lineages may have altered social phenotypes (Chalfin et al., 2014; Künzl et al., 2003; Künzl and Sachser, 1999), and behavioral and endocrine responses have evolved in contexts characterized by multiple stressors. In addition, most in situ studies have been conducted on highly social mammals. For instance, while it is generally accepted that social isolation is a risk factor for mortality, and affiliative behavior lowers stress levels in social species (House et al., 1988; Sapolsky et al., 1997; Holt-Lunstad et al., 2010; Cruces et al., 2014), little is known about the effects of the social environment on individuals of less social species (Ferron, 1985; Maher, 2009; Yoerg, 1999). In fact, Blumstein et al. (2018) found that the strength of affiliative social relationships is negatively associated with longevity in yellow-bellied marmots (*Marmota flaviventer*), emphasizing that studies on less social species may provide insightful perspectives in this field.

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Furthermore, physiological and behavioral responses to social conditions have been mostly studied in males (Palanza et al., 2001; Palanza and Parmigiani, 2017; Pittet et al., 2017; Taylor et al., 2000). Yet, sex differences in the perception of the social environment are expected due to sex-specific social roles (Kudielka and Kirschbaum, 2005; Palanza et al., 2001; Palanza and Parmigiani, 2017; Stroud et al., 2002). Since female mammals generally invest more in their offspring, female responsiveness to social variation may be essential for the wellbeing of both females and their young (Taylor et al., 2000). In this respect, the strength and stability of female social bonds may be key predictors of fitness (Palanza and Parmigiani, 2017; Stroud et al., 2002; Taylor et al., 2000).

Exposure to challenging conditions that disrupt homeostasis and cause mental or physical tension promotes the activation of pathways that culminate in the production of glucocorticoid hormones (Anisman and Merali, 1999; Cruces et al., 2014; Sapolsky et al., 2000; Sheriff et al., 2010). Due to their role in coping responses, glucocorticoid hormones (GC) and their metabolites are often used to measure stress levels in ecological and psychosocial studies (Sheriff et al., 2010; Sheriff and Love, 2013; Wey et al., 2015). Here, we evaluate how both affiliative and agonistic aspects of the social environment are associated with maternal fecal glucocorticoid metabolites (FGM) levels in facultatively social, yellow-bellied marmots.

Wild female marmots can live alone or form kin groups (Armitage and Schwartz, 2000). While large group sizes are characterized by more agonistic interactions and reproductive suppression (Armitage and Schwartz, 2000), group living may also have advantages since solitary females occupy suboptimal environments (Armitage and Downhower, 1974) and exhibit more vigilant behavior than females in groups (Mady and Blumstein, 2017). Group living may be particularly important to mothers and their offspring (Blumstein et al., 2016) because pups are extremely vulnerable to predators (Wey and Blumstein, 2012). Although Wey and Blumstein (2012) found no relationship between social context and FGM levels in females, their analysis did not use information on reproductive status, which may be an important determinant of female sensitivity to variation in the social environment (Ralph and Tilbrook, 2016; Reeder et al., 2004; Tu et al., 2005).

Assuming that GC levels vary according to the magnitude of environmental challenges, and that individuals facing greater challenges have lower fitness, the cort-fitness hypothesis predicts that baseline GC levels will be negatively associated with long-term survival and/or reproductive success (Bonier et al., 2009a, 2010). Due to conflicting results in different taxa (Bonier et al., 2009b; Dipietro et al., 2006; Maguire and Mody, 2016; Sapolsky et al., 2000; Whirledge and Cidlowski, 2010), the efficiency of GC as a proxy of fitness should be validated in each species (Bonier et al., 2010). In yellow-bellied marmots, high concentrations of FGM are associated with an increased risk of mortality (Wey et al., 2015). However, females with higher FGM levels wean larger litters (Blumstein et al., 2016), indicating a higher reproductive output. Because about half of the pups are either killed by predators or fail to survive their first winter (Armitage and Downhower, 1974), the relationship between maternal FGM levels and offspring survival should be further investigated for a better understanding of female reproductive success under sub-optimal conditions.

Maternal response to sub-optimal conditions may be adaptive when it reliably prepares offspring for their future environments (Sheriff and Love, 2013). Cues received during early life trigger developmental plasticity that may be beneficial later in life (Bateson et al., 2014). In this regard, maternal care may provide such cues (Klaus et al., 2013) and is known to directly affect offspring development and brain function (Curley et al., 2011; Curley and Champagne, 2016; Luby et al., 2012; Weaver et al., 2004). Female yellow-bellied marmots engage in maternal care by grooming and interacting affiliatively with offspring, as well as by alarm calling (Blumstein et al., 1997). Female marmot FGM levels have been shown to be significantly associated with litter size, offspring sex ratio, dispersal behavior and personality (Monclús et al., 2011; Petelle et al., 2017). We ask whether maternal care, measured through female-pup interactions and maternal antipredator behavior, is associated with maternal FGM levels.

Here, we use long-term data on a wild population to study the association between the social environment and FGM levels of facultatively social females, and the impact of their FGM levels on offspring fitness. To do so, we (1) estimated the association between maternal FGM levels and social network metrics calculated from affiliative and agonistic interactions; (2) investigated whether female FGM levels are associated with their antipredator behavior and interactions with pups; and (3) measured the association between maternal FGM and both offspring FGM levels and offspring survival.

2. Methods

2.1. Data collection

We analyzed 10 years (2005–2012, 2014, 2015) of behavioral and trapping data collected from females and their offspring in free-living yellow-bellied marmots in and around the Rocky Mountain Biological Laboratory (38°57′N, 106°59′W; 2900 m elevation; Gunnison County, Colorado, USA; Blumstein, 2013). This research was performed under the University of California, Los Angeles Institutional Animal Care and Use protocol (2001–191-01, renewed annually) and with permits from Colorado Parks and Wildlife (TR917, renewed annually).

Trapping sessions occurred biweekly from May to mid-September (details in Blumstein et al., 2016; Wey et al., 2015). We trapped marmots with Tomahawk live traps (Tomahawk Live Trap Co., Tomahawk, WI) baited with Purina Omolene 100 Horse Feed (Purina Mills, LLC, Gray Summit, MO). At each capture, we recorded individual identification through fur mark and two uniquely numbered metal ear-tags (Monel self-piercing fish tags #3, National Band and Tag, Newport, KY, United States); individual sex and reproductive status; time and date of capture. We also collected hair and, when available, fecal material (Armitage, 1982). Fecal samples were collected from May to the end of July, only when feces were both fresh and could be clearly associated with the trapped individual, and were stored on ice and frozen at −20 °C within 2 h. Unique dorsal fur marks were drawn using black Nyanzol fur dye to identify individuals from afar during behavioral observations.

Weather permitting, we performed daily behavioral observations during times of peak activity (0700–1000, and 1600–1900 h; Armitage, 1962) from mid-April to mid-September. Observers used binoculars and 15–45× spotting scopes, being positioned 20–150 m from marmot groups (Blumstein et al., 2009; Yang et al., 2017). Observers recorded individual identity, type of behavior, location and predator presence in an all-occurrence sampling scheme (details in Blumstein et al., 2009). Behavior types included alarm calling and 32 types of intraspecific interactions, grouped as agonistic for aggressive behaviors or affiliative for amicable ones (ethogram in Blumstein et al., 2009). For each interaction, we recorded the identity of initiators and recipients. We also quantified time allocated to antipredator vigilance with 2-min focal observations on foraging individuals (details in Blumstein et al., 2004, 2010). Focals were scored in JWatcher 1.0 (Blumstein and Daniel, 2007).

To test how maternal fecal glucocorticoid metabolites (FGM) levels are associated with the social context and pup care, FGM and survival, we fitted (generalized) linear mixed effects models. The variables included in the models are explained below.

Maternal and pup FGM – Free glucocorticoid hormones (GC), which are biologically active, are metabolized by the liver and excreted via urine and feces (Palme, 2005; Touma and Palme, 2005). Because of the accumulation of GC metabolites in the gut, FGM concentrations are less sensitive to episodic fluctuations in the hypothalamo-pituitary-adrenal axis activity, thus being less influenced by short-term stressors (Sheriff et al., 2010; Touma and Palme, 2005). Therefore, FGM concentrations

are an easy, effective, and non-invasive method to access changes in plasma free-GC concentrations in response to conditions that may last from several hours to days (Palme, 2005; Sheriff et al., 2010; Smith et al., 2012; Touma and Palme, 2005).

Smith et al. (2012) validated this method in both captive and wild yellow-bellied marmots, describing significant rise in FGM levels with stressors (biological and physiological validation) and significant effects of year, season, time of day, sex, age, reproductive status and individual identity. We use extensive, long-term individual level data collected from this yellow-bellied marmot population (Blumstein, 2013) to control for these additional effects on GC levels that could confound our ability to isolate an effect of interest (Djurhuus et al., 2004; Palme, 2005; Sapolsky et al., 2000). Thus, we used FGM concentrations as a proxy of baseline stress levels (Palme, 2005; Sapolsky et al., 2000; Sheriff et al., 2010; Smith et al., 2012; Touma and Palme, 2005). Marmot FGM levels rise 24 h after capture (Smith et al., 2012), therefore we only analyzed samples collected during an individual's first capture of each trapping session. FGMs were extracted using a double-antibody ^{125}I radioimmunoassay kit (RIA; MP Biomedicals, Costa Mesa, CA) from samples within 6 months of collection (Smith et al., 2012). Details are described in Appendix A of Blumstein et al. (2006). Briefly, we boiled 0.2 g of thawed feces in 90% aqueous ethanol at 80 °C, centrifuged the solution, decanted the supernatant and repeated the procedure. Supernatants were dried in a vacuum centrifuge and reconstituted with 1 ml of Absolute ethanol. We used 12.5 μl of this concentrated solution in a corticosterone RIA that was cross-reactive with cortisol but not other steroid hormones. RIAs were run in duplicate and if the CV was > 10% we reran samples.

FGM concentration data were used differently depending on the model fitted. We used: (1) FGM concentrations measured from each fecal sample; (2) the mean of FGM concentrations from samples collected before a given female's litter emerged (hereafter, FGM before emergence mean); and (3) the mean of FGM concentrations from samples collected after a given female's litter emerged (hereafter, FGM after emergence mean).

Pup care – Female-pup interaction and antipredator behavior were used as proxies for pup care. In marmot matrilines, more than one female can breed in a season, and these share burrows and nurse non-offspring (Armitage and Gurri-Glass, 1994). Consequently, interactions among any female and any pup in a social group may represent pup care and have an important role in offspring phenotypic variation, as observed in other species (Bauer et al., 2015; Birnie et al., 2013; Branchi et al., 2006; Curley et al., 2009; Fleming et al., 2002; Sayler and Salmon, 1969). Thus, we measured whether reproductive females were observed interacting with pups or not during a season, including both mother-offspring and potential alloparental interactions (Blumstein and Armitage, 1999). Vigilance was measured as the proportion of time mothers spent vigilant in each 2-min focal animal sample. Propensity to alarm call was quantified based on observations of mothers seen calling or not during a season.

Maternal age and litter emergence date – We analyzed only mothers of known age (i.e., they had been monitored since birth). We also recorded the emergence date for each litter, defined as the first day in which the first pup from a litter was seen above ground. Because females are observed and trapped frequently, we are able to detect lactating females and potential burrows with pups. Groups with lactating females are observed closely, and most litters were found as soon as they emerged.

Social attributes – We used observed social interactions between all females older than one year to create two separate social networks, one that only considered affiliative interactions and one that only considered agonistic interactions. From each social network, we calculated 10 social attributes that captured various dimensions of marmot social behavior (Fuong et al., 2015; Wey and Blumstein, 2012), which are *degree* (in and out), *strength* (in and out), *closeness* (in and out), *betweenness centrality*, *eigenvector centrality*, *embeddedness*, and *clustering coefficient*:

- *Degree* represents the number of social partners an individual has. It is a directed measure such that in-degree represents the number of individuals that initiated an interaction with the focal animal while out-degree represents the number of individuals with which the focal animal initiated an interaction (Wasserman and Faust, 1994).
- *Strength* represents the total number of interactions the focal individual received (in-strength) or initiated (out-strength) regardless of the number of social partners (Barrat et al., 2004).
- *Closeness* represents how closely the focal individual is connected to every other individual in the network and is calculated as the reciprocal of the sum of the shortest path lengths between a focal and every other individual (Wasserman and Faust, 1994; Wey et al., 2008). A shortest path length of 1 represents individuals that interacted directly with each other while a shortest path length of 2 represents individuals that are most closely connected to each other through one other individual. By including indirect social connections, *closeness* is a measure that reflects how a focal individual can influence (out-closeness) or be influenced by (in-closeness) individuals in the social network, even if they do not interact directly. The closer individuals are connected, the more influence they will have on one another.
- *Betweenness centrality* estimates how much focal individuals connect all other individuals in a network, and is therefore a measure of an individual's importance in maintaining group stability as well as its role as a potential "bridge" in information transfer. It is calculated as the proportion of shortest path lengths in the network that include the focal individual (Wey et al., 2008).
- *Eigenvector centrality* ranks individuals based on the quality of their connections. An individual has a high eigenvector centrality if it is connected to other high-ranking individuals. It is calculated as the eigenvector associated with the maximal eigenvalue of an adjacency matrix (Bonacich, 2007).
- *Embeddedness* is a measure of integration into a group. It identifies the greatest size of the cohesive subgroups to which the focal individual belongs (Moody and White, 2003). Cohesive subgroups are groups within the greater social network in which each individual is independently connected to every other individual.
- *Clustering coefficient* reflects how densely the network is clustered around a focal individual. It uses the number of observed connections among the focal individual's neighbors over the total possible connections (Wey et al., 2008).

Although the social networks included non-reproductive females, only reproductive females were considered focal individuals. All social attributes were calculated in igraph package 0.7.0 (Csardi and Nepusz, 2006) and R 3.3.1 (R Development Core Team, 2016). Because many of these social attributes are correlated (Wey and Blumstein, 2012), we used a principal component analysis to reduce them into uncorrelated measures (extractions based on eigenvalue > 1 with varimax rotation; SPSS 21.0; IBM Corp., Armonk, NY; IBM Corp 2012).

Group size – Social groups are formed by individuals living in close proximity and more associated among themselves than with other individuals in the population (Maldonado-Chaparro et al., 2015). We used data from individuals seen or trapped at least five times in a given year. We calculated the simple ratio index (SRI, Cairns and Schwager, 1987) from live-trapping and observation data for each pair of marmots using Socprog (Whitehead, 2009). We established the number and identity of individuals belonging to social groups with the random walk algorithm in Map Equation (Rosvall et al., 2009).

Litter size – Estimated during behavioral observations and trapping sessions. Relationships were further confirmed with parentage analysis, using DNA extracted from hair samples. We genotyped pups and females with 8–12 microsatellite markers and estimated mother-offspring pairs using CERVUS 3.0 (Kalinowski et al., 2007). Details can be found in Blumstein et al. (2010).

Pup survival – We obtained three estimates of pup survival rates per litter: summer, winter, and annual survival. Summer survival was calculated as the number of pups that survived until the end of the active season divided by litter size. Winter survival was the number of pups observed or trapped in the following summer divided by the number of pups that survived their first summer. Annual survival was the number of pups observed or trapped in the following summer divided by litter size.

Predator index – Following Monclús et al. (2011), we calculated predator index as the proportion of observation sessions in which predators were recorded, from April to June. Locations were classified as having high or low predator presence based on a median cut. Predators included: red fox (*Vulpes vulpes*), coyote (*Canis latrans*), American badger (*Taxidea taxus*), American black bear (*Ursus americanus*), domestic dog (*Canis lupus familiaris*), and several species of raptors and mustelids (Van Vuren, 2001).

2.2. Statistical analysis

According to the distribution of the dependent variable, we fitted linear mixed effects models (LMMs) or generalized linear mixed effects models with binomial distribution (binomial GLMMs). We used a top-down approach for model selection following Zuur et al. (2009). Initial models contained random effects (individual identity and year), fixed effects and interactions (Table S1), which were selected based on data availability and biological relevance. The optimal fixed component was found by dropping variables one-by-one and, in each turn, performing likelihood ratio tests (LMM) or analysis of deviance tests (binomial GLMM) to compare nested models. We computed the marginal and conditional R squared values for all final models to understand how much of the data variance is being explained by fixed and random effects.

We transformed variables as necessary, and scaled and centered continuous explanatory variables so that estimates would be comparable. Specific data transformations in each model can be found in the Supplementary File S2. We evaluated the assumptions of the models by visually inspecting the correlation among variables, qq plots of residuals, plots of residuals with fitted values, and plots of residuals with each explanatory variable (present or not in the final model). We also calculated Cook's distance to look for influential observations and checked model fit by plotting fitted with observed values of the dependent variable. We used standardized residuals for LMMs, and deviance residuals for binomial GLMMs. For a better evaluation of plots with residuals from the binomial GLMMs, residuals were separated in groups of five based on the order of fitted values, and the average of these groups were used in the plots. Analyses were carried out in R 3.4.4 (R Core Team, 2018) using the packages lme4 1.1–14 (Bates et al., 2015), and lmerTest 3.0–1 (Kuznetsova et al., 2017). Plots were generated using the packages ggplot2 3.0.0 (Wickham, 2016) and ggpubr 0.1.7. (Kassambara, 2018).

To test how the social context is associated with maternal FGM levels, we fitted separate LMMs for affiliative and agonistic interactions. Affiliative interactions are more common in yellow-bellied marmots than agonistic behavior, so we analyzed the data separately because of the differences in sample size. The fixed effects of the initial models (i.e., first step of the model selection) were group size, litter size, metrics from social networks, predator index, maternal age and time/day of capture in which the fecal sample was collected. Interactions between fixed effects can be found in Table S1. The dependent variable was maternal FGM concentrations measured from each fecal sample (ng/ml).

To test the association between maternal FGM levels and pup care, we fitted binomial GLMMs for each measure of pup care: alarm calling, vigilance and female-pup social interactions. For the models using alarm calling and female-pup interactions, fixed effects were maternal FGM after emergence, litter size, maternal age, predator index and total time spent observing each social group. For the model using vigilance data, fixed effects included: maternal FGM after emergence, litter size, maternal age, predator index, and time/day in which the focal was performed. Interactions between fixed effects can be found in Table S1.

To study the influence of maternal FGM levels on pup survival, we fitted binomial GLMMs for each measure of pup survival: summer, winter, and annual survival. The models included maternal FGM after emergence, litter size, maternal age, and predator index as fixed effects. Finally, to investigate the effect of maternal FGM levels on pup FGM levels, we fitted two LMMs to the pup FGM concentration data (ng/ml). In one model we used maternal FGM before emergence and, in the other, maternal FGM after emergence mean as fixed effects. Common fixed effects for both models were litter size, predator index, maternal age, and time/date of capture in which the fecal sample was collected.

3. Results

3.1. Maternal fecal glucocorticoid metabolites (FGM) levels

3.1.1. How are maternal FGM levels associated with affiliative interactions?

The female-affiliative social network analysis included 51 unique females and represented 87 breeding events over 10 years. Females had an average of 2.52 FGM measurements per active season (SD = 1.41, n = 219 individual measurements). Social groups averaged 13.36 individuals (SD = 7.92; range 3–34), litter size averaged 4.36 pups (SD = 1.81, range 1–10), and the average maternal age was 4.37 years (SD = 2.51, range 2–12). The principal components analysis explained 74.6% of the variation in affiliative social metrics. The principal components can be interpreted as popularity ($PC1_{AFF}$), which included degree (in and out), closeness (in and out), and eigenvector centrality; relationship strength ($PC2_{AFF}$), which included strength (in and out);

Table 1
Factor loadings for each component of the principal component analysis on social metrics from affiliative and agonistic networks. High loading values are in bold.

Social metrics	Affiliative networks			Agonistic networks		
	PC1 (popularity)	PC2 (relationship strength)	PC3 (group cohesiveness)	PC1 (aggression initiated)	PC2 (aggression received)	PC3 (group cohesiveness)
In-degree	**0.85**	0.18	−0.14	0.18	**0.93**	0.11
Out-degree	**0.84**	0.28	0.01	**0.95**	−0.02	0.00
In-strength	0.21	**0.90**	0.07	−0.19	**0.88**	0.14
Out-strength	0.20	**0.91**	0.10	**0.52**	−0.02	0.38
In-closeness	**0.87**	0.12	−0.13	0.55	**0.68**	0.23
Out-closeness	**0.84**	0.21	0.15	**0.88**	0.01	0.12
Betweenness centrality	0.19	−0.19	**−0.63**	0.43	−0.15	**−0.81**
Eigenvector centrality	**0.85**	−0.04	0.12	**0.68**	0.29	−0.12
Embeddedness	0.21	0.28	**0.72**	0.22	0.36	**0.75**
Clustering coefficient	0.02	−0.35	**0.80**	0.20	0.03	**0.84**

Table 2
Results from linear mixed effects models explaining variation in maternal stress levels (measured through fecal glucocorticoid metabolite concentrations) in a wild yellow-bellied marmot population. Models differed by the inclusion of social network metrics based on affiliative or agonistic interactions.

Affiliative networks			Agonistic networks		
Fixed effect	Estimate ± SE	P	Fixed effect	Estimate ± SE	P
Intercept	−0.11 ± 0.26	0.681	Intercept	−0.03 ± 0.36	0.937
$PC3_{AFF}$	−0.03 ± 0.07	0.681	$PC1_{AGO}$	0.20 ± 0.08	0.014
Litter size	0.19 ± 0.06	0.001	Litter size	0.22 ± 0.07	0.004
Predator index (low)	0.03 ± 0.14	0.843	$PC2_{AGO}$	0.21 ± 0.08	0.007
Time of capture	0.16 ± 0.05	0.001	Time of capture	0.19 ± 0.07	0.005
Day of capture	−0.13 ± 0.05	0.005	Day of capture	−0.22 ± 0.06	< 0.001
$PC3_{AFF}$: Predator index	−0.25 ± 0.11	0.020			
Affiliative networks			Agonistic networks		
Random effect	Variance	SD	Random effect	Variance	SD
Mother identity	0.02	0.13	Mother identity	0.01	0.10
Year	0.60	0.78	Year	0.97	0.98

and group cohesiveness ($PC3_{AFF}$), which included clustering, embeddedness, and betweenness (Table 1).

Our final model included $PC3_{AFF}$ (group cohesiveness), litter size, predator index, time/date of individual capture, and the interaction between $PC3_{AFF}$ and predator index (Table 2). The interaction between $PC3_{AFF}$ and predator index explained significant variation in maternal FGM levels ($p = 0.020$, Table 2). In low predation pressure environments, females with high group cohesion had somewhat lower FGMs than females with low group cohesion (Fig. 1). In contrast, there was no apparent association between group cohesion and FGMs in high predation environments. Litter size was positively associated with maternal FGM ($p = 0.001$), and both day and time of capture affected FGM levels (Table 2). Female identity and year (random effects) explained much of the variation in maternal FGM, because this model had a marginal R^2 of 0.10 and a conditional R^2 of 0.66. By comparing estimates in this model, we infer that the interaction between group cohesiveness and predator index was the fixed effect with the strongest effect on maternal FGM, followed by litter size (Table 2).

3.1.2. How are maternal FGM levels associated with agonistic interactions?

The female-agonistic dataset included 32 unique females that represented 42 breeding events over 8 years. Females had an average of 2.74 FGM measurements per season ($SD = 1.52$, $n = 115$ individual FGM samples). Groups averaged 15.22 individuals ($SD = 8.60$; range 3–34). The principal components analysis explained 75.5% of the variation in affiliative social metrics. The principal components can be interpreted as aggression initiated ($PC1_{AGO}$), which included out-degree, out-closeness, out-strength, and eigenvector centrality; aggression received ($PC2_{AGO}$), which included in-degree, in-closeness, and in-strength; and group cohesiveness ($PC3_{AGO}$), which included clustering, embeddedness, and betweenness (Table 1).

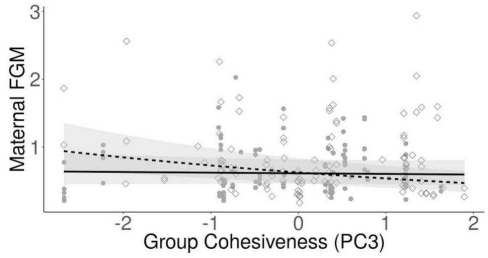

Fig. 1. Relationship between maternal fecal glucocorticoid metabolite concentrations (ng/ml) and group cohesiveness, a principle component characterized by affiliative clustering, embeddedness, and betweenness. The dashed line and diamond shaped points illustrate environments with low predator indices, while the continuous line and the filled circles illustrate environments with high predator indices. Points represent the actual data, while lines were generated with predicted probabilities from a linear mixed effects model. To generate predictions from the model, all other variables were set to the mean. Buffers illustrate the 95% confidence intervals. Importantly, observed values do not control for significant variation explained by the model.

Our final model included $PC1_{AGO}$ (aggression initiated), $PC2_{AGO}$ (aggression received), litter size, and time/day of individual capture (Table 2). After controlling for FGM seasonal and daily variation, maternal FGM levels were positively associated with aggression initiated, aggression received and litter size ($p = 0.014$, 0.007 and 0.004, respectively; Table 2, Fig. 2). Female identity and year explained a significant portion of the variation in maternal FGM levels; this model had a marginal R^2 of 0.13 and a conditional R^2 of 0.74. In addition to day of capture, litter size was the fixed effect with highest estimates, followed by $PC2_{AGO}$ and $PC1_{AGO}$, respectively (Table 2).

3.2. Pup care

3.2.1. Does maternal FGM explain variation in the probability of a mother emitting an alarm call?

This dataset included 59 unique mothers involved in 91 breeding events. We only positively identified 30 mothers that alarm called following pup emergence. Our final model was no different from the null model, and annual effects explained more of the variation than maternal identity (Table 3).

3.2.2. Does female FGM explain variation in whether females are ever seen interacting with pups?

From the 90 reproductive events of 58 females observed during 10 years, females were observed interacting with pups in 49 events. In our final model, maternal FGM after emergence and predator index were negatively associated with the occurrence of female-pup interactions ($p = 0.032$ and 0.001, respectively; Table 3). This result suggests that mothers are less likely to interact with pups when exhibiting high FGM levels or at locations with high predation pressure. Individual identity and year seem not to have an effect on female-pup interactions (Table 3). This model had a marginal R^2 of 0.22 and a conditional R^2 of 0.22.

3.2.3. Does maternal FGM explain variation in time mothers allocate to vigilance?

We collected 71 focals from 24 females with pups over 7 years, with an average of 2.09 focals per female per year. The final model had only

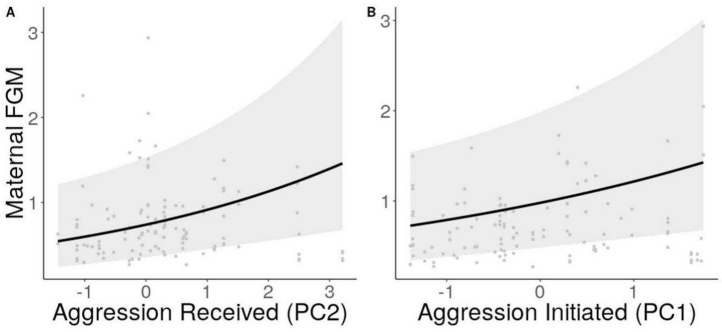

Fig. 2. Relationship between maternal fecal glucocorticoid metabolite concentrations (ng/ml) and aggression received (A) and aggression initiated (B). Aggression received (PC2) was inferred from a principal component on which in-degree, in-closeness, and in-strength were loaded; and aggression initiated (PC1) was defined by out-degree, out-closeness, out-strength, and eigenvector centrality. Points represent the actual data, while lines were generated with predicted probabilities from a linear mixed effects model. To generate predictions from the model, all other variables were set to the mean. Buffers illustrate the 95% confidence intervals. Importantly, the points of observed values are not controlled for significant variation explained by the model.

one fixed effect, the predator index, suggesting that mothers spend less time being vigilant in environments with lower predator pressure ($p = 0.028$; Table 3). The random effects explained no variation in vigilance. This model had a marginal R^2 of 0.20 and a conditional R^2 of 0.20.

3.3. Pup FGM levels

The model using the average maternal FGM before emergence included 227 FGM measurements from 148 pups of 43 mothers across 7 years, while the model using maternal FGM after emergence included 217 FGM measurements from 139 pups of 40 mothers across 7 years. Interestingly, maternal FGM was positively associated with pup FGM levels only when it was calculated as a mean before emergence ($p = 0.028$; Table 4). Mothers with high FGM levels before pup emergence had pups with high FGM, while maternal FGM levels measured after pup emergence had no effect on pup FGM levels. The random effect of year explained more of the variation in pup FGM levels than maternal or pup identity. The model using maternal FGM before emergence had a marginal R^2 of 0.05 and a conditional R^2 of 0.65.

3.4. Offspring survival

Offspring survival was measured for 91 litters from 59 females across 10 years. In the final models, maternal FGM after emergence was negatively associated with measurements of winter and annual survival ($p < 0.001$ and $= 0.001$, respectively; Table 5, Fig. 3). For the model fitted to summer survival data, we found a negative effect of predator index ($p = 0.031$), where pups were more likely to survive in environments with low predator pressure, and a significant effect of the interaction between maternal FGM after emergence and litter size ($p = 0.011$, Table 5). During the active season, large litters from females with low FGM levels had higher chance of survival than smaller litters, but this pattern was inverted if females had high FGM concentration (Fig. 4). In addition, the random effects of mother identity and year explained no variation in any measurements of survival. The marginal R^2 for the summer survival model, winter survival model and annual survival model were 0.57, 0.23 and 0.25, respectively.

4. Discussion

4.1. Associations between social interactions and maternal fecal glucocorticoid metabolites (FGM) levels

Overall, we found that social conditions are associated with maternal FGM levels in wild female yellow-bellied marmots. These results contrast with Wey and Blumstein (2012), who conducted similar social network measurements on a smaller sample from the same yellow-bellied marmot population and found no association of social interactions with female FGM levels. Wey and Blumstein (2012) analyzed females at different life stages but did not include information about individual reproductive status in the models, which is an important factor that explains female sensitivity to environmental and social conditions in other species (Mora et al., 1996; Palanza et al., 2001; Ralph and Tilbrook, 2016; Reeder et al., 2004; Tu et al., 2005). The discrepancy of our results suggests that reproductive female marmots are more responsive to their social environment, however this should be tested directly.

Maternal FGM levels were associated with position in agonistic networks; mothers both initiating and receiving agonistic interactions had increased FGM levels, so agonistic behavior clearly has an important role in female fitness and marmot social organization. Lea et al. (2010) found that measures of initiated aggression were positively associated with both longevity and lifetime reproductive success, while

Table 3
Results from generalized linear mixed effects models explaining variation in pup care in a wild yellow-bellied marmot population. Models differed by the proxies used to access pup care: alarm calling, female-pup interactions, and proportion of time allocated to vigilance while foraging.

Alarm calling			Female-pup interactions			Vigilance		
Fixed effect	Estimate ± SE	P	Fixed effect	Estimate ± SE	P	Fixed effect	Estimate ± SE	P
Intercept	−1.63 ± 0.94	0.084	Intercept	−0.52 ± 0.31	0.092	Intercept	−1.15 ± 0.43	0.008
–	–	–	Maternal FGM after emergence	−0.52 ± 0.24	0.032	Predator index (low)	−1.85 ± 0.85	0.028
–	–	–	Predator index (low)	1.57 ± 0.48	0.001	–	–	–
Alarm calling			**Female-pup interactions**			**Vigilance**		
Random effect	Variance	SD	Random effect	Variance	SD	Random effect	Variance	SD
Mother identity	0.01	0.09	Mother identity	0.00	0.00	Mother identity	0.00	0.00
Year	5.65	2.38	Year	0.00	0.00	Year	0.00	0.00

Table 4
Results from linear mixed effects models explaining variation in pup stress levels (measured through fecal glucocorticoid metabolite concentrations - FGM) in a wild yellow-bellied marmot population. Models differed by the calculation of the fixed effect related to maternal stress levels: maternal FGM before emergence (included only samples preceding pup emergence) and maternal FGM after emergence (included only samples following pup emergence).

Maternal FGM before emergence			Maternal FGM after emergence		
Fixed effect	Estimate ± SE	P	Fixed effect	Estimate ± SE	P
Intercept	−0.57 ± 0.17	0.013	Intercept	−0.50 ± 0.20	0.044
Maternal FGM before emergence	0.12 ± 0.05	0.028	–	–	–
Random effect	Variance	SD	Random effect	Variance	SD
Mother identity	0.02	0.15	Mother identity	0.02	0.15
Pup identity	0.01	0.08	Pup identity	0.01	0.10
Year	0.17	0.41	Year	0.25	0.50

received agonistic interactions were negatively correlated to lifetime reproductive success. Female marmots can be hierarchically ranked based solely on the relative number of wins against opponents in agonistic interactions, and dominant females exhibit lower FGM levels (Blumstein et al., 2016).

Under low predation pressure, group cohesiveness was weakly associated with reduced maternal FGM, but may potentially act as a social buffer by reducing effects of external stressors (Cohen, 2004; Kiyokawa and Hennessy, 2018). The conditional and weak relationship between social buffering and predator pressure represented in Fig. 1 suggests that affiliative bonds may not significantly alter maternal FGM levels under some scenarios. Maldonado-Chaparro et al. (2015) found that yellow-bellied marmots lose influence over conspecifics as group size increases, indicating some fragility of social bonds in this socially flexible rodent.

The affiliative metrics associated with maternal FGM were undirected and unweighted, indicating that a female marmot can be affected or affect a social network by simply being connected to others, independently of the number of interactions or the identity/dominance rank of individuals. Although evidently there are limitations to the effects of social bonds in marmots, there may be collective context-specific benefits, which could explain previous observations of marmots exhibiting higher sociality in harsher environments (Armitage, 2014; Barash, 1974; Blumstein, 2013). Sociality can buffer individuals from the impact of environmental changes in other taxa (Keiser et al., 2014; Pinter-Wollman et al., 2009), and the benefits of marmot social behavior may occur not only during hibernation (as reported in Alpine marmots, *M. marmota*, but not yellow-bellied marmots–e.g., Allainé, 2000; Arnold, 1988; Ruf and Arnold, 2000) but also in the active season.

Recent studies of yellow-bellied marmots have identified overwinter survival (Yang et al., 2017) and longevity costs (Blumstein et al., 2018) of sociality. The potential positive fitness effects of social buffering could balance out these negative effects and be a factor that explains affiliative social interactions in yellow-bellied marmots. Despite the restricted number of behavioral studies with less social mammals, some species seem to benefit from social behavior. Young 13-lined ground squirrels (*Ictidomys tridecemlineatus*)—an undomesticated and asocial species—have capacity for social reward (Lahvis et al., 2015). Social contact is also important for the maintenance of female estrous cycle in solitary kangaroo rats (*Dipodomys heermanni*; Yoerg, 1999). Previously isolated kangaroo rats behave more socially after several encounters with conspecifics (Yoerg, 1999). In more social species, positive outcomes of social bonds on female fitness are more pronounced (Cameron et al., 2009; Silk et al., 2003, 2009, 2010; Vander Wal et al., 2015). Since advantages of sociality are context dependent (Lahvis et al., 2015; Silk, 2007), identifying which conditions differ among less and more social species may reveal the conditions that have selected for more complex social systems in ancestral species.

4.2. Associations between female FGM levels and pup care

The influence of adult stress on parental care is still underexplored when compared to the effects of individual early experiences on phenotypes (Darnaudéry and Maccari, 2008; Pittet et al., 2017). In wild marmots, we found that FGM levels are associated with pup care: females with high FGM levels are less likely to interact with pups. Previous studies reported similar findings on other species. For instance, mothers in low quality environments or with high stress levels engage in more offspring-directed aggression in guinea pigs and rats (*Rattus*

Table 5
Results from generalized linear mixed effects models explaining variation in offspring survival in a wild yellow-bellied marmot population. Models differed by the different calculations of survival: summer survival, winter survival and annual survival.

Summer survival			Winter survival			Annual survival		
Fixed effect	Estimate ± SE	P	Fixed effect	Estimate ± SE	P	Fixed effect	Estimate ± SE	P
Intercept	0.02 ± 0.33	0.964	Intercept	0.25 ± 0.23	0.281	Intercept	−0.40 ± 0.24	0.098
Maternal FGM after emergence	−0.74 ± 0.30	0.015	Maternal FGM after emergence	−0.98 ± 0.27	< 0.001	Maternal FGM after emergence	−1.06 ± 0.30	0.001
Litter size	−0.66 ± 0.35	0.061	–	–	–	–	–	–
Predator index (low)	1.11 ± 0.51	0.031	–	–	–	–	–	–
Maternal FGM after emergence: Litter size	−1.09 ± 0.43	0.011	–	–	–	–	–	–
Summer survival			Winter survival			Annual survival		
Random effect	Variance	SD	Random effect	Variance	SD	Random effect	Variance	SD
Mother identity	0.00	0.00	Mother identity	0.00	0.00	Mother identity	0.00	0.00
Year	0.00	0.00	Year	0.00	0.00	Year	0.00	0.00

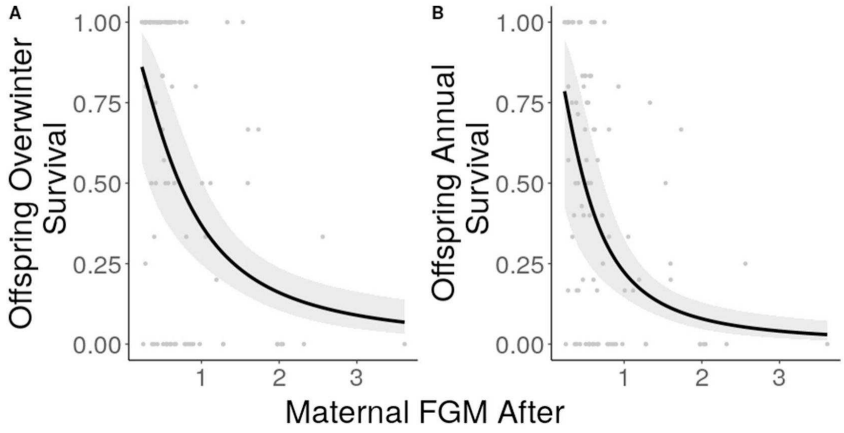

Fig. 3. Relationship of offspring overwinter (A) and annual (B) survival with maternal FGM measured after pups emerged (fecal glucocorticoid metabolite concentrations (ng/ml)). Points represent the actual data, while lines were generated with predicted probabilities from a linear mixed effects model. Buffers illustrate the 95% confidence intervals. Importantly, the points of observed values are not controlled for significant variation explained by the model.

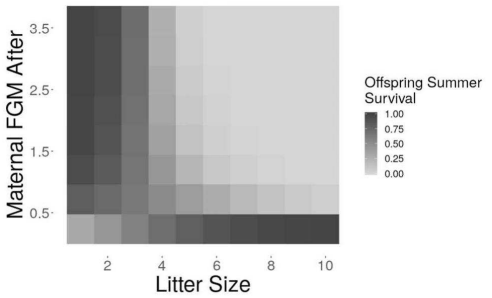

Fig. 4. Relationship between offspring summer survival, litter size and maternal FGM measured after pups emerged (fecal glucocorticoid metabolite concentrations (ng/ml)). The heatmap was generated with predicted probabilities from a linear mixed effects model. To generate predictions from the model, all other variables were set to the mean if continuous or to a fixed value if categorical (Predator index set as high).

rattus and *Cavia aperea*; Klaus et al., 2013; Rosenblum and Andrews, 1994). The artificial shortening of photoperiod, restraining experiments and administration of corticosterone reduce maternal care in rats, leading to a decrease of frequency and duration of nursing and licking/grooming interactions (Brummelte and Galea, 2010; Champagne and Meaney, 2006; Toki et al., 2007). Pittet et al. (2017) found no effects of chronic social partner instability on rat maternal care. However, when exposed to male intruders, females increased maternal care, indicating a variation in maternal behavior response according to stressor type or combination.

Our measures of female-pup interactions were taken at least 30 days after birth, which may not have been the most sensitive period for behavioral imprinting, since significant effects of pup care on pup development occur within the first weeks of life (Curley and Champagne, 2016) and the frequency of female-pup interactions decreases with pup age (Peña and Champagne, 2013). However, if the strength of female-pup bonds decrease at a similar rate among litters, the differences in pup care found after pup emergence may still reflect differences in within-burrow pup care among litters.

4.3. Associations between female FGM levels and pup FGM levels

Female yellow-bellied marmots produce at most one litter per year, reproduce as soon as they emerge from hibernation, have approximately a 30-day long gestation, and lactate another 25–30 days (Armitage, 2014; Blumstein and Armitage, 1998). Pups emerge from burrows nearly weaned (Frase and Hoffmann, 1980), so maternal FGM measured before pup emergence may quantify stress during both pregnancy and lactation. Only maternal stress measured during this period was significantly associated with pup FGM levels, suggesting that events during pregnancy and lactation may have long-term effects on pup behavior and neuroendocrine development (Curley and Champagne, 2016; Darnaudéry and Maccari, 2008). This finding is consistent with previous studies in yellow-bellied marmots, where lactating females with high FGM levels produced less docile pups (Petelle et al., 2017), a personality trait stable across life stages (Petelle et al., 2013).

Therefore, perinatal maternal FGM has an overall positive association with offspring FGM levels in marmots, but it is important to consider that differences in the type and intensity of stressors, pup sex (Curley and Champagne, 2016; Darnaudéry and Maccari, 2008), pup genotype (Pan et al., 2018), and within-litter variation in maternal care (Hasselt et al., 2012; Pan et al., 2014) are expected to modify the intensity and direction of maternal effects on pup stress responses. Since glucocorticoid hormones are involved in many metabolic pathways, the mechanism behind the influence of maternal FGM on offspring traits may essentially involve any pathway known to affect pup development, which can include hormone transmission via the placenta (Mairesse et al., 2007) and milk (Dettmer et al., 2018; Hinde et al., 2015), microbiota sharing via nursing (Daft et al., 2015; Ubeda et al., 2012), and behavioral imprinting (Bauer et al., 2015; Curley and Champagne, 2016; Darnaudéry and Maccari, 2008; Liu et al., 1997).

4.4. Associations between maternal FGM levels and pup survival

Although females with high FGM levels wean larger litters (Blumstein et al., 2016), their pups are significantly less likely to survive. Mothers with high FGM levels produced offspring with lower winter and annual survival. Random effects of mother identity explained no variation in the models, suggesting that external factors have an important role in pup survival. In addition to having a low annual reproductive success, mothers with high FGM levels have low

overwinter survival (Wey et al., 2015), which reinforces that FGMs are reliable proxies for female fitness in free-living yellow-bellied marmots and supports the cort-fitness hypothesis (Bonier et al., 2009a, 2010). A negative association between glucocorticoid baseline levels and fitness has been found in other species, such as mice (Maguire and Mody, 2016), barn swallows (*Hirundo rustica*; Jenni-Eiermann et al., 2008), and tufted puffins (*Fratercula cirrhata*; Williams et al., 2008). However, associations between fitness and baseline glucocorticoids give conflicting results depending on the species, individual life history stage and sex (Bonier et al., 2009a, b).

In addition, the effects of litter size on offspring summer survival are contingent on maternal FGM levels: when mothers have high FGM levels, individuals born into small litters have higher summer survival than individuals born into larger litters. Interestingly, Monclús et al. (2011) reported that, under stressful conditions, older females were more likely to produce smaller litters than younger mothers. By using both results together, it is possible to hypothesize that older females are able to increase offspring survival likelihood by programming litter sizes according to environmental conditions. This suggested hypothesis follows the rationale of the experience constraint hypothesis (Curio, 1983; Forslund and Part, 1995), where female marmots could learn from experience how to use physiological or environmental cues to increase pup fitness. We may not expect that females are able to cognitively determine the number of pups they will give birth to, however pups spend approximately a month after birth within the burrow, and mothers could actively reduce their litter size by not feeding or killing some young.

Experienced mothers have increased reproductive success compared to inexperienced females in other species (Broussard et al., 2008; Colas, 1999; Dwyer and Lawrence, 2000; Künkele and Kenagy, 1997; Lunn et al., 1994; Schino and Troisi, 2005; Snyder et al., 2016; Sunderland et al., 2008; Wang and Novak, 1994; Zedrosser et al., 2009). Previous work has shown that the interaction between maternal age and FGM also determines offspring personality (Petelle et al., 2017), and, if changes on offspring phenotype enhance their fitness, marmot maternal behavior may support the Predictive Adaptive Response (PAR) hypothesis. According to the PAR hypothesis, maternal stress responses may be adaptive if stress levels reliably prepare offspring for their future environments, which has been observed across mammals, reptiles and fishes (Bateson et al., 2014; Sheriff and Love, 2013). Since this hypothesis was not directly tested in this study, future work should test whether older female marmots actually adopt strategies to enhance litter survival.

4.5. Conclusions

Physiological stress, measured through fecal glucocorticoid metabolites (FGM) levels, in free-living mothers of a facultatively social mammal is seemingly associated with both affiliative and agonistic interactions with conspecifics, which may have critical consequences for offspring survival. Agonistic interactions are key for yellow-bellied marmot social organization and have a positive association with maternal FGM. Female affiliative behavior may be collectively advantageous depending on the social group's environmental context. An increase in maternal FGM levels during pregnancy and lactation is associated with high FGM levels in pups, which may cause life-long changes in offspring behavior. Litters in social groups with mothers exhibiting high FGM levels throughout the active season received less maternal and/or alloparental care. Females with high FGM levels had offspring with lower annual survival and therefore have an overall lower reproductive success, which supports the cort-fitness hypothesis. Mothers under adverse conditions may, however, maximize offspring fitness by producing small litters.

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Declaration of competing interest

None.

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References

Allainé, D., 2000. Sociality, mating system and reproductive skew in marmots: evidence and hypotheses. Behav. Process. 51, 21–34. https://doi.org/10.1016/S0376-6357(00)00116-9.

Anisman, H., Merali, Z., 1999. Understanding stress: characteristics and caveats. Alcohol Res. Heal. 23, 241–249. https://doi.org/10.1053/meta.2002.33184.

Armitage, K.B., 1962. Social behaviour of a colony of the yellow-bellied marmot (*Marmota flaviventris*). Anim. Behav. 10, 319–331. https://doi.org/10.1016/0003-3472(62)90055-6.

Armitage, K.B., 1982. Yellow-bellied marmot. In: Davis, D.E. (Ed.), CRC Handbook of Census Methods for Terrestrial Vertebrates. CRC Press, Inc, Boca Raton, Florida, USA, pp. 148–149.

Armitage, K.B., 2014. Marmot Biology: Sociality, Individual Fitness, and Population Dynamics. Cambridge University Press, Cambridge.

Armitage, K.B., Downhower, J.F., 1974. Demography of yellow-bellied marmot populations. Ecology 55, 1233–1245. https://doi.org/10.2307/1935452.

Armitage, K.B., Gurri-Glass, G.E., 1994. Communal nesting in yellow-bellied marmots. In: Rumiantsev, V.Y. (Ed.), Actual Problems of Marmots Investigation. ABF Publishing House, Moscow, pp. 14–26.

Armitage, K.B., Schwartz, O.A., 2000. Social enhancement of fitness in yellow-bellied marmots. Proc. Natl. Acad. Sci. 97, 12149–12152. https://doi.org/10.1073/pnas.200196097.

Arnold, W., 1988. Social thermoregulation during hibernation in alpine marmots (*Marmota marmota*). J. Comp. Physiol. B. 158, 151–156. https://doi.org/10.1007/BF01075828.

Barash, D.P., 1974. The evolution of marmot societies: a general theory. Science 185, 415–420. https://doi.org/10.1126/science.185.4149.415.

Barrat, A., Barthelemy, M., Vespignani, A., 2004. Modeling the evolution of weighted networks. Phys. Rev. 70, 1–12. https://doi.org/10.1103/PhysRevE.70.066149.

Bates, D., Maechler, M., Bolker, B., 2015. Package "lme4" (Version 1.1–12). http://lme4.r-forge.r-project.org.

Bateson, P., Gluckman, P., Hanson, M., 2014. The biology of developmental plasticity and the predictive adaptive response hypothesis. J. Physiol. 592, 2357–2368. https://doi.org/10.1113/jphysiol.2014.271460.

Bauer, C.M., Hayes, L.D., Ebensperger, L.A., Ramírez-estrada, J., León, C., Davis, G.T., Romero, L.M., 2015. Maternal stress and plural breeding with communal care affect development of the endocrine stress response in a wild rodent. Horm. Behav. 75, 18–24. https://doi.org/10.1016/j.yhbeh.2015.07.021.

Beery, A.K., Kaufer, D., 2015. Stress, social behavior, and resilience: insights from rodents. Neurobiol. Stress 1, 116–127. https://doi.org/10.1016/j.ynstr.2014.10.004.

Birnie, A.K., Taylor, J.H., Cavanaugh, J., French, J.A., 2013. Quality of maternal and paternal care predicts later stress reactivity in the cooperatively-breeding marmoset (*Callithrix geoffroyi*). Psychoneuroendocrinology 38, 3003–3014. https://doi.org/10.1016/j.psyneuen.2013.08.011.

Blumstein, D.T., 2013. Yellow-bellied marmots: insights from an emergent view of sociality. Phil. Trans. R. Soc. B 368, 20120349. https://doi.org/10.1098/rstb.2012.0349.

Blumstein, D.T., Armitage, K.B., 1998. Life history consequences of social complexity: a comparative study of ground dwelling sciurids. Behav. Ecol. 9, 1–7. https://doi.org/10.1093/beheco/9.1.8.

Blumstein, D.T., Armitage, K.B., 1999. Cooperative breeding in marmots. Oikos 84, 369–382. https://doi.org/10.2307/3546418.
Blumstein, D.T., Daniel, J.C., 2007. Quantifying Behavior the JWatcher Way. Sinauer Associates, Inc., Sunderland, MA.
Blumstein, D.T., Steinmetz, J., Armitage, K.B., Daniel, J.C., 1997. Alarm calling in yellow-bellied marmots: II. The importance of direct fitness. Anim. Behav. 53, 173–184. https://doi.org/10.1006/anbe.1996.0286.
Blumstein, D.T., Runyan, A., Seymour, M., Ozgul, A., Ransler, F., Im, S., Stark, T., 2004. Locomotor ability and wariness in yellow-bellied marmots. Ethology 110, 615–634. https://doi.org/10.1111/j.1439-0310.2004.01000.x.
Blumstein, D.T., Patton, M.L., Saltzman, W., 2006. Faecal glucocorticoid metabolites and alarm calling in free-living yellow-bellied marmots. Biol. Lett. 2, 29–32. https://doi.org/10.1098/rsbl.2005.0405.
Blumstein, D.T., Wey, T.W., Tang, K., 2009. A test of the social cohesion hypothesis: interactive female marmots remain at home. Proc. R. Soc. B 276. https://doi.org/10.1098/rspb.2009.0703.
Blumstein, D.T., Lea, A.J., Olson, L.E., Martin, J.G.A., 2010. Heritability of anti-predatory traits: vigilance and locomotor performance in marmots. J. Evol. Biol. 23, 879–887. https://doi.org/10.1111/j.1420-9101.2010.01967.x.
Blumstein, D.T., Keeley, K.N., Smith, J.E., 2016. Fitness and hormonal correlates of social and ecological stressors of female yellow-bellied marmots. Anim. Behav. 112, 1–11. https://doi.org/10.1016/j.anbehav.2015.11.002.
Blumstein, D.T., Williams, D.M., Lim, A.N., Kroeger, S., Martin, J.G.A., 2018. Strong social relationships are associated with decreased longevity in a facultatively social mammal. Proc. R. Soc. B 285, 20171934. https://doi.org/10.1098/rspb.2017.1934.
Bonacich, P., 2007. Some unique properties of eigenvector centrality. Soc. Networks 29, 555–564. https://doi.org/10.1016/j.socnet.2007.04.002.
Bonier, F., Martin, P.R., Moore, I.T., Wingfield, J.C., 2009a. Do baseline glucocorticoids predict fitness? Trends Ecol. Evol. 24, 634–642. https://doi.org/10.1016/j.tree.2009.04.013.
Bonier, F., Moore, I.T., Martin, P.R., Robertson, R.J., 2009b. The relationship between fitness and baseline glucocorticoids in a passerine bird. Gen. Comp. Endocrinol. 163, 208–213. https://doi.org/10.1016/j.ygcen.2008.12.013.
Bonier, F., Martin, P.R., Moore, I.T., Wingfield, J.C., 2010. Clarifying the Cort-Fitness hypothesis: a response to Dingemanse et al. Trends Ecol. Evol. 25, 262–263. https://doi.org/10.1016/j.tree.2010.01.009.
Branchi, I., Andrea, I.D., Fiore, M., Fausto, V. Di, Aloe, L., Alleva, E., 2006. Early social enrichment shapes social behavior and nerve growth factor and brain-derived neurotrophic factor levels in the adult mouse brain. Biol. Psychiatry 60, 690–696. https://doi.org/10.1016/j.biopsych.2006.01.005.
Broussard, D.R., Dobson, F.S., Murie, J.O., 2008. Previous experience and reproductive investment of female Columbian ground squirrels. J. Mammal. 89, 145–152. https://doi.org/10.1644/06-MAMM-A-357.1.
Brummelte, S., Galea, L.A.M., 2010. Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. Horm. Behav. 58, 769–779. https://doi.org/10.1016/j.yhbeh.2010.07.012.
Cairns, S.J., Schwager, S.J., 1987. A comparison of association indices. Anim. Behav. 35, 1454–1469. https://doi.org/10.1016/S0003-3472(87)80018-0.
Cameron, E.Z., Setsaas, T.H., Linklater, W.L., 2009. Social bonds between unrelated females increase reproductive success in feral horses. Proc. Natl. Acad. Sci. 106, 13850–13853. https://doi.org/10.1073/pnas.0900639106.
Chalfin, L., Dayan, M., Levy, D.R., Austad, S.N., Miller, R.A., Iraqi, F.A., Dulac, C., Kimchi, T., 2014. Mapping ecologically relevant social behaviours by gene knockout in wild mice. Nat. Commun. 5, 1–10. https://doi.org/10.1038/ncomms5569.
Champagne, F.A., Meaney, M.J., 2006. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. Biol. Psychiatry 59, 1227–1235. https://doi.org/10.1016/j.biopsych.2005.10.016.
Cohen, S., 2004. Social relationships and health. Am. Psychol. 59, 676–684. https://doi.org/10.1037/0003-066X.59.8.676.
Colas, S., 1999. Evidence for sex-biased behavioral maternal investment in the gray mouse lemur (*Microcebus murinus*). Int. J. Primatol. 20, 911–926. https://doi.org/10.1023/A:1020878618941.
Cruces, J., Venero, C., Pereda-pérez, I., Fuente, M. De, 2014. The effect of psychological stress and social isolation on neuroimmunoendocrine communication. Curr. Pharm. Des. 20, 4608–4628. https://doi.org/10.2174/1381612820666140130205822.
Csardi, G., Nepusz, T., 2006. The igraph software package for complex network research. InterJournal Complex Syst 1695.
Curio, E., 1983. Why do young birds reproduce less well? In: Ibis (Lond. 1859). vol. 125. pp. 400–404. https://doi.org/10.1111/j.1474-919X.1983.tb03130.x.
Curley, J.P., Champagne, F.A., 2016. Influence of maternal care on the developing brain: mechanisms, temporal dynamics and sensitive periods. Front. Neuroendocrinol. 40, 52–66. https://doi.org/10.1016/j.yfrne.2015.11.001.Influence.
Curley, J.P., Davidson, S., Bateson, P., Champagne, F.A., 2009. Social enrichment during postnatal development induces transgenerational effects on emotional and reproductive behavior in mice. Front. Behav. Neurosci. 3, 1–14. https://doi.org/10.3389/neuro.08.025.2009.
Curley, J.P., Jensen, C.L., Mashoodh, R., Champagne, F.A., 2011. Social influences on neurobiology and behavior: epigenetic effects during development. Psychoneuroendocrinology 36, 352–371. https://doi.org/10.1016/j.psyneuen.2010.06.005.
Daft, J.G., Ptacek, T., Kumar, R., Morrow, C., Lorenz, R.G., 2015. Cross-fostering immediately after birth induces a permanent microbiota shift that is shaped by the nursing mother. Microbiome 3, 17. https://doi.org/10.1186/s40168-015-0080-y.
Darnaudéry, M., Maccari, S., 2008. Epigenetic programming of the stress response in male and female rats by prenatal restraint stress. Brain Res. Rev. 57, 571–585. https://doi.org/10.1016/j.brainresrev.2007.11.004.
Dettmer, A.M., Murphy, A.M., Guitarra, D., Slonecker, E., Suomi, S.J., Rosenberg, K.L., Novak, M.A., Meyer, J.S., Hinde, K., 2018. Cortisol in neonatal mother's milk predicts later infant social and cognitive functioning in rhesus monkeys. Child Dev. 89, 525–538. https://doi.org/10.1111/cdev.12783.
Dipietro, J.A., Novak, M.F.S.X., Costigan, K.A., Atella, L.D., Reusing, S.P., 2006. Maternal psychological distress during pregnancy in relation to child development at age two. Child Dev. 77, 573–587. https://doi.org/10.1111/j.1467-8624.2006.00891.x.
Djurhuus, C.B., Gravholt, C.H., Nielsen, S., Pedersen, S.B., Møller, N., Schmitz, O., 2004. Additive effects of cortisol and growth hormone on regional and systemic lipolysis in humans. AJP Endocrinol. Metab. 286, E488–E494. https://doi.org/10.1152/ajpendo.00199.2003.
Dwyer, C.M., Lawrence, A.B., 2000. Maternal behaviour in domestic sheep (*Ovis aries*): Constancy and change with maternal experience. Behaviour 137, 1391–1413. https://psycnet.apa.org/doi/10.1163/156853900501999.
Ferron, J., 1985. Social behavior of the golden-mantled ground squirrel (*Spermophilus lateralis*). Can. J. Zool. 63, 2529–2533. https://doi.org/10.1139/z85-375.
Fleming, A.S., Kraemer, G.W., Gonzalez, A., Lovic, V., Rees, S., Melo, A., 2002. Mothering begets mothering: the transmission of behavior and its neurobiology across generations. Pharmacol. Biochem. Behav. 73, 61–75. https://doi.org/10.1016/S0091-3057%2802%2900793-1.
Forslund, P., Part, T., 1995. Age and reproduction in birds - hypotheses and tests. Trends Ecol. Evol. 10, 374–378. https://doi.org/10.1016/S0169-5347(00)89141-7.
Frase, B.A., Hoffmann, R.S., 1980. Marmota flaviventris. Mamm. Species (135), 1–8. https://doi.org/10.2307/3503965.
Fuong, H., Maldonado-Chaparro, A., Blumstein, D.T., 2015. Are social attributes associated with alarm calling propensity? Behav. Ecol. 26, 587–592. https://doi.org/10.1093/beheco/aru235.
Hall, F.S., 1998. Social deprivation of neonatal, adolescent, and adult rats has distinct neurochemical and behavioral consequences. Crit. Rev. Neurobiol. 12, 129–162. https://doi.org/10.1615/CritRevNeurobiol.v12.i1-2.50.
Hasselt, F.N. Van, Tieskens, J.M., Trezza, V., Krugers, H.J., Vanderschuren, L.J.M.J., Joëls, M., 2012. Within-litter variation in maternal care received by individual pups correlates with adolescent social play behavior in male rats. Physiol. Behav. 106, 701–706. https://doi.org/10.1016/j.physbeh.2011.12.007.
Hinde, K., Skibiel, A.L., Foster, A.B., Rosso, D., Sally, P., 2015. Cortisol in mother's milk across lactation reflects maternal life history and predicts infant temperament. Behav. Ecol. 26, 269–281. https://doi.org/10.1093/beheco/aru186.
Holt-Lunstad, J., Smith, T.B., Layton, J.B., 2010. Social relationships and mortality risk: a meta-analytic review. PLoS Med. 7, e1000316. https://doi.org/10.1371/journal.pmed.1000316.
House, J.S., Landis, K.R., Umberson, D., 1988. Social relationships and health. Science 241, 540–545. https://doi.org/10.1126/science.3399889.
Iossa, G., Soulsbury, C.D., Baker, P.J., Edwards, K.J., Harris, S., 2008. Behavioral changes associated with a population density decline in the facultatively social red fox. Behav. Ecol. 20, 385–395. https://doi.org/10.1093/beheco/arn149.
Jenni-Eiermann, S., Glaus, E., Gru, M., Schwabl, H., Jenni, L., 2008. Glucocorticoid response to food availability in breeding barn swallows (*Hirundo rustica*). Gen. Comp. Endocrinol. 155, 558–565. https://doi.org/10.1016/j.ygcen.2007.08.011.
Kalinowski, S.T., Taper, M.L., Marshall, T.C., 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16, 1099–1106. https://doi.org/10.1111/j.1365-294X.2007.03089.x.
Kassambara, A., 2018. Ggpubr: "ggplot2" based publication ready plots. R package version 0.1.7. https://CRAN.R-project.org/package=ggpubr.
Keiser, C.N., Modlmeier, A.P., Singh, N., Jones, D.K., Pruitt, J.N., 2014. Exploring how a shift in the physical environment shapes individual and group behavior across two social contexts. Ethology 120, 825–833. https://doi.org/10.1111/eth.12256.
Kiyokawa, Y., Hennessy, M.B., 2018. Comparative studies of social buffering: a consideration of approaches, terminology, and pitfalls. Neurosci. Biobehav. Rev. 86, 131–141. https://doi.org/10.1016/j.neubiorev.2017.12.005.
Klaus, T., Schöpper, H., Huber, S., 2013. Effects of chronic stress during pregnancy on maternal performance in the guinea pig (*Cavia aperea f. porcellus*). Behav. Process. 94, 83–88. https://doi.org/10.1016/j.beproc.2012.12.006.
Klein, E.S., Barbier, M.R., Watson, J.R., 2017. The dual impact of ecology and management on social incentives in marine common-pool resource systems. R. Soc. Open Sci. 4, 170740. https://doi.org/10.1098/rsos.170740.
Kudielka, B.M., Kirschbaum, C., 2005. Sex differences in HPA axis responses to stress: a review. Biol. Psychol. 69, 113–132. https://doi.org/10.1016/j.biopsycho.2004.11.009.
Künkele, J., Kenagy, G.J., 1997. Inefficiency of lactation in primiparous rats: the costs of first reproduction. Physiol. Zool. 70, 571–577. https://doi.org/10.1086/515862.
Künzl, C., Sachser, N., 1999. The behavioral endocrinology of domestication: a comparison between the domestic guinea pig (*Cavia aperea f. porcellus*) and its wild ancestor, the cavy (*Cavia aperea*). Horm. Behav. 35, 28–37. https://doi.org/10.1006/hbeh.1998.1493.
Künzl, C., Kaiser, S., Meier, E., Sachser, N., 2003. Is a wild mammal kept and reared in captivity still a wild animal? Horm. Behav. 43, 187–196. https://doi.org/10.1016/S0018-506X(02)00017-X.
Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2017. lmerTest package: tests in linear mixed effects models. J. Stat. Softw. 82, 1–26. https://doi.org/10.18637/jss.v082.i13.
Lahvis, G.P., Panksepp, J.B., Kennedy, B.C., Wilson, C.R., Merriman, D.K., 2015. Social conditioned place preference in the captive ground squirrel (*Ictidomys Tridecemlineatus*): social reward as a natural phenotype. J. Comp. Psychol. 129, 291–303. https://doi.org/10.1037/a0039435.
Lea, A.J., Blumstein, D.T., Wey, T.W., Martin, J.G.A., 2010. Heritable victimization and the benefits of agonistic relationships. Proc. Natl. Acad. Sci. 107, 21587–21592.

https://doi.org/10.1073/pnas.1009882107.
Liu, D., Diorio, J., Tannenbaum, B., Caldji, C., Francis, D., Freedman, A., Sharma, S., Pearson, D., Plotsky, P.M., Meaney, M.J., 1997. Maternal care, hippocampal glucocorticoid receptors, and hypothalamic-pituitary-adrenal responses to stress. Science 277, 1659–1662. https://doi.org/10.1126/science.277.5332.1659.
Luby, J.L., Barch, D.M., Belden, A., Gaffrey, M.S., Tillman, R., Babb, C., Nishino, T., Suzuki, H., Botteron, K.N., 2012. Maternal support in early childhood predicts larger hippocampal volumes at school age. Proc. Natl. Acad. Sci. 109, 2854–2859. https://doi.org/10.1073/pnas.1118003109.
Lunn, N.J., Boyd, I.L., Croxall, J.P., 1994. Reproductive performance of female Antarctic fur seals: the influence of age, breeding experience, environmental variation and individual quality. J. Anim. Ecol. 63, 827–840. https://doi.org/10.2307/5260.
Mady, R.P., Blumstein, D.T., 2017. Social security: are socially connected individuals less vigilant? Anim. Behav. 134, 79–85. https://doi.org/10.1016/j.anbehav.2017.10.010.
Maguire, J., Mody, I., 2016. Behavioral deficits in juveniles mediated by maternal stress hormones in mice. Neural Plast 25016, 2762518. https://doi.org/10.1155/2016/2762518.
Maher, C.R., 2009. Effects of relatedness on social interaction rates in a solitary marmot. Anim. Behav. 78, 925–933. https://doi.org/10.1016/j.anbehav.2009.06.027.
Mairesse, J., Lesage, J., Breton, C., Bre, B., Hahn, T., Darnaude, M., Dickson, S.L., Seckl, J., Blondeau, B., Vieau, D., Maccari, S., Viltart, O., 2007. Maternal stress alters endocrine function of the feto-placental unit in rats. Am. J. Physiol. Endocrinol. Metab. 292, 1526–1533. https://doi.org/10.1152/ajpendo.00574.2006.
Maldonado-Chaparro, A.A., Hubbard, L., Blumstein, D.T., 2015. Group size affects social relationships in yellow-bellied marmots (*Marmota flaviventris*). Behav. Ecol. 26, 909–915. https://doi.org/10.1093/beheco/arv034.
Monclús, R., Tiulim, J., Blumstein, D.T., 2011. Older mothers follow conservative strategies under predator pressure: the adaptive role of maternal glucocorticoids in yellow-bellied marmots. Horm. Behav. 60, 660–665. https://doi.org/10.1016/j.yhbeh.2011.08.019.
Moody, J., White, D.R., 2003. Structural cohesion and embeddedness: a hierarchical concept of social groups. Am. Sociol. Rev. 68, 103–127. https://doi.org/10.2307/3088904.
Mora, S., Dussaubat, N., Díaz-Véliz, G., 1996. Effects of the estrous cycle and ovarian hormones on behavioral indices of anxiety in female rats. Psychoneuroendocrinology 21, 609–620. https://doi.org/10.1016/S0306-4530(96)00015-7.
Negrin, A., Fuentes, C., Espinosa, D., Dias, P., 2016. The loss of behavioral diversity as a consequence of anthropogenic habitat disturbance: the social interactions of black howler monkeys. Primates 57, 9–15. https://doi.org/10.1007/s10329-015-0503-1.
Ondrasek, N.R., Wade, A., Burkhard, T., Hsu, K., Nguyen, T., Post, J., Zucker, I., 2015. Environmental modulation of same-sex affiliative behavior in female meadow voles (*Microtus pennsylvanicus*). Physiol. Behav. 140, 118–126. https://doi.org/10.1016/j.physbeh.2014.12.021.
Palanza, P., Parmigiani, S., 2017. How does sex matter? Behavior, stress and animal models of neurobehavioral disorders. Neurosci. Biobehav. Rev. 76, 134–143. https://doi.org/10.1016/j.neubiorev.2017.01.037.
Palanza, P., Gioiosa, L., Parmigiani, S., 2001. Social stress in mice: gender differences and effects of estrous cycle and social dominance. Physiol. Behav. 73, 411–420. https://doi.org/10.1016/S0031-9384(01)00494-2.
Palme, R., 2005. Measuring fecal steroids: guidelines for practical application. Ann. N. Y. Acad. Sci. 1046, 75–80. https://doi.org/10.1196/annals.1343.007.
Pan, P., Fleming, A.S., Lawson, D., Jenkins, J.M., Mcgowan, P.O., 2014. Within- and between-litter maternal care alter behavior and gene regulation in female offspring. Behav. Neurosci. 128, 736–748. https://doi.org/10.1037/bne0000014.
Pan, P., Lawson, D.O., Dudin, A., Vasquez, O.E., Sokolowski, M.B., Fleming, A.S., McGowan, P.O., 2018. Both maternal care received and genotype influence stress - related phenotype in female rats. Dev. Psychobiol. 60, 889–902. https://doi.org/10.1002/dev.21770.
Pasinelli, G., Walters, J.R., 2002. Social and environmental factors affect natal dispersal and philopatry of male red-cockaded woodpeckers. Ecology 83, 2229–2239. https://doi.org/10.1890/0012-9658(2002)083[2229:SAEFAN]2.0.CO;2.
Peña, C.J., Champagne, F.A., 2013. Implications of temporal variation in maternal care for the prediction of neurobiological and behavioral outcomes in offspring. Behav. Neurosci. 127, 33–46. https://doi.org/10.1037/a0031219.
Petelle, M.B., McCoy, D.E., Alejandro, V., Martin, J.G.A., Blumstein, D.T., 2013. Development of boldness and docility in yellow-bellied marmots. Anim. Behav. 86, 1147–1154. https://doi.org/10.1016/j.anbehav.2013.09.016.
Petelle, M.B., Dang, B.N., Blumstein, D.T., 2017. The effect of maternal glucocorticoid levels on juvenile docility in yellow-bellied marmots. Horm. Behav. 89, 86–91. https://doi.org/10.1016/j.yhbeh.2016.12.014.
Pinter-Wollman, N., Isbell, L.A., Hart, L.A., 2009. The relationship between social behaviour and habitat familiarity in African elephants (*Loxodonta africana*). Proc. R. Soc. B 276, 1009–1014. https://doi.org/10.1098/rspb.2008.1538.
Pittet, F., Babb, J.A., Carini, L., Nephew, B.C., 2017. Chronic social instability in adult female rats alters social behavior, maternal aggression and offspring development. Dev. Psychobiol. 59, 291–302. https://doi.org/10.1002/dev.21491.
R Core Team, 2018. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
R Development Core Team, 2016. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Ralph, C.R., Tilbrook, A.J., 2016. The hypothalamo-pituitary-adrenal (HPA) axis in sheep is attenuated during lactation in response to psychosocial and predator stress. Domest. Anim. Endocrinol. 55, 66–73. https://doi.org/10.1016/j.domaniend.2015.11.003.
Reeder, D.M., Kunz, T.H., Widmaier, E.P., 2004. Baseline and stress-induced glucocorticoids during reproduction in the variable flying fox, Pteropus hypomelanus (Chiroptera: Pteropodidae). J. Exp. Zool. Part A 301, 682–690. https://doi.org/10.1002/jez.a.58.
Rosenblum, L.A., Andrews, M.W., 1994. Influences of environmental demand on maternal behavior and infant development. Acta Paediatr. Suppl. 397, 57–63. https://doi.org/10.1111/j.1651-2227.1994.tb13266.x.
Rosvall, M., Axelsson, D., Bergstrom, C.T., 2009. The map equation. Eur. Phys. J. Spec. Top. 178, 13–23. https://doi.org/10.1140/epjst/e2010-01179-1.
Ruf, T., Arnold, W., 2000. Mechanisms of social thermoregulation in hibernating Alpine marmots (*Marmota marmota*). In: Heldmaier, G., Klingenspor, M. (Eds.), Life in the Cold. Springer, pp. 81–94.
Sapolsky, R.M., Alberts, S.C., Altmann, J., 1997. Hypercortisolism associated with social subordinance or social isolation among wild baboons. Arch. Gen. Psychiatry 54, 1137–1143. https://doi.org/10.1001/archpsyc.1997.01830240097014.
Sapolsky, R.M., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89. https://doi.org/10.1210/er.21.1.55.
Sayler, A., Salmon, M., 1969. Communal nursing in mice: influence of multiple mothers on the growth of the young. Science 164, 1309–1310. https://doi.org/10.1126/science.164.3885.1309.
Schino, G., Troisi, A., 2005. Neonatal abandonment in Japanese macaques. Am. J. Phys. Anthropol. 126, 447–452. https://doi.org/10.1002/ajpa.20078.
Sheriff, M.J., Love, O.P., 2013. Determining the adaptive potential of maternal stress. Ecol. Lett. 16, 271–280. https://doi.org/10.1111/ele.12042.
Sheriff, M.J., Krebs, C.J., Boonstra, R., 2010. Assessing stress in animal populations: do fecal and plasma glucocorticoids tell the same story? Gen. Comp. Endocrinol. 166, 614–619. https://doi.org/10.1016/j.ygcen.2009.12.017.
Silk, J.B., 2007. The adaptive value of sociality in mammalian groups. Phil. Trans. R. Soc. B 362, 539–559. https://doi.org/10.1098/rstb.2006.1994.
Silk, J.B., Alberts, S.C., Altmann, J., 2003. Social bonds of female baboons. Science 302, 1231–1235. https://doi.org/10.1126/science.1088580.
Silk, J.B., Beehner, J.C., Bergman, T.J., Crockford, C., Engh, A.L., Moscovice, L.R., Wittig, R.M., Seyfarth, R.M., Cheney, D.L., 2009. The benefits of social capital: close social bonds among female baboons enhance offspring survival. Proc. R. Soc. B 276, 3099–3104. https://doi.org/10.1098/rspb.2009.0681.
Silk, J.B., Beehner, J.C., Bergman, T.J., Crockford, C., Engh, A.L., Moscovice, L.R., Wittig, R.M., Seyfarth, R.M., Cheney, D.L., 2010. Strong and consistent social bonds enhance the longevity of female baboons. Curr. Biol. 20, 1359–1361. https://doi.org/10.1016/j.cub.2010.05.067.
Smith, J.E., Monclús, R., Wantuck, D., Florant, G.L., Blumstein, D.T., 2012. Fecal glucocorticoid metabolites in wild yellow-bellied marmots: experimental validation, individual differences and ecological correlates. Gen. Comp. Endocrinol. 178, 417–426. https://doi.org/10.1016/j.ygcen.2012.06.015.
Snyder, R.J., Perdue, B.M., Zhang, Z., Maple, T.L., Charlton, B.D., 2016. Giant panda maternal care: a test of the experience constraint hypothesis. Sci. Rep. 6, 27509. https://doi.org/10.1038/srep27509.
Stroud, L.R., Salovey, P., Epel, E.S., 2002. Sex differences in stress responses: social rejection versus achievement stress. Biol. Psychiatry 52, 318–327. https://doi.org/10.1016/S0006-3223(02)01333-1.
Sunderland, N., Heffernan, S., Thomson, S., Hennessy, A., 2008. Maternal parity affects neonatal survival rate in a colony of captive bred baboons (*Papio hamadryas*). J. Med. Primatol. 37, 223–228. https://doi.org/10.1111/j.1600-0684.2007.00277.x.
Taylor, S.E., Klein, L.C., Lewis, B.P., Gruenewald, T.L., Gurung, R.A.R., Updegraff, J.A., 2000. Biobehavioral responses to stress in females: tend-and-befriend, not fight-or-flight. Psychol. Rev. 107, 411–429. https://psycnet.apa.org/doi/10.1037/0033-295X.107.3.411.
Toki, S., Morinobu, S., Imanaka, A., Yamamoto, S., Yamawaki, S., Honma, K., 2007. Importance of early lighting conditions in maternal care by dam as well as anxiety and memory later in life of offspring. Eur. J. Neurosci. 25, 815–829. https://doi.org/10.1111/j.1460-9568.2007.05288.x.
Touma, C., Palme, R., 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Ann. N. Y. Acad. Sci. 1046, 54–74. https://doi.org/10.1196/annals.1343.006.
Tu, T.M., Lupien, S.J., Walker, C.D., 2005. Measuring stress responses in postpartum mothers: perspectives from studies in human and animal populations. Stress 8, 19–34. https://doi.org/10.1080/10253890500103806.
Ubeda, C., Lipuma, L., Gobourne, A., Viale, A., Leiner, I., Equinda, M., Khanin, R., Pamer, E.G., 2012. Familial transmission rather than defective innate immunity shapes the distinct intestinal microbiota of TLR-deficient mice. J. Exp. Med. 209, 1445–1456. https://doi.org/10.1084/jem.20120504.
Van Vuren, D.H., 2001. Predation on yellow-bellied marmots (*Marmota flaviventris*). Am. Midl. Nat. 145, 94–100.
Vander Wal, E., Festa-Bianchet, M., Réale, D., Coltman, D.W., Pelletier, F., 2015. Sex-based differences in the adaptive value of social behavior contrasted against morphology and environment. Ecology 96, 631–641. https://doi.org/10.1890/14-1320.1.
Wang, Z., Novak, M.A., 1994. Parental care and litter development in primiparous and multiparous prairie voles (*Microtus ochrogaster*). J. Mammal. 75, 18–23. https://doi.org/10.2307/1382232.
Wasserman, S., Faust, K., 1994. Social Network Analysis: Methods and Applications. Cambridge University Press, New York.
Weaver, I.C.G., Cervoni, N., Champagne, F.A., Alessio, A.C.D., Sharma, S., Seckl, J.R., Dymov, S., Szyf, M., Meaney, M.J., 2004. Epigenetic programming by maternal behavior. Nature 7, 847–854. https://doi.org/10.1038/nn1276.
Wey, T.W., Blumstein, D.T., 2012. Social attributes and associated performance measures in marmots: bigger male bullies and weakly affiliating females have higher annual reproductive success. Behav. Ecol. Sociobiol. 66, 1075–1085. https://doi.org/10.

1007/s00265-012-1358-8.
Wey, T., Blumstein, D.T., Shen, W., Jordán, F., 2008. Social network analysis of animal behaviour: a promising tool for the study of sociality. Anim. Behav. 75, 333–344. https://doi.org/10.1016/j.anbehav.2007.06.020.
Wey, T.W., Lin, L., Patton, M.L., Blumstein, D.T., 2015. Stress hormone metabolites predict overwinter survival in yellow-bellied marmots. Acta Ethol 18, 181–185. https://doi.org/10.1007/s10211-014-0204-6.
Whirledge, S., Cidlowski, J.A., 2010. Glucocorticoids, stress, and fertility. Minerva Endocrinol. 35, 109–125. https://doi.org/10.1586/eem.10.1.
Whitehead, H., 2009. SOCPROG programs: Analysing animal social structures. Behav. Ecol. Sociobiol. 63, 765–778. https://doi.org/10.1007/s00265-008-0697-y.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Williams, C.T., Kitaysky, A.S., Kettle, A.B., Buck, C.L., 2008. Corticosterone levels of tufted puffins vary with breeding stage, body condition index, and reproductive performance. Gen. Comp. Endocrinol. 158, 29–35. https://doi.org/10.1016/j.ygcen.2008.04.018.
Yang, W.J., Maldonado-Chaparro, A.A., Blumstein, D.T., 2017. A cost of being amicable in a hibernating mammal. Behav. Ecol. 28, 11–19. https://doi.org/10.1093/beheco/arw125.
Yoerg, S.I., 1999. Solitary is not asocial: effects of social contact in kangaroo rats (heteromyidae: *Dipodomys heermanni*). Ethology 105, 317–333. https://doi.org/10.1046/j.1439-0310.1999.00392.x.
Zedrosser, A., Dahle, B., Swenson, J.E., 2009. The effects of primiparity on reproductive performance in the brown bear. Oecologia 160, 847–854. https://doi.org/10.1007/s00442-009-1343-8.
Zuur, A.F., Ieno, E.N., Walker, N.J., Saveliev, A.A., Smith, G.M., 2009. Models and Extensions in Ecology with R. Springer, New York.

CHAPTER 3

Hibernation slows epigenetic aging in yellow-bellied marmots

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Abstract

Species that hibernate live longer than would be expected based solely on their body size. Hibernation is characterized by long periods of metabolic suppression (torpor) interspersed by short periods of increased metabolism (arousal). The torpor-arousal cycles occur multiple times during hibernation, and it has been suggested that processes controlling the transition between torpor and arousal states cause aging suppression. Metabolic rate is also a known correlate of longevity, we thus proposed the 'hibernation-aging hypothesis' whereby aging is suspended during hibernation. We tested this hypothesis in a well-studied population of yellow-bellied marmots (*Marmota flaviventer*), which spend 7-8 months per year hibernating. We used two approaches to estimate epigenetic age: the epigenetic clock and the epigenetic pacemaker. Variation in epigenetic age of 149 samples collected throughout the life of 73 females were modeled using generalized additive mixed models (GAMM), where season (cyclic cubic spline) and chronological age (cubic spline) were fixed effects. As expected, the GAMM using epigenetic ages calculated from the epigenetic pacemaker was better able to detect nonlinear patterns in epigenetic age change over time. We observed a logarithmic curve of epigenetic age with time, where the epigenetic age increased at a higher rate until females reached sexual maturity (2-years old). With respect to circannual patterns, the epigenetic age increased during the summer and essentially stalled during the winter. Our enrichment analysis of age-related CpG sites revealed pathways related to development and cell differentiation, while the season-related CpGs enriched pathways related to central carbon metabolism, immune system, and circadian clock. Taken together, our results are consistent with the hibernation-aging hypothesis and may explain the enhanced longevity in hibernators.

Key words: DNA methylation, epigenetic clock, epigenetic pacemaker, seasonality, torpor

Introduction

Aging is a poorly understood natural phenomenon, characterized by an age-progressive decline in intrinsic physiological function[1,2]. The high variation in disease and functional impairment risk among same-age individuals shows that biological age is uncoupled from chronological age[3–5]. Some individuals age at faster rates than others, and little is known about the causes of this inter-individual variance in biological aging rates[6,7]. To this end, researchers have been attempting to develop biomarkers of aging[4,8]. DNA methylation (DNAm) based age estimators, also known as epigenetic clocks (ECs), are arguably the most accurate molecular estimators of age[3,9–12]. An EC is usually defined as a penalized regression, where chronological age is regressed on methylation levels of individual cytosines[13]. The EC has been successfully used to study human aging, and is becoming increasingly used to study aging in other species[14–20].

Biological processes underpinning ECs remain to be characterized[11]. Age-adjusted estimates of epigenetic age (epigenetic age acceleration) are associated with a host of age related conditions and stress factors, such as cumulative lifetime stress[21], smoking habits[22,23], all-cause mortality[24–28], and age-related conditions/diseases[13,26,29,30]. These associations suggest that epigenetic age is an indicator of biological age[5,31]. In fact, measures of epigenetic aging rates are associated with longevity at the individual level as well as across mammalian species[6,17]. Several studies present evidence that long-lived species age more slowly at an epigenetic level[19,20,31–33]. The link between the epigenetic aging and biological aging is further reinforced by the observation that treatments known to increase lifespan significantly slow the EC[15,17].

Longevity is related to body size, but some species have longer lifespans than expected based on their body size[34,35]. A characteristic of long-lived species is the ability to engage in bouts of torpor[36,37]. Torpor is a hypometabolic state characterized by a dramatic decrease in gene transcription and translation rates[38–43]. During hibernation, torpor bouts are interspersed by short periods of euthermy (< 24 h), when gene expression occurs and metabolism is fully recovered[41,44]. Some of the physiological stresses from the cyclic transition between deep torpor and euthermy are similar to the ones experienced by the aging body (e.g., oxidative stress), and promote responses in cellular signaling pathways that are essential for both longevity and torpor survival[36,45]. Thus, the cellular and molecular stress responses associated with torpor-arousal cycles and long periods of inactivity may suppress aging[36,45].

Hibernation is mostly comprised of long periods of metabolic suppression, and overall metabolic rate reduction is associated with longevity[45–47]. Therefore, we hypothesize that aging is reduced during hibernation which we refer to as the hibernation-aging hypothesis. Specifically, a species that engages in torpor may periodically "suspend" aging, as previously suggested[36]. With this rationale, we predict that the epigenetic aging is faster during the active season and slower during hibernation. We test this prediction in yellow-bellied marmots (*Marmota flaviventer*), which spend 7-8 months per year hibernating[48]. Torpor bouts represent 88.6% of the yellow-bellied marmot hibernation period, resulting in an average energy saving of 83.3% when compared to the energetic expenditure of an euthermic adult[48,49].

Methods

All samples were collected as part of a long-term study of a free-living population of yellow-bellied marmots in the Gunnison National Forest, Colorado (USA), where marmots were

captured and blood samples collected biweekly during the their active season (May to August[50]). Data and samples were collected under the UCLA Institutional Animal Care and Use protocol (2001-191-01, renewed annually) and with permission from the Colorado Parks and Wildlife (TR917, renewed annually).

Individuals were monitored throughout their lives, and chronological age was calculated based on the date at which juveniles first emerged from their natal burrows. We only used female samples because precise age for most adult males is unavailable since males are typically immigrants born elsewhere[51,52]. We selected 160 whole blood samples from 78 females with varying ages. From these, DNA methylation (DNAm) profiling worked well for 149 samples from 73 females with ages varying from 0.01 to 12.04 years.

Genomic DNA was extracted with Qiagen DNeasy blood and tissue kit and quantified with Qubit. DNAm profiling was performed with the custom Illumina chip HorvathMammalMethylChip40[53]. This array, referred to as mammalian methylation array, profiles 36 thousand CpG sites in conserved genomic regions across mammals. From all probes, 31,388 mapped uniquely to CpG sites (and its respective flanking regions) in the yellow-bellied marmot assembly (GenBank assembly accession: GCA_003676075.2). We used the SeSaMe normalization method to estimate β values for each CpG site[54].

Two model approaches were used to study epigenetic aging in marmots: the epigenetic clock[9,10] and the epigenetic pacemaker[55–58]. Both models are described below.

Epigenetic clock (EC)

Under the EC a linear correlation with age is determined by attempting to fit a single coefficient to each CpG site. We fitted a generalized linear model with elastic-net penalization[59] to the

chronological-age and β-value data sets using the glmnet v.4.0-2 package in R[60]. Alpha was set to 0.5, which assigns ridge and lasso penalties with same weight. The elastic-net penalization limits the impact of collinearity and shrinks irrelevant coefficients to zero. This method estimates coefficients that minimize the mean squared error between chronological and predicted ages, and performs an automatic selection of CpG sites for age prediction. We applied a 10-fold cross validation to select the model with lowest error based on the training set. Predicted ages were scored for samples not included in the training set of the model (code is available in the supplementary material). In this respect, the predicted age was estimated for groups of ~14 samples, resulting in 11 EC models. These models comprised 360 sites, and the average coefficient per site and intercept are available in the supplementary material.

Epigenetic pacemaker

While ECs are used to estimate the age of a sample based on weighted sums of methylation values, the epigenetic pacemaker (EPM) models the dynamics of methylation across the genome. To accomplish this, it models each individual CpG site as a linear function of an underlying epigenetic state of an individual. This epigenetic state changes with time in a nonlinear fashion, and we are therefore able to use this paradigm to identify periods with variable rates of methylation changes throughout lifespan. The EPM assumes that the relative increase/decrease rate of methylation levels among sites remains constant, but the absolute rates can be modified when rates at all sites change in synchrony[56–58]. The optimum values of methylation change rate and initial methylation level per site, as well as the epigenetic state per sample, are calculated through iterations implemented in a fast conditional expectation maximization algorithm[61] to minimize the residual sum of squares error between known and estimated methylation levels (β

values). Thus, the epigenetic state is an estimate of age that, given the methylation rates and initial methylation levels for each site, minimizes the differences between known and estimated methylation levels in a specific sample for all sites included in the model. We selected sites to use in the EPM based on the absolute Pearson correlation coefficients (r) between chronological age and methylation levels per site[57,58]. All sites with $r > 0.7$ were included, which resulted in 309 sites. A 10-fold cross validation was used to estimate epigenetic states (supplementary material). We report the rate and intercept values per site from the EPM using all data as training set (no cross validation, supplementary material).

Hibernation-aging hypothesis

We fitted two Generalized Additive Mixed Models (GAMM) with the EPM- or the EC-estimated epigenetic age as dependent variable. For both GAMMs, fixed effects included a cubic spline function for chronological age, and a cyclic cubic spline function for day of year. We also tested for the interaction between these two variables (using tensor product interaction with a cubic spline). Individual identity was added as random effect. Day of year varied from 1 to 365, with 1 representing 1 May and 365 representing 30 April.

We used simulations to estimate the type 1 error and the potential power to detect a hibernation-aging effect given the limitations of our sample collection. Specifically, blood samples could only be collected during the active season, instead of throughout the year. Our earliest sample was collected on 27 April and the latest on 20 August. We simulated two different traits: (1) a trait that increases linearly with age independently of the season; and (2) a trait that increases during the summer but not during the winter. The daily rate of increase for the first trait was set at 0.004, to simulate data with a similar range to the observed EPM data. For the second trait, the

rate of increase was set to zero during winter (16 Sept to 17 April, days 139-352 using 1 May as reference). The simulation assumed that the active season was 150 days long starting on 18 April (day 353) and finishing on 15 Sept (day 138). The rate of increase during the active season was set as 0.0164 (0.004 / 365 * 150) so that the annual rate of increase was similar between the two simulated traits. Our simulation was parameterized using among-individual and residual variance from the EPM. We performed these simulations using field data (day of sample collection, age in days, birth date, and number of samples), and estimated the significance of the seasonal effect (cyclic spline with days since 1 May). We repeated this procedure 1000 times for both traits. The proportion of simulations on trait 1 (no seasonal effect) that were significant indicated our type 1 error. The proportion of simulations on trait 2 (seasonal effect) that were significant was an indication of the power to detect this effect.

We evaluated GAMMs by checking convergence, concurvity between fixed effects and the autocorrelation of deviance residuals. We also checked model fit by plotting fitted with observed epigenetic state and visually inspected qq plots and histograms of deviance residuals, plots of deviance residuals with fitted values, and plots of deviance residuals with explanatory variables. GAMMs were fitted and checked using the mgcv R package v.1.8[62]. All analysis and figures were developed in R v.3.6.3[63] in RStudio v. 1.2.5033[64], python v.3.7.4[65], Jupyter notebook v.6.0.3[66], ggplot2 v.3.3.0.9[67], and ggpubr v.0.2.5[68].

Influence of chronological age and seasons on methylation levels per CpG site

We performed additional analyses to identify which CpG sites were associated with age and seasonality. We fitted a Generalized Additive Model (GAM) per CpG site, where methylation

level was the dependent variable. The independent variables were a cubic spline function for chronological age and a cyclic cubic spline function for day of year.

Since epigenome wide association studies of age (EWAS) have been more commonly used to identify CpG sites related to chronological age, we performed a linear regression per CpG site. Each model had methylation level as dependent variable and chronological age as independent variable. Significance thresholds were set to $1x10^{-5}$.

CpG site enrichment analysis

Gene enrichment was performed with the Genomic Regions Enrichment Tool v.3.0.0 (GREAT hypergeometric test[69]). GREAT analyzes the potential cis-regulatory role of the non-coding regions with CpG sites of interest, and identifies which pathways are overrepresented in the data. To associate CpGs with genes, we used the "Basal plus extension" association with a maximum window distance between the CpG and the genes of 50 kb. GREAT tests the observed distribution of CpG neighboring genes against the expected number of sites associated with each pathway due to their representation in the mammalian array (background set). Since GREAT requires a high quality annotation, we used the respective locations of the marmot sites on the human assembly (GRCh37), and therefore only used sites mapped to conserved genes between marmots and humans. Two data sets were analyzed: sites associated with chronological age and with day of year. The alignment and annotation methods are described in the mammalian methylation array method paper[53].

Results

The epigenetic aging models developed with the epigenetic clock (EC) and the epigenetic pacemaker (EPM) were both highly accurate (Figure 1), showing high correlations between

epigenetic and chronological age (r = 0.98 and 0.92, respectively). The EC used 360 CpG sites, whereas the EPM used 309 sites. There was an overlap of 72 sites between the two models.

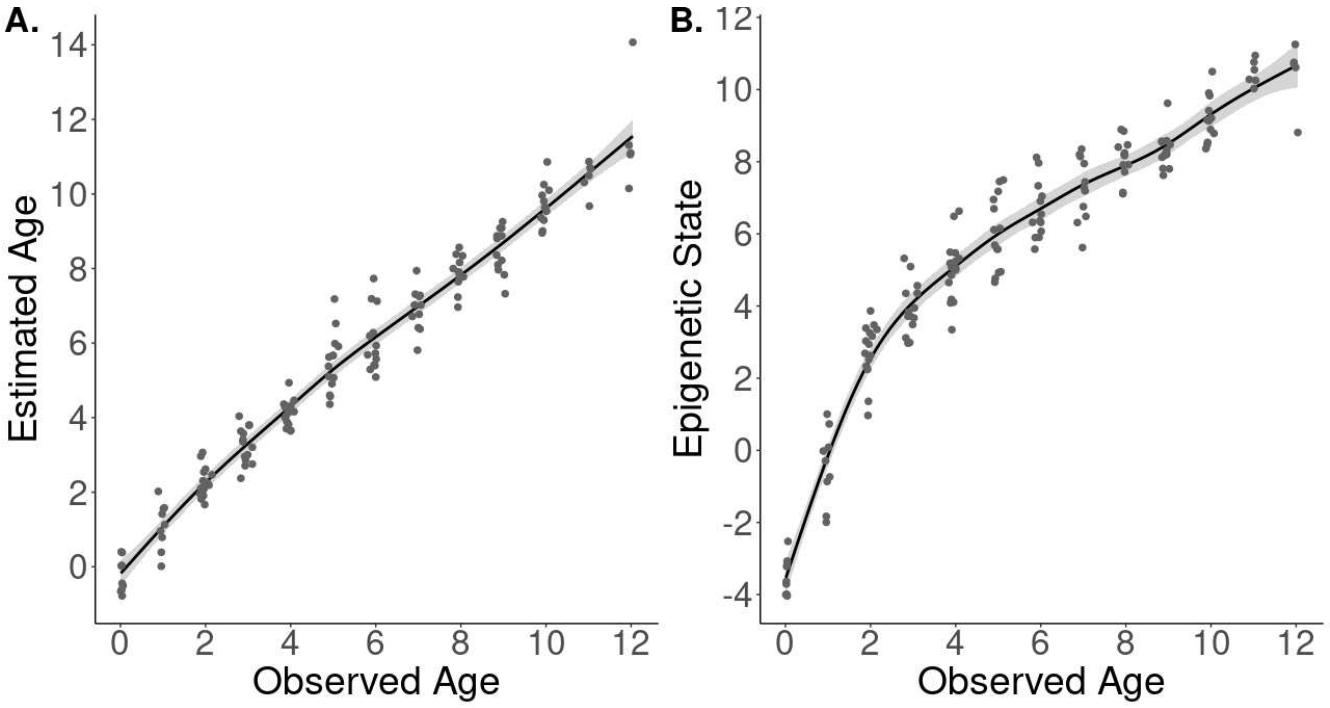

Figure 1: Epigenetic aging models for a wild population of yellow-bellied marmots developed from the epigenetic clock (A), and the epigenetic pacemaker (B). Points represent samples from individuals of known age at the sampling moment (Observed Age), and y-axis represent the epigenetic age calculated by each model. Trend lines were developed by fitting cubic splines.

The GAMM fitted to the predicted age from the EC explained 96.6% of the variation (Adj. R^2) and had a residual variance of 0.346. The random effect of individual identity had an intercept variance of 0.009. The age spline was significant ($F = 1225.76$, $p < 0.0001$) and the cyclic spline for days since 1 May was not significant ($p = 0.78$). The tensor interaction smooths was also not significant ($p = 0.11$). Details of this model are described in the supplementary material.

The GAMM fitted to the epigenetic state data explained 95.6% of the variation and had a residual variance of 0.284. The random effect of individual identity had an intercept variance of

0.332. Both smooth terms significantly influenced marmot epigenetic state ($p < 0.005$, Table 1), but the interaction between them was not significant ($p = 0.44$). The effect of chronological age and day of year result in a particular pattern of epigenetic state change (Figure 2A). The partial effect of day on epigenetic state shows an increase in epigenetic state during the summer and suggests a reversal of such changes during the winter (Figure 2B). Moreover, the rate of epigenetic state increase is the highest in the mid-point of the active season. The partial effect of chronological age shows that the epigenetic state increases at a higher rate until females reach 2-years old, followed by a deceleration as individuals become older (Figure 2C).

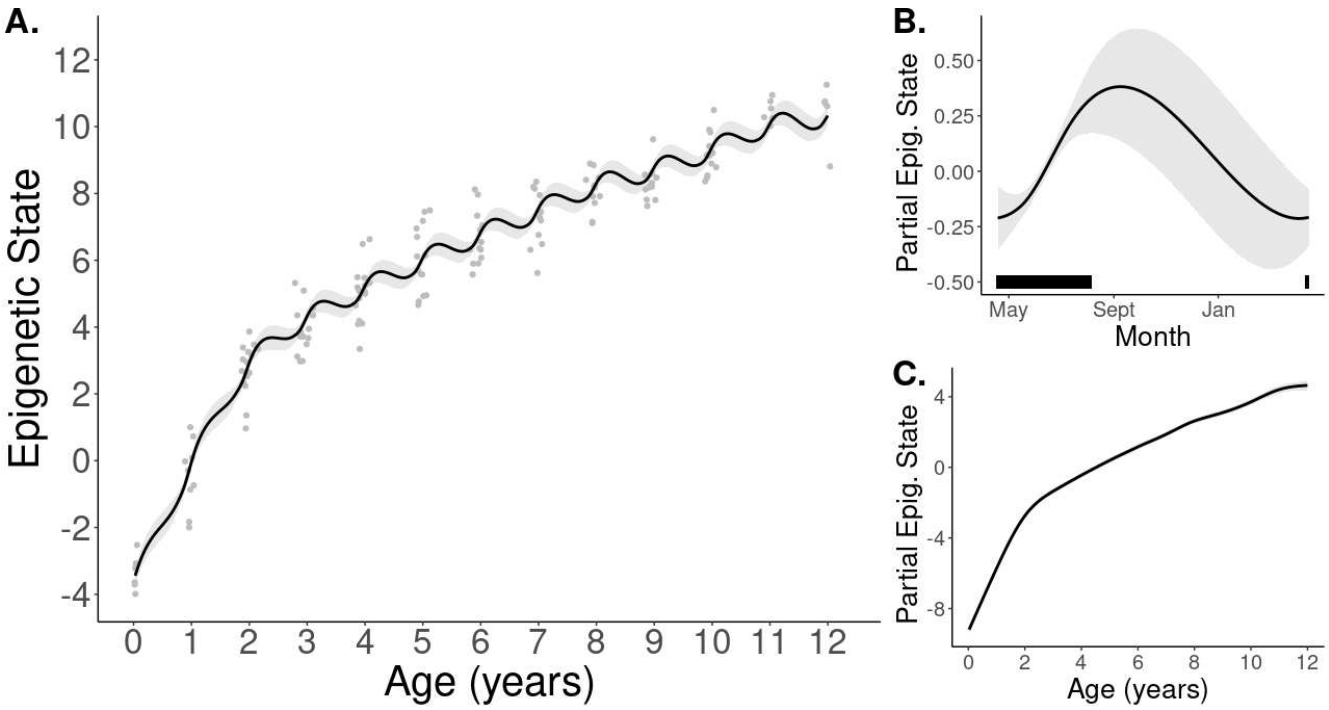

Figure 2. Visualization of the generalized additive mixed model with epigenetic states generated from the epigenetic pacemaker model using CpG sites highly correlated to chronological age (absolute $r > 0.7$). A) Changes in the epigenetic state (or epigenetic age) as individuals age. Points are actual data, while lines are the predictions from the model. B) Predictions generated with the partial effect of date of year (cyclic cubic smoother spline) on epigenetic state. The black horizontal bar represents when samples were collected and most of the marmot active

season. C) Predictions generated with the partial effect of chronological age (cubic smoother spline) on epigenetic state. Buffers illustrate the 95% confidence intervals.

Table 1. Output from the generalized additive mixed model using epigenetic states (or epigenetic ages) as dependent variable. Epigenetic states were estimated from epigenetic pacemaker models (EPM). Age: individual chronological age in years calculated from the first time an individual emerged from their mother's burrow to the date they were captured. Date: day of the year (values varied from 1 to 365, with 1 representing 1 May and 365 representing 30 April).

	Estimate	Std. Error	t value	p-value
Intercept	5.53	0.09	63.39	<0.0001
Smooth terms:				
	edf	**Ref.df**	**F**	**p-value**
Age (cubic spline)	8.43	8.43	291.82	<0.0001
Date (cyclic spline)	1.39	8.00	1.08	0.003
Age (cubic spline) × Date (cyclic spline)	0.00	12.00	0.00	0.440
Random effect:				
	Intercept	**SD Residual**		
Animal ID	0.576	0.532		

Simulation

From the 1000 GAMMs fitted to data simulated with a seasonal effect, 76.5% found a significant effect of seasons, indicating high power to detect a seasonal effect given the simulated parameters and our data structure. From the 1000 GAMMs fitted to data simulated with a constant linear age effect, 7.3% had a significant season effect, indicating a slightly higher type 1 error than expected (5%). Based on this result from the simulations with no seasonal effect, we

calculated a new critical value for the probability that respects the 5% type 1 error rate by estimating the 0.05 quantile of the p-value distribution from a null model. The 0.05 quantile was 0.0344, which can be taken as the critical value with which to estimate the significance of a seasonal effect. The p-value for seasonal effects on our data is < 0.0344 and therefore is considered significant. From this, we concluded that our results were not driven by our sampling.

Age-related CpGs

In the EWAS of chronological age, the methylation level of 6,364 CpGs were significantly ($p < 10^{-5}$) associated with chronological age. In the GAMs per site, the age effect (cubic smoother spline) was significant in 6,303 sites, which largely overlapped with EWAS of age (Figure 3D). From the 5,841 sites that overlapped between the EWAS and the age effect, 66% (3,827 sites) had effective degrees of freedom (edf) values larger than 2 for the age effect in the GAMs. The edf measures the complexity of the curve, and these results imply that most CpG sites have a non-linear relationship with chronological age. Top age-related CpGs in both EWAS and GAMs were located on NR2F1 and EVX2 downstream regions (Figure 3AB). The promoters of EN1 and HOXD10 were also hypermethylated with age. Age-related sites uniquely identified by GAMs were proximal to FAM172A intron (hypermethylated), and hypomethylated in both CSNK1D 3`UTR and HNRNPC intron.

The 3,914 CpGs used in the enrichment analysis were located in both genic and intergenic regions relative to transcriptional start sites, with a higher proportion located at promoter regions than in the background (supplementary material). Most CpGs in promoter regions were hypermethylated with age (supplementary material). DNAm aging in marmots was proximal to polycomb repressor complex targets (PRC2, EED) with H3K27ME3 marks (supplementary

material), which is a consistent observed pattern in all mammals[70]. The enriched pathways were largely associated with development, cell differentiation and homeostasis.

Season-related CpGs

The seasonal effect in the GAMs per site, measured with a cyclic cubic spline function of day of the year, was significantly associated with methylation in 47 CpG sites proximal to 37 genes. Most of the season-related CpGs were also associated with age (Figure 3D). Some of the top season- and age-related CpGs are proximal to FILIP1 exon, ARHGEF12 intron, ZNF521 intron, JARID2 exon, and AHDC1 intron (Figure 3C). The top season sites with no association with age are proximal to AHDC1 intron, MAZ exon, CTNNA1 exon, AUTS2 intron, and EFNA5 exon (Figure 3C). The AHDC1 intron seems to be an interesting region for further exploration because it is proximal to sites solely affected by season, to sites only related with age, and those influenced by both. Mutations in AHDC1 are implicated in obstructive sleep apnea (PMID 31737670), so this gene may play a role in sleep processes, and potentially hibernation.

Since the seasonal effect size is smaller and more nonlinear than the age effect (Figure 2), our power to identify sufficient season CpGs for enrichment analysis was limited by our sample size. Thus, we performed a second enrichment analysis in a CpG site set selected using a less conserved false detection rate correction (Benjamini–Hochberg FDR[71]). With this method, 206 CpGs were significantly affected by season, and 126 were used in the enrichment analysis. Some interesting biological functions in this set included pyruvate metabolism (GO:0006090), transporters of monocarboxylates (GO:0008028, GO:0015355), leukocyte migration (GO:0050900), and the circadian clock system (GO:0032922, P00015, MP:0002562).

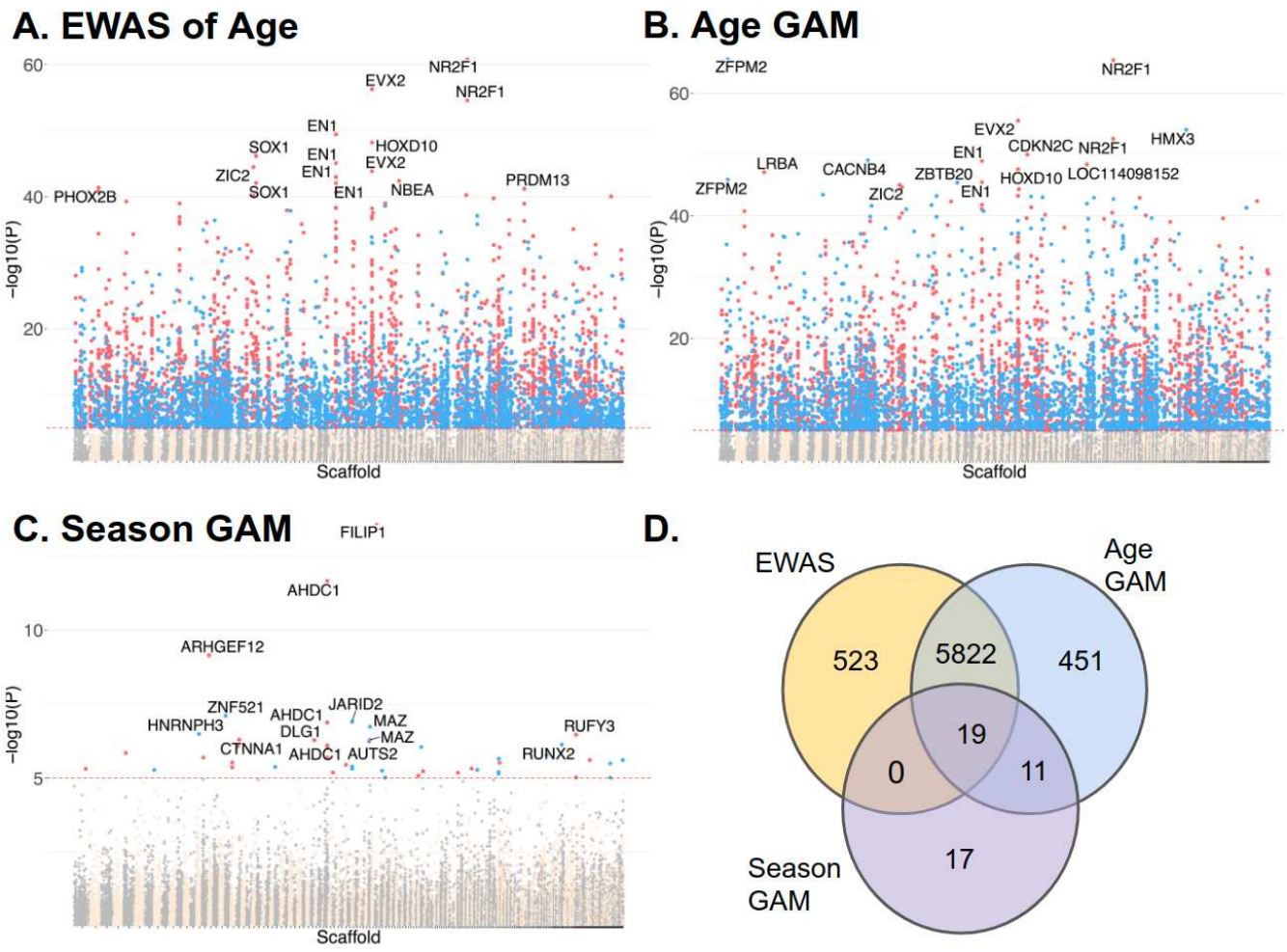

Figure 3. Associations of CpG sites with chronological age and seasons (day of the year) in blood of yellow bellied marmots (*Marmota flaviventer*). A, C) Manhattan plots visualizing log transformed p-values. The y-axis reports p values for two fixed effects of the Generalized Additive Models of individual cytosines (dependent variable): (A) chronological age (cubic spline function) and (C) day of year (cyclic cubic spline function). B) The y-axis reports p values for the epigenome-wide association (EWAS) of chronological age. The CpG sites coordinates were estimated based on the alignment of Mammalian array probes to yellow-bellied marmot genome assembly. The direction of associations with chronological age is highlighted for the significant sites ($p < 10^{-5}$) with red for hypermethylated and blue for hypomethylated sites. Note that the season effect is cyclical, and we show the direction od association with chronological age for the active season. D) Venn diagram showing the overlap of significant CpG sites between EWAS and GAMs.

Discussion

Acquiring chronological-age data from wildlife is a daunting task, but age data has fundamental applications to behavioral ecology, evolutionary biology, and animal conservation[7,72]. Epigenetic clocks (ECs) promise to inform age estimates in wild and non-model organisms[14,18,72]. This is the first study to present epigenetic aging models for marmots, a fascinating animal model to study hibernation. We applied a validated platform for measuring methylation levels (mammalian methylation array[53]) to a unique collection of tissues—blood samples from known age, free-living animals—to investigate how aging is affected by active-hibernation cycles.

The epigenetic pacemaker (EPM) results showed a rapid change in epigenetic age until marmots reached 2-years old, their age of sexual maturity[51,73]. After reaching adulthood, epigenetic age change was more linear and slower, which is similar to the pattern observed in humans older than 20 years[57]. The pattern observed in marmot epigenetic aging is consistent with the notion that methylation remodeling is associated with key physiological milestones[33]. A logarithmic relationship between methylation change rate and chronological age may be a shared trait in mammals, and such a relationship has been described for multiple human tissues[9,55,57] and species, including dogs[33], mice[15], and yellow-bellied marmots.

With regard to active and hibernation seasons, the EC model was unable to capture seasonal effects because it uses a penalized regression to relate the dependent variable (chronological age) to cytosines. The EPM is better equipped to detect non-linear and potentially cyclic patterns because it estimates the epigenetic state by minimizing the error between estimated and measured methylation levels[57,58], which allows for a non-linear relationship of methylation levels with chronological age. Since aging rate is not constant throughout an individual's lifespan[74,75],

the EPM is possibly more influenced by factors associated with biological aging[57]. In fact, methylation levels in most CpG sites had a non-linear relationship with chronological age in our models per CpG site.

According to the model that used EPM-estimated epigenetic age, biological aging slows during hibernation. Specifically, the clear delay in epigenetic-state changes during hibernation supports our hibernation-aging hypothesis. Interestingly, this hypothesis does not seem to hold for individuals prior to sexual maturity. Even though we observed a non-significant interaction between chronological age and day of year, our model predictions indicated a weaker deceleration in aging during hibernation for individuals in their first and second years of life (Figure 2A). Compared to adults, young marmots spend less time torpid during hibernation, have higher daily mass loss in deep torpor[48], and may immerge into hibernation weeks later[76–78]. Indeed, thermoregulatory support from adults increases overwinter survival of young alpine marmots[79–81]. Thus, a weaker effect of slowed aging during hibernation in younger animals may be explained by their later hibernation start date in addition to an overall higher metabolic rate during hibernation.

Some of the physiological stresses experienced by individuals during hibernation are similar to those observed with aging, and therefore the molecular and physiological responses required for an individual to successfully hibernate may prevent aging[36,45]. Additionally, hibernation combines conditions known to promote longevity[36,45,82], such as food deprivation (calorie restriction[83–85]), low body temperature[82,86–88], and reduced metabolic rates[45]. Conceivably, these factors may also be associated with the slower marmot aging observed in the beginning and end of their active season (Figure 2B). Marmots in early Spring and late Fall have limited calorie

intake[78,89], reduced overall activity[89–91], and lower metabolic rate[92] than during Summer. Because molecular and physiological events associated with hibernation are similar among mammals[36,41,44,93], the within active season variation in epigenetic aging rate may occur in other mammals. For instance, free-living arctic ground squirrels begin dropping body temperature 45 days before hibernation[94], 13-lined ground squirrels drops food consumption by 55% prior to hibernation[95], and some species exhibit short and shallow torpor bouts before and after hibernation[96].

DNA methylation (DNAm) aging in marmots was related to genes involved in several developmental and differentiation processes—as seen in other mammals[17,19,72,97]. This common enrichment across mammals implies an evolutionary conservation in the biological processes underpinning aging. This inference has been further reinforced by a recent study developing ECs capable of accurately predicting chronological age in distantly related species and, in theory, in any mammal species[70]. These "universal clocks" for eutherians can be used in any tissue sample and are developed from CpG sites located in conserved genomic regions across mammals[53].

Seasonally dynamic methylation levels were identified in 47 CpG sites. Although few CpGs were identified in our analysis per site, the effect of season was detected by the EPM algorithm, which represents methylation changes in all sites correlated ($r > 0.7$) with chronological age[63,64]. Thus, seasonality probably influences many more CpGs in common with aging than we were able to detect. Nevertheless, many of the top season-related sites were proximal to genes with circannual patterns in other species. For instance, AUTS2 is differently expressed among seasons and within hibernation in brown adipose tissue of 13-lined ground squirrels[98] and its proximal CpGs are differentially methylated in blood and liver throughout the reproductive season of great

tits[99]. JARID2 is differentially expressed within hibernation in the cerebral cortex of 13-lined ground squirrels[100] and seasonally expressed in human peripheral blood mononuclear cells[101]. RUFY3 is differentially expressed between active and hyperphagia phases in the subcutaneous adipose tissue of grizzly bears[102] and is close to season-related CpGs in great tits[103]. Methylation levels of sites close to FILIP1, AHDC1, ARHGEF12, ZNF521, CTNNA1 and AUTS2 vary seasonally in great tits[103]. ARHGEF12 is also upregulated in songbirds exhibiting migratory behavior[104]. The expression of these genes may thus be of some importance to species with seasonal behavior, including in hibernating and non-hibernating species.

Since hibernation depends on the synchrony of all regulatory stages[45] and profoundly alters physiology, most pathways are affected by season in hibernating species. However, little is known about the molecular regulation of seasonal rhythms, and our results imply a role for DNA methylation in regulating some circannual processes, as previously suggested[105]. Seasonal changes in central carbon metabolism and immune responses are expected because immune function is downregulated during hibernation[106], and the reliance on carbohydrates as energy source is switched for lipid metabolism[44,45]. Remarkably, the circadian clock system was enriched by CpGs related to seasonality. Seasonal changes in photoperiod are encoded in the circadian clock, and modify gene expression in core-clock genes as well as in clock-controlled genes[107–109].

In sum, our main finding was the little to no change in epigenetic aging during hibernation. While hibernation may increase longevity by protecting individuals from predators and diseases[37], we suggest that the biological processes involved in hibernation are important contributors to the long lifespan seen in hibernators. Since the reduction of metabolic rates is reached through similar molecular and biochemical patterns across the animal kingdom[110], the

wide inter- and intra-specific variation of torpor use in nature should be explored for more insights about the interplay between aging and torpor.

Data, Code and Supplementary Material availability

Data, code and all supplementary files are available at http:://doi.org/10.17605/OSF.IO/E42ZV Epigenetic data also is deposited in the Gene Expression Omnibus GSE174544

Competing Interest Statement

SH is a founder of the non-profit Epigenetic Clock Development Foundation which plans to license several patents from his employer UC Regents. These patents list SH as inventor. The other authors declare no conflicts of interest.

Author contributions

GMP, SH and DTB conceived the study. GMP, JGAM, CF and AH analyzed data. GMP, AH, DTB, JGAM and SH wrote the manuscript. The remaining authors helped with data generation, statistical analysis and critical feedback. All authors reviewed and edited the article.

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References

1. Flatt, T. A new definition of aging? *Front. Genet.* **3**, 148 (2012).
2. Berdasco, M. & Esteller, M. Hot topics in epigenetic mechanisms of aging: 2011. *Aging Cell* **11**, 181–186 (2012).
3. Jylhävä, J., Pedersen, N. L. & Hägg, S. Biological Age Predictors. *EBioMedicine* **21**, 29–36 (2017).
4. Wagner, K. H., Cameron-Smith, D., Wessner, B. & Franzke, B. Biomarkers of aging: From function to molecular biology. *Nutrients* **8**, 338 (2016).
5. Field, A. E. *et al.* DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Mol. Cell* **71**, 882–895 (2018).
6. Horvath, S. *et al.* Decreased epigenetic age of PBMCs from Italian semi-supercentenarians and their offspring. *Aging* **7**, 1159–1170 (2015).
7. Nussey, D. H., Froy, H., Lemaitre, J. F., Gaillard, J. M. & Austad, S. N. Senescence in natural populations of animals: Widespread evidence and its implications for bio-gerontology. *Ageing Res. Rev.* **12**, 214–225 (2013).
8. Johnson, T. E. Recent results: Biomarkers of aging. *Exp. Gerontol.* **41**, 1243–1246 (2006).
9. Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol.* **14**, R115 (2013).
10. Hannum, G. *et al.* Genome-wide Methylation Profiles Reveal Quantitative Views of Human Aging Rates. *Mol Cell* **49**, 359–367 (2013).

11. Unnikrishnan, A. *et al.* The role of DNA methylation in epigenetics of aging. *Pharmacol. Ther.* **195**, 172–185 (2019).

12. Bocklandt, S. *et al.* Epigenetic Predictor of Age. *PLoS One* **6**, e14821 (2011).

13. Horvath, S. & Raj, K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nat. Rev. Genet.* **19**, 371–384 (2018).

14. Polanowski, A. M., Robbins, J., Chandler, D. & Jarman, S. N. Epigenetic estimation of age in humpback whales. *Mol. Ecol. Resour.* **14**, 976–987 (2014).

15. Petkovich, D. A. *et al.* Using DNA Methylation Profiling to Evaluate Biological Age and Longevity Interventions. *Cell Metab.* **25**, 954–960 (2017).

16. Stubbs, T. M. *et al.* Multi-tissue DNA methylation age predictor in mouse. *Genome Biol.* **18**, 68 (2017).

17. Wang, T. *et al.* Epigenetic aging signatures in mice livers are slowed by dwarfism, calorie restriction and rapamycin treatment. *Genome Biol.* **18**, 57 (2017).

18. Ito, G., Yoshimura, K. & Momoi, Y. Analysis of DNA methylation of potential age-related methylation sites in canine peripheral blood leukocytes. *J. Vet. Med. Sci.* **79**, 745–750 (2017).

19. Thompson, M. J., von Holdt, B., Horvath, S. & Pellegrini, M. An epigenetic aging clock for dogs and wolves. *Aging* **9**, 1055–1068 (2017).

20. Lowe, R. *et al.* Ageing-associated DNA methylation dynamics are a molecular readout of lifespan variation among mammalian species. *Genome Biol.* **19**, 22 (2018).

21. Zannas, A. S. *et al.* Lifetime stress accelerates epigenetic aging in an urban, African American cohort: Relevance of glucocorticoid signaling. *Genome Biol.* **16**, 266 (2015).

22. Zaghlool, S. B. *et al.* Association of DNA methylation with age, gender, and smoking in an Arab population. *Clin. Epigenetics* **7**, 6 (2015).

23. Gao, X., Zhang, Y., Breitling, L. P. & Brenner, H. Relationship of tobacco smoking and smoking-related DNA methylation with epigenetic age acceleration. *Oncotarget* **7**, 46878–46889 (2016).

24. Marioni, R. E. *et al.* The epigenetic clock and telomere length are independently associated with chronological age and mortality. *Int. J. Epidemiol.* **45**, 424–432 (2016).

25. Marioni, R. E. *et al.* DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol.* **16**, 25 (2015).

26. Perna, L. *et al.* Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clin. Epigenetics* **8**, 64 (2016).

27. Chen, B. H. *et al.* DNA methylation based measures of biological age: meta analysis predicting time to death. *Aging* **8**, 1844–1859 (2016).

28. Christiansen, L. *et al.* DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell* **15**, 149–154 (2016).

29. Horvath, S. & Levine, A. J. HIV-1 infection accelerates age according to the epigenetic clock. *J. Infect. Dis.* **212**, 1563–1573 (2015).

30. Horvath, S. *et al.* Accelerated epigenetic aging in Down syndrome. *Aging Cell* **14**, 491–495 (2015).

31. Parrott, B. B. & Bertucci, E. M. Epigenetic Aging Clocks in Ecology and Evolution. *Trends Ecol. Evol.* **34**, 767–770 (2019).

32. Wagner, W. Epigenetic aging clocks in mice and men. *Genome Biol.* **18**, 107 (2017).

33. Wang, T. *et al.* Quantitative Translation of Dog-to-Human Aging by Conserved Remodeling of the DNA Methylome. *Cell Syst.* **11**, 1–10 (2020).

34. Wilkinson, G. S. & Adams, D. M. Recurrent evolution of extreme longevity in bats. *Biol. Lett.* **15**, 20180860 (2019).

35. Austad, S. N. Comparative biology of aging. *J. Gerontol. A Biol. Sci. Med. Sci.* **64**, 199–201 (2009).

36. Wu, C. W. & Storey, K. B. Life in the cold: Links between mammalian hibernation and longevity. *BioMol. Concepts* **7**, 41–52 (2016).

37. Turbill, C., Bieber, C. & Ruf, T. Hibernation is associated with increased survival and the evolution of slow life histories among mammals. *Proc. R. Soc. B* **278**, 3355–3363 (2011).

38. Chen, Y. *et al.* Mechanisms for increased levels of phosphorylation of elongation factor-2 during hibernation in ground squirrels. *Biochemistry* **40**, 11565–11570 (2001).

39. Knight, J. E. *et al.* mRNA stability and polysome loss in hibernating Arctic ground squirrels (*Spermophilus parryii*). *Mol. Cell. Biol.* **20**, 6374–6379 (2000).

40. Yan, J., Barnes, B. M., Kohl, F. & Marr, T. G. Modulation of gene expression in hibernating arctic ground squirrels. *Physiol. Genomics* **32**, 170–181 (2008).

41. Van Breukelen, F. & Martin, S. L. Molecular adaptations in mammalian hibernators: unique adaptations or generalized responses? *J. Appl. Physiol.* **92**, 2640–2647 (2002).

42. Morin, P. & Storey, K. B. Evidence for a reduced transcriptional state during hibernation in ground squirrels. *Cryobiology* **53**, 310–318 (2006).

43. van Breukelen, F. & Martin, S. L. Reversible depression of transcription during hibernation. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **172**, 355–361 (2002).

44. Carey, H. V., Andrews, M. T. & Martin, S. L. Mammalian hibernation: Cellular and molecular responses to depressed metabolism and low temperature. *Physiol. Rev.* **83**, 1153–1181 (2003).

45. Al-attar, R. & Storey, K. B. Suspended in time: Molecular responses to hibernation also promote longevity. *Exp. Gerontol.* **134**, 110889 (2020).

46. Azzu, V. & Valencak, T. G. Energy Metabolism and Ageing in the Mouse: A Mini-Review. *Gerontology* **63**, 327–336 (2017).

47. Schrack, J. A., Knuth, N. D., Simonsick, E. M. & Ferrucci, L. 'IDEAL' aging is associated with lower resting metabolic rate: The baltimore longitudinal study of aging. *J. Am. Geriatr. Soc.* **62**, 667–672 (2014).

48. Armitage, K. B., Blumstein, D. T. & Woods, B. C. Energetics of hibernating yellow-bellied marmots (*Marmota flaviventris*). *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* **134**, 101–114 (2003).

49. Armitage, K. B. Phylogeny and patterns of energy conservation in marmots. in *Molecules to migration: the pressures of life* (eds. Morris, S. & Vosloo, A.) 591–602 (Bologna: Medimond Publishing, 2008).

50. Blumstein, D. T. Yellow-bellied marmots: insights from an emergent view of sociality. *Philos. Trans. R. Soc. B* **368**, 20120349 (2013).

51. Armitage, K. B. Reproductive strategies of yellow-bellied marmots: energy conservation and differences between the sexes. *J. Mammal.* **79**, 385–393 (1998).

52. Armitage, K. B. & Downhower, J. F. Demography of yellow-bellied marmot populations. *Ecology* **55**, 1233–1245 (1974).

53. Arneson, A. *et al.* A mammalian methylation array for profiling methylation levels at conserved sequences. *bioRxiv* doi: 10.1101/2021.01.07.425637 (2021) doi:https://doi.org/10.1101/2021.01.07.425637.

54. Zhou, W., Triche, T. J., Laird, P. W. & Shen, H. SeSAMe: reducing artifactual detection of DNA methylation by Infinium BeadChips in genomic deletions. *Nucleic Acids Res.* **46**, e123 (2018).

55. Snir, S., VonHoldt, B. M. & Pellegrini, M. A Statistical Framework to Identify Deviation from Time Linearity in Epigenetic Aging. *PLoS Comput. Biol.* **12**, e1005183 (2016).

56. Snir, S., Wolf, Y. I. & Koonin, E. V. Universal Pacemaker of Genome Evolution. *PLoS Comput. Biol.* **8**, e1002785 (2012).

57. Snir, S., Farrell, C. & Pellegrini, M. Human epigenetic ageing is logarithmic with time across the entire lifespan. *Epigenetics* **14**, 912–926 (2019).

58. Farrell, C., Snir, S. & Pellegrini, M. The Epigenetic Pacemaker: modeling epigenetic states under an evolutionary framework. *Bioinformatics* **36**, 4662–4663 (2020).

59. Zou, H. & Hastie, T. Regularization and variable selection via the elastic net. *J. R. Stat. Soc. B* **67**, 301–320 (2005).

60. Friedman, J., Hastie, T. & Tibshirani, R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *J. Stat. Softw.* **33**, 1–22 (2010).

61. Snir, S. & Pellegrini, M. An epigenetic pacemaker is detected via a fast conditional expectation maximization algorithm. *Epigenomics* **10**, 695–706 (2018).

62. Wood, S. N. Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *J. R. Statist. Soc. B.* **73**, 3–36 (2011).

63. R Core Team. *R: A language and environment for statistical computing*. *R Foundation for Statistical Computing* (2020).

64. RStudio Team. *RStudio: Integrated Development Environment for R. RStudio, Inc.* (2019).

65. Van Rossum, G. & Drake, F. L. *Python 3 Reference Manual*. (CreateSpace, 2009).

66. Kluyver, T. *et al. Jupyter Notebooks—a publishing format for reproducible computational workflows*. *Positioning and Power in Academic Publishing: Players, Agents and Agendas* (IOS Press, 2016). doi:10.3233/978-1-61499-649-1-87.

67. Wickham, H. *ggplot2: Elegant Graphics for Data Analysis.* (Springer-Verlag, 2016).

68. Kassambara, A. ggpubr: 'ggplot2' Based Publication Ready Plots. *https://cran.r-project.org/package=ggpubr* (2020).

69. Mclean, C. Y. *et al.* GREAT improves functional interpretation of cis-regulatory regions. *Nat Biotechnol* **28**, 495–501 (2010).

70. Mammalian Consortium *et al.* Universal DNA methylation age across mammalian tissues. *bioRxiv* doi: 10.1101/2021.01.18.426733 (2021).

71. Benjamini, Y. & Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Statist. Soc. B* **57**, 289–300 (1995).

72. De Paoli-Iseppi, R. *et al.* Measuring animal age with DNA methylation: From humans to wild animals. *Front. Genet.* **8**, 106 (2017).

73. Armitage, K. B. Reproductive competition in female yellow-bellied marmots. in *Adaptive strategies and diversity in marmots* (eds. Ramousse, R., Allainé, D. & Le Berre, M.) 133–142 (International Marmot Network, 2003).

74. Marioni, R. E. *et al.* Tracking the epigenetic clock across the human life course: A meta-analysis of longitudinal cohort data. *J. Gerontol. A Biol. Sci. Med. Sci.* **74**, 57–61 (2019).

75. El Khoury, L. Y. *et al.* Systematic underestimation of the epigenetic clock and age acceleration in older subjects. *Genome Biol.* **20**, 283 (2019).

76. Kilgore, D. L. & Armitage, K. B. Energetics of Yellow-Bellied Marmot Populations. *Ecology* **59**, 78–88 (1978).

77. Armitage, K. B. Social and population dynamics of yellow-bellied marmots: results from long-term research. *Annu. Rev. Ecol. Syst.* **22**, 379–407 (1991).

78. Webb, D. R. Environmental harshness, heat stress, and *Marmota flaviventris*. *Oecologia* **44**, 390–395 (1980).

79. Armitage, K. B. Evolution of sociality in marmots. *J. Mammal.* **80**, 1–10 (1999).

80. Allainé, D. Sociality, mating system and reproductive skew in marmots: Evidence and hypotheses. *Behav. Processes* **51**, 21–34 (2000).

81. Arnold, W. The evolution of marmot sociality: II. Costs and benefits of joint hibernation. *Behav. Ecol. Sociobiol.* **27**, 239–246 (1990).

82. Keil, G., Cummings, E. & Magalhães, J. P. Being cool: how body temperature influences ageing and longevity. *Biogerontology* **16**, 383–397 (2015).

83. Means, L. W., Higgins, J. L. & Fernandez, T. J. Mid-life onset of dietary restriction extends life and prolongs cognitive functioning. *Physiol. Behav.* **54**, 503–508 (1993).

84. Speakman, J. R. & Mitchell, S. E. Caloric restriction. *Mol. Aspects Med.* **32**, 159–221 (2011).

85. Walford, R. L. & Spindler, S. R. The response to calorie restriction in mammals shows features also common to hibernation: A cross-adaptation hypothesis. *Journals Gerontol. Biol. Sci.* **52A**, B179–B183 (1997).

86. Conti, B. *et al.* Transgenic mice with a reduced core body temperature have an increased life span. *Science* **314**, 825–828 (2006).

87. Conti, B. Considerations on temperature, longevity and aging. *Cell. Mol. Life Sci.* **65**, 1626–1630 (2008).

88. Gribble, K. E., Moran, B. M., Jones, S., Corey, E. L. & Mark Welch, D. B. Congeneric variability in lifespan extension and onset of senescence suggest active regulation of aging in response to low temperature. *Exp. Gerontol.* **114**, 99–106 (2018).

89. Johns, D. W. & Armitage, K. B. Behavioral ecology of alpine yellow-bellied marmots. *Behav. Ecol. Sociobiol.* **5**, 133–157 (1979).

90. Armitage, K. B. Social behaviour of a colony of the yellow-bellied marmot (*Marmota flaviventris*). *Anim. Behav.* **10**, 319–331 (1962).

91. Armitage, K. B. Vernal behaviour of the yellow-bellied marmot (*Marmota flaviventris*). *Anim. Behav.* **13**, 59–68 (1965).

92. Armitage, K. B., Melcher, J. C. & Ward, J. M. Oxygen consumption and body temperature in yellow-bellied marmot populations from montane-mesic and lowland-xeric environments. *J. Comp. Physiol. B* **160**, 491–502 (1990).

93. Villanueva-Cañas, J. L., Faherty, S. L., Yoder, A. D. & Albà, M. M. Comparative genomics of mammalian hibernators using gene networks. *Integr. Comp. Biol.* **54**, 452–462 (2014).

94. Sheriff, M. J., Williams, C. T., Kenagy, G. J., Buck, C. L. & Barnes, B. M. Thermoregulatory changes anticipate hibernation onset by 45 days: data from free-living arctic ground squirrels. *J. Comp. Physiol. B* **182**, 841–847 (2012).

95. Schwartz, C., Hampton, M. & Andrews, M. T. Hypothalamic gene expression underlying pre-hibernation satiety. *Genes, Brain Behav.* **14**, 310–318 (2015).

96. Geiser, F. Metabolic rate and body temperature reduction during hibernation and daily torpor. *Annu. Rev. Physiol.* **66**, 239–274 (2004).

97. Maegawa, S. *et al.* Widespread and tissue specific age-related DNA methylation changes in mice. *Genome Res.* **20**, 332–340 (2010).

98. Hampton, M., Melvin, R. G. & Andrews, M. T. Transcriptomic analysis of brown adipose tissue across the physiological extremes of natural hibernation. *PLoS One* **8**, e85157 (2013).

99. Lindner, M. *et al.* Temporal changes in DNA methylation and RNA expression in a small song bird: within- and between-tissue comparisons. *BMC Genomics* **22**, 36 (2021).

100. Schwartz, C., Hampton, M. & Andrews, M. T. Seasonal and Regional Differences in Gene Expression in the Brain of a Hibernating Mammal. *PLoS One* **8**, e58427 (2013).

101. Dopico, X. C. *et al.* Widespread seasonal gene expression reveals annual differences in human immunity and physiology. *Nat. Commun.* **6**, 7000 (2015).

102. Jansen, H. T. *et al.* Hibernation induces widespread transcriptional remodeling in metabolic tissues of the grizzly bear. *Commun. Biol.* **2**, 336 (2019).

103. Viitaniemi, H. M. *et al.* Seasonal Variation in Genome-Wide DNA Methylation Patterns and the Onset of Seasonal Timing of Reproduction in Great Tits. *Genome Biol. Evol.* **11**, 970–983 (2019).

104. Johnston, R. A., Paxton, K. L., Moore, F. R., Wayne, R. K. & Smith, T. B. Seasonal gene expression in a migratory songbird. *Mol. Ecol.* **25**, 5680–5691 (2016).

105. Stevenson, T. J. Epigenetic Regulation of Biological Rhythms: An Evolutionary Ancient Molecular Timer. *Trends Genet.* **34**, 90–100 (2018).

106. Bouma, H. R., Carey, H. V. & Kroese, F. G. M. Hibernation: the immune system at rest? *J. Leukoc. Biol.* **88**, 619–624 (2010).

107. Coomans, C. P., Ramkisoensing, A. & Meijer, J. H. The suprachiasmatic nuclei as a seasonal clock. *Front. Neuroendocrinol.* **37**, 29–42 (2015).

108. Sumová, A., Bendová, Z., Sládek, M., Kováčikova, Z. & Illnerová, H. Seasonal Molecular Timekeeping Within the Rat Circadian Clock. *Physiol. Res.* **53**, S167–S176 (2004).

109. Meijer, J. H., Michel, S. & Vansteensel, M. J. Processing of daily and seasonal light information in the mammalian circadian clock. *Gen. Comp. Endocrinol.* **152**, 159–164 (2007).

110. Storey, K. B. & Storey, J. M. Metabolic rate depression in animals: Transcriptional and translational controls. *Biol. Rev.* **79**, 207–233 (2004).

CHAPTER 4

Epigenetic models developed for plains zebras predict age in domestic horses and endangered equids

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Abstract

Effective conservation and management of threatened wildlife populations require an accurate assessment of age structure to estimate demographic trends and population viability. Epigenetic aging models are promising developments because they estimate individual age with high accuracy, accurately predict age in related species, and do not require invasive sampling or intensive long-term studies. Using blood and biopsy samples from known age plains zebras (*Equus quagga*), we model epigenetic aging using two approaches: the epigenetic clock (EC) and the epigenetic pacemaker (EPM). The plains zebra EC has the potential for broad application within the genus *Equus* given that five of the seven extant wild species of the genus are threatened. We test the EC's ability to predict age in sister taxa, including two endangered species and the more distantly related domestic horse, demonstrating high accuracy in all cases. By comparing chronological and estimated age in plains zebras, we investigate age acceleration as a proxy of health status. An interaction between chronological age and inbreeding is associated with age acceleration estimated by the EPM, suggesting a cumulative effect of inbreeding on biological aging throughout life.

Introduction

Effective management of threatened species relies on the ability to estimate demographic trends, which depend, in turn, on accurate information about age distributions within populations[1]. Growth rates shape age distributions, can reflect past and current environmental and anthropogenic perturbances[2,3] and can also be used to predict future population growth[4]. However, age is challenging to quantify in wild animals. Age estimation typically requires either investment in long-term field studies or invasive approaches that may not be feasible in live animals[4,5]. Another problem is the limited accuracy of some methods, which may negatively impact conservation efforts[2]. The challenges and importance of obtaining accurate age information have motivated efforts to develop an accurate and non-invasive approach to aging wild animals[4,6].

Epigenetic aging models, particularly epigenetic clocks (ECs), promise to improve the aging of wild animals and thereby make valuable contributions to wildlife conservation and population biology[4,6]. These highly accurate clocks use information from genomic methylation patterns and have been studied extensively in humans[7-9] and mice[10-12]. The development and use of epigenetic models in other species are still limited but are becoming increasingly common[6]. A critical limitation to developing epigenetic aging models for wildlife is that these models need to be trained on samples from individuals of known age. Therefore populations of non-model organisms with known-age individuals are of extreme importance[13]. Here, we develop epigenetic models for a wild equid (plains zebras, *Equus quagga*) using both blood and remote biopsy samples collected from known-age individuals in a captive-bred population.

Besides their high accuracy, four other features of epigenetic aging models should make them attractive for wildlife managers. First, they can be developed from many different tissue types [6,14]. Second, they can be created based on very few genomic sites. The most accurate clocks for humans involve only a few hundred CpG sites[14,15], and far fewer CpGs have been used to build epigenetic clocks in some wild vertebrates[3,16,17]. Third, an accurate clock can be developed using relatively few individuals of known age[3,16,17]. Finally, epigenetic clocks developed for one species have been shown to predict age accurately in closely related taxa (e.g., humans and chimps;[14]), and therefore can be developed for a less threatened species with the intent of using them in threatened sister species. Using this rationale, we aim to test the performance of the EC developed for plains zebras to predict age in domestic horses (*E. caballus*) and two threatened species: Grevy's zebras (*E. grevyi*) and Somali asses (*E. africanus somaliensis*).

Since individual chronological ages are known for our plains zebra population, it is possible to estimate how fast individuals are aging compared to others by the discrepancy between epigenetic age and chronological age, dubbed age acceleration. Positive age acceleration indicates that an individual is biologically older than expected based on its chronological age. Age acceleration is predictive of all-cause mortality in humans[18-22], which suggests that epigenetic models can be a powerful approach to study the impact of different factors on biological aging. Age-acceleration has also been associated with stress and adversity[23-25], elevated glucocorticoids[26,27], and inbreeding[28-31], all of which are relevant for managing wild populations. The plains zebra population sampled in this study has a complex pedigree due to semi-captive breeding, which creates the opportunity to test whether inbreeding is associated with accelerated epigenetic aging in this population. The strong correlation of ECs to age can sometimes negatively affect their ability to detect age acceleration associated with biological

variation[32,33]. We therefore also develop a second model for the plains zebra, the epigenetic pacemaker model (EPM). The EPM has previously been found to be useful for investigating how environmental and life-history factors influence aging [34,35].

Our main goals for this study are to (1) develop epigenetic aging models for plains zebras; (2) test the ability of an EC developed for plains zebras to predict age in three equid species; and (3) estimate the influence of inbreeding levels on plains zebra aging patterns by correlating it with age acceleration predicted by both an EC and EPM. Our main results include an epigenetic clock that predicts age accurately in plains zebras and three congeners tested, including domestic horse and two endangered sister species. We further show that inbreeding associated age acceleration increases with age, suggesting that inbreeding may have a cumulative effect on age acceleration througout life. The development of epigenetic aging models in a wild equid stands to have broad impact because the crown group of the genus *Equus* comprises a closely related group of 6 extant species, 5 of which range from near threatened to critically endangered[36,37].

Results

Epigenetic aging models

We developed epigenetic models using methylation data profiles from three plains zebra data sets: (1) 76 blood samples, (2) 20 biopsy samples, and (3) 96 blood and biopsy samples combined (Table 1). For each data set we developed both an epigenetic clock (EC) and an epigenetic pacemaker (EPM). We evaluated the effectiveness of applying the blood-based zebra EC to predict age in other equids using known-age domestic horses (*E. caballus*, n=188), Grevy's zebras (*E. grevyi,* n=5), and Somali asses (E. *africanus somaliensis,* n=7).

Table 1. Description of the zebra data. We restrict the description to animals whose ages could be estimated with high confidence (90% or higher). Tissue type, N=Total number of samples/arrays. Number of females. Age: mean, minimum and maximum.

Tissue	N	No. Female	Mean Age	Min. Age	Max. Age
Blood	76	42	5.21	0.156	20.2
Biopsy	20	9	5.87	0.162	24.8

To develop the ECs we fit a generalized linear model with elastic-net penalization using leave-one-out (LOO) cross-validation. To improve EC fit[14] we square root transformed chronological age prior to fitting the models. The blood EC (Pearson's r = 0.96, median absolute error (MAE) = 0.56 years, Fig. 1a) and the combined tissue EC (r = 0.89, MAE = 0.62, Fig. 1c) predicted age more accurately than the biopsy EC (r = 0.62, MAE = 1.79 years, Fig. 1b). The blood EC selected 70 CpG sites, the biopsy clock 31 CpGs, and the combined clock selected 99 CpGs. The biopsy EC had no CpG sites in common with the blood and combined ECs. The blood EC and combined tissue EC shared only two CpGs. We report coefficients, intercepts, and lambdas in Supplementary Data 1 and present the results from using untransformed ages in Supplementary Fig. 1.

Cross-species predictive ability was high (Fig. 1d-f). The zebra-blood EC predicted horse age with high accuracy (r = 0.93, MAE = 1.82). The error when predicting the ages of horses younger than 15 years is lower (MAE 1.15) than when predicting the ages of older horses (MAE = 3.97). While prediction errors for Grevy's zebra and Somali wild ass ages were even lower (MAE of 1.08 and 1.15 respectively), this should be viewed with caution as we had limited sample sizes from Grevy's zebra (n = 5) and Somali wild ass (n = 7).

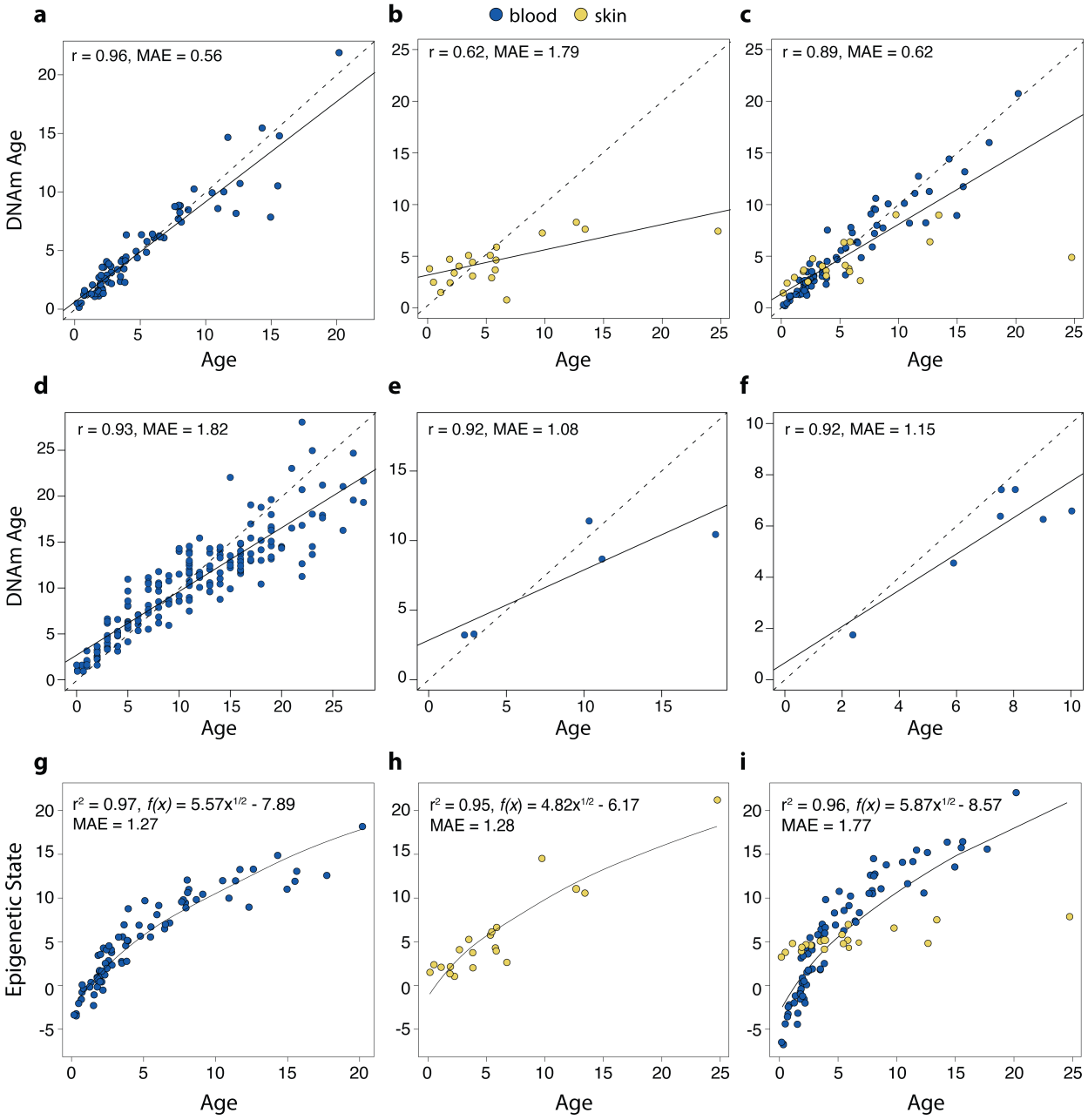

Figure 1. Predictive Ability of Epigenetic Aging Models. **a-c** Plains zebra epigenetic clocks (EC). We developed 3 ECs for zebras using square-root transformed ages: **a** blood samples (n=76), **b** biopsy samples (n=20), and **c** combined tissue types. Leave-one-sample-out (LOO) estimate of DNA methylation age are plotted against chronological age. Linear regressions of epigenetic age are indicated by a solid line while the diagonal dashed line depicts y=x. **d-f** Tests of the ability of the plains zebra blood clock to predict chronological age in other equids: **d** domestic horse n=188, **e** Grevy's zebra n=5, **f** Somali wild ass n=7. **g-i** Epigenetic pacemaker (EPM) models for plains zebras. Epigenetic states (or epigenetic age) of plains zebras predicted from the EPM using **g** blood (n=76), **h** remote biopsy tissue (n=20), and **i** both sample types combined. Predictions are based on 76 blood samples and 20 biopsy samples. The equation of

the fitted curve (solid line) is described for each plot. MAE are based on ages translated by the equation.

To construct plains zebra EPMs we used sites in which methylation levels were highly correlated with individual chronological age. Epigenetic states were estimated using a leave-one-out cross-validation. The blood, biopsy, and combined-tissue EPMs included 391, 242, and 248 CpG sites, respectively. We provide details of the CpGs selected by each model in Supplementary Data 1. Epigenetic state was strongly correlated with chronological age in both tissue types: blood (r = 0.97, Fig. 1g) and biopsy (r = 0.95, Fig. 1h). EPMs based on the two tissues used largely distinct sets of CpG sites, sharing only 40 sites. Despite retaining a strong overall correlation in the combined EPM (r = 0.96**)**, the difference in the model's performance for the two sample types is apparent (Fig. 1i). The combined tissue pacemaker shared 138 sites with blood and only 34 with biopsies.

Association of inbreeding with biological aging

We derived the genotypes used to estimate inbreeding (F and F_{ROH}) from two sources: RAD sequencing data (42 samples) and genotypes imputed at the same set of loci from low-coverage whole-genome sequencing data using GLIMPSE[38] (28 additional samples). GLIMPSE produced high-quality imputations (mean dosage r^2 of 80%; mean concordance between imputed and true genotype of 92%) as assessed by running leave-one-out imputations on 35 samples present in both the RADseq and low coverage data sets (Supplementary Fig. 2a, b). The Mendelian error rate across loci averaged 0.06 (+/- 0.06) in the full data set. ROH were discoverable across 90% of the genome and ranged in size from 1.5 MB (minimum allowed size) up to > 60 MB (Supplementary Fig. 2c). ROH over 10 MB are expected to reflect inbreeding loops occurring

within the last five generations[39,40]. Individuals from more recent generations showed a pattern of excessive total ROH relative to the number of ROH segments as indicated by an upward shift in ROH size relative to number (Supplementary Fig. 2d;[39]). F_{ROH} ranged from 0 to 0.37, and F statistics ranged from -0.21 to 0.32 (one outlier with an extreme negative F value was removed from analysis). The Pearson correlation between F and F_{ROH} was 0.84.

We used multiple linear regressions to assess whether inbreeding is associated with age acceleration in the plains zebra population. Inbreeding was estimated both as the inbreeding coefficient F and by the proportion of the genome in runs of homozygosity (F_{ROH}). Age acceleration was calculated as the residuals of chronological age regressed on predicted age, and was calculated separately for the EPM and EC. Sex was added to the linear models as a covariate. EPM age acceleration was significantly associated with an interaction between chronological age and both F and F_{ROH} (Fig. 2, Supplementary Table 1a, c), indicating that the impacts of inbreeding on biological age increase with chronological age. EC age acceleration was not associated with either measure of inbreeding. Sex was not associated with age acceleration in any model. A re-run of our analyses using only the RADseq samples gave similar results (Supplementary Table 1e-h).

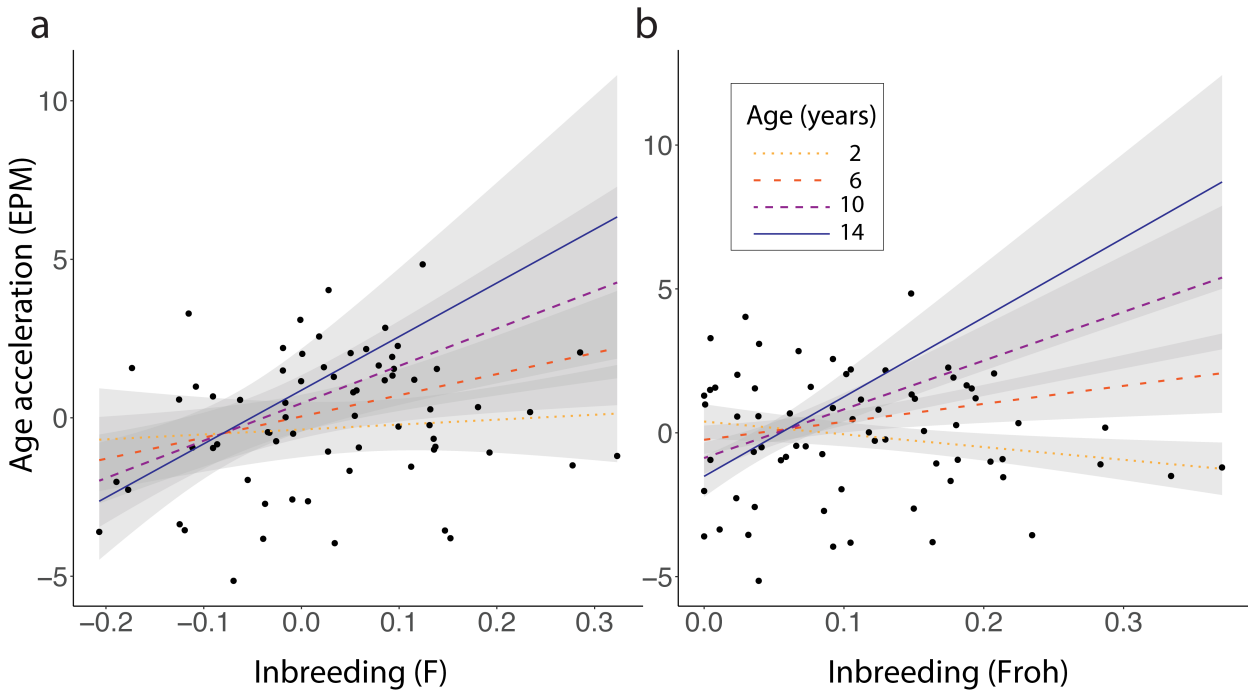

Figure 2. Relationship between Epigenetic Age acceleration calculated from the epigenetic pacemaker (EPM) model and inbreeding in plains zebras. Lines represent the predicted age acceleration for individuals with different chronological ages and different levels of inbreeding. Gray areas show 95% confidence intervals. Black dots represent the individual plains zebra data. Inbreeding was calculated in PLINK as **a** F and **b** F_{ROH}.

EWAS and functional analysis of plains zebra tissues

We performed the EWAS analysis on the 31,836 probes that could be uniquely aligned to specific adjacent loci in the horse genome. Since the mammalian methylation array is based on stretches of DNA that are conserved in all mammals, the horse annotation can be applied to the zebra data[41]. At a nominal $p<10^{-4}$, a total of 9757 and 331 probes were related to age in blood (n = 76, age-range 0.15-20.2 years) and biopsies (n = 20, age-range 0.16-24.8 years) respectively (Fig. 3a). The top age-related changes per tissue are as follows (Fig. 3a): blood, hypomethylation in *FANCL* upstream, *MAF* downstream, *ZNF608* upstream, and *PBX3* intron; biopsy, hypermethylation in *PLCB1*, *NEUROD1*, and *BARHL2* upstream. DNAm aging was distributed

in both genic and intergenic regions relative to transcriptional start sites (Fig. 3b). Promoters and 5'UTR regions, which can be considered expression regulatory regions, mainly gained methylation with aging in both tissues. This observation paralleled a systematic positive correlation of CpG islands with age (Fig. 3c).

The association of CpG sites with chronological age in blood and biopsy was relatively similar, with a moderate positive correlation between the z-scores from the EWAS for each tissue type (r = 0.25, Fig. 3e). Of the CpGs with significant association with chronological age, only 81 overlapped between blood and biopsy samples (Fig. 3d). Some of these shared CpGs include hypermethylation in *PLCB1* exon, *RIMS1* exon, and hypomethylation in *NOVA2* intron and *NFIA* intron (Supplementary Fig. 3a, b). In contrast to results based on specific genes, functional enrichment analysis using GREAT [42] identified that age-related CpGs in both blood and biopsies were significantly related with regard to biological pathways, specifically development (e.g., nervous system) and survival, and were enriched with polycomb repressor complex 2 (e.g. *EED, SUZ12, PCR2*) target genes (Supplementary Fig. 4d).

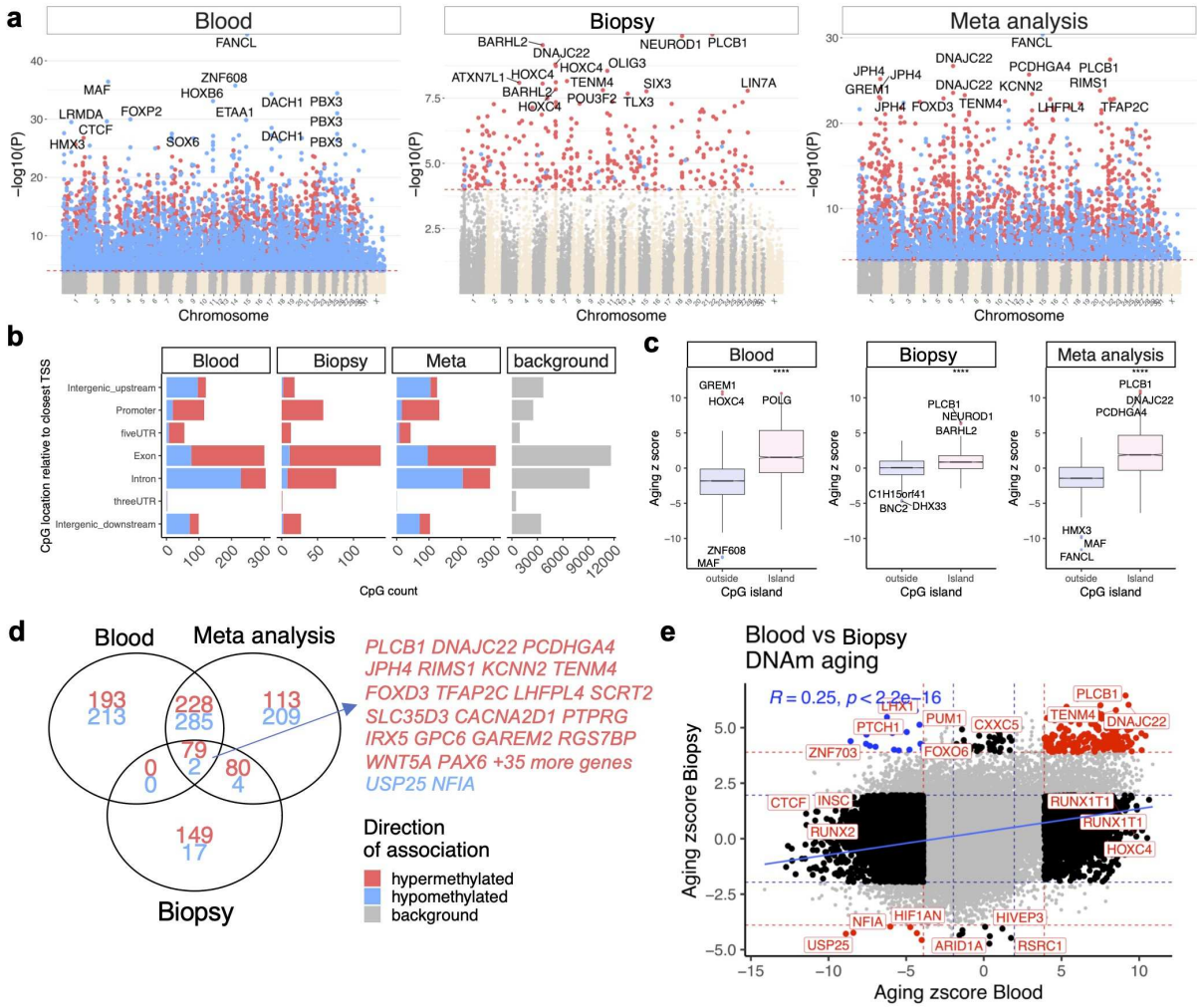

Figure 3. Epigenome wide association study (EWAS) of chronological age in blood and skin of plains zebras. **a** Manhattan plots of the EWAS of chronological age. Since a genome assembly was not available for zebra, the coordinates are estimated based on the alignment of Mammalian array probes to EquCab3.0.100 (domestic horse) genome. The direction of associations with $p < 10^{-4}$ (red dotted line) is highlighted by red (hypermethylated) and blue (hypomethylated) colors. The top 15 CpGs were labeled by the neighboring genes. **b** Location of top age-related CpGs in each tissue relative to the closest transcriptional start site. Top CpGs were selected at $p < 10^{-4}$ and further filtering based on z-score of association with chronological age for up to 500 in a positive and negative direction. The number of selected CpGs: blood, 1000; biopsy, 331; meta-analysis, 1000. The grey color represents the location of 3,1836 mammalian BeadChip array probes mapped to EquCab3.0.100 genome. **c** Box plot of z-scores from a correlation of age with CpG location (within or outside CpG islands). The median Z statistics are significantly different ($p < 10^{-4}$). **d** Venn diagram of the top age-related CpGs in blood and biopsy samples from plains zebras. **e** Sector plot of DNA methylation aging in plains zebra blood and biopsy tissues. Red

dotted line: $p<10^{-4}$; blue dotted line: $p>0.05$; Red dots: shared CpGs; black dots: tissue specific changes; blue dots: CpGs whose age correlation differs between blood and biopsy tissue.

Discussion

To the best of our knowledge, this is the first study to present DNA methylation-based age estimators for any wild equid. The high accuracy of the epigenetic clock (EC) models reflects that we used a custom array that profiled 36 thousand probes that were highly conserved across numerous mammalian species. These robust data allowed us to construct highly accurate epigenetic aging models for plains zebras. The best model to predict chronological age was the EC developed from blood samples, which predicted individual age with a 6-month error.

Developing a highly accurate biopsy-based EC may be challenging due to the variability of tissue types within such a sample. Biopsy samples consist of three skin layers - the epidermis, the dermis, and the hypodermis - and may even contain deeper tissues. These individual layers can exhibit different methylation patterns[43] and are often present in different proportions across samples because they vary in thickness across the body and among individuals. A comparison between blood and skin-based (dermis and epidermis only) odontocete clocks also found the skin-based clocks to be less accurate[44]. Despite the inherent difficulties of using biopsy samples and the small sample size and skewed age distribution of the biopsy samples (Supplementary Fig. 5), our biopsy clock predicted age with an error of +/- 2 years.

The plains zebra blood EC accurately predicted the chronological ages of horses, Grevy's zebras, and Somali wild asses. This was expected since caballine and non-caballine equids are somewhat closely related (4-4.5 MYA)[45,46]. Non-caballine species of equids are more closely related to plains zebras (1.28-1.75 MYA) than domestic horses, which may explain the lower errors found

for age estimation in Grevy's zebras and Somali asses. In fact, chimpanzees and bonobos have a similar divergence time to those observed within the non-caballine equids[47] and align more closely to each other in DNAm age than either does with humans[14].

The extraordinary accuracy of ECs stems from their utilization of sites that maximize a linear relationship between epigenetic age and chronological age. This accuracy can sometimes negatively affect their ability to detect age acceleration associated with biological variation[32,33]. Methylation levels change in a non-linear fashion throughout individuals' lifetimes in several species, with accelerated changes in early life and slower changes once individuals reach adulthood[14,34]. The epigenetic pacemaker (EPM) was developed to model these non-linear changes [34,48,49]. The EPM estimates epigenetic age by maximizing the similarity between estimated and observed methylation levels, and therefore does not make any assumptions about linearity but rather identifies the shape of the relationship between age and methylation directly from the data. In this sense, the EPM is potentially more associated with biological than chronological aging[34], which may be particularly useful for investigating how environmental and life-history factors influence aging[35].

The EPM models developed here reveal that epigenetic changes occur in a non-linear fashion throughout the lifespan of plains zebras. As has been found in humans and other species[34,35], young zebras undergo faster epigenetic changes than adult zebras. We also observed that the variance in the estimates of epigenetic age is lower in young compared to old zebras. Increased variation in epigenetic age in adults is observed in humans and other species as well, and may be a consequence of lifetime accumulation of environmental and physiological factors on the epigenome[7,14]. In agreement with the idea of cumulative effects on aging, the effects of

inbreeding on epigenetic aging were more apparent in older individuals. We found a significant effect of the interaction between inbreeding measures and chronological age on age acceleration estimated from the blood EPM, wherein inbred individuals would exhibit a higher age acceleration at older chronological ages. The association between inbreeding and DNA methylation has been described in plants[28,30], chickens[31], and salmon [29]. Increases in inbreeding effects with age, such as those we describe, are predicted by theory[50] and have been shown empirically in an insect[51] and mammal[52] species.

Because the probes in the mammalian array were selected based on conservation in mammalian genomes, we expect our findings will have high translatability into humans and other mammals. The age-related gain of methylation in promoters (Fig. 3b) is consistent with observations in humans and many other species[53,54]. The low overlap of significant CpGs between tissues (Fig. 3d) may reflect the relatively low sample size (n=20 skin biopsy samples) or biological differences between the biopsy and whole blood samples. Some of the genes close to the most significant CpGs in the EWAS of blood and biopsy samples play key roles in the DNA damage pathway and maintenance of genomic integrity (*FANCL*)[55], regulation of cellular and/or developmental processes (*MAF*[56], *PBX3*[57], *NEUROD1*[58], *BARHL2*[59,60], *NOVA2*[61,62], *NFIA*[63,64]), and extra- and/or intra- cellular signaling (*PLCB1*[65,66], *RIMS1*[67,68]).

Most of these genes are near to the top significant CpG sites from an EWAS that included multiple mammalian species and tissues: *MAF*, *NEUROD1*, *BARHL2*, *NFIA*, *PLCB1*, and *RIMS1*[69]. In mice, *MAF* promotes osteoblast differentiation and may be an important gene for age-related bone disease therapy[70,71]. *PBX3* is at the center of the most enriched network associated with aging based on differentially methylated regions in human blood[72], and its

expression levels in rat frontal cortex with aging may depend on the amount of stress experienced by previous generations[73]. Many of these genes are potential targets for therapies to prevent and treat age-related cognitive decline and neurodegeneration. *BARHL2* is differentially expressed in the hippocampus of young and old rats[74], and methylation levels in nearby CpGs are associated with aging across different tissues in naked mole rats [75]. *PLCB1'*s expression levels are associated with aging in the human prefrontal cortex[76]. SNPs near *ZNF608* have been associated with early stage of cognitive decline[77], Alzheimer's disease risk[78], and body mass index[79,80] in humans. *NEUROD1* is differentially expressed with aging in the mouse hippocampus and is a critical regulator of neurogenesis[81]. *RIMS1* is important for synaptic transmission at neuromuscular junctions in mammals[82]. The abundance of the *NOVA2* protein in the cytoplasm decreases with aging in human neurons[83]. *NFIA* modulates the plasticity of local circuits in the adult hippocampus and may be involved in the cortical atrophy associated with Alzheimer's disease[84] In addition to the EWAS results, the GREAT enrichment analysis implicated developmental pathways, bivalent chromatin, and regions suppressed by polycomb repressor complex 2. These results are consistent with observations in many other mammalian species and corroborate findings on DNAm aging in many other mammalian species[3,5,14,17,32,35,69,75,85].

We expect that epigenetic aging models will be valuable for ecological studies and population management in wild species since age estimates can be used to assess reproductive potential and population viability. In known age populations, both ECs[27,41,86,87] and EPMs[34,48,49] have the potential to identify causes of individual accelerated aging. These models can be especially useful when combined with ecological or other genetic data[4]. Given the small number of CpG sites required, an aging project in a wild population could be done relatively inexpensively using bisulfite sequencing or pyrosequencing[88-90], and the costs of the mammalian array are decreasing.

While epigenetic models based on blood are more accurate and less invasive than many other options for aging mammals, there are drawbacks in that animals must be immobilized to obtain the samples. Because biopsy samples can be obtained with minimal disruption[91], a highly reliable clock based on biopsy samples will be a worthwhile direction for future research. Since the methods for extracting genomic DNA from feces have improved[92-94], it will be worthwhile to explore whether epigenetic aging models can be adapted to this non-invasive source of DNA.

Methods

Samples

We obtained both whole blood (96) and remote biopsy (24) samples from a captive population of zebras maintained in a semi-wild state by the Quagga Project[95] in the Western Cape of South Africa. The population was founded in 1989 with 19 wild individuals (9 from Etosha National Park in Namibia and 10 from the Kwazulu-Natal in South Africa). Since its inception, the population has undergone artificial selection to reproduce the phenotype of the extinct quagga subspecies: no stripes on legs and hindquarters, and thinner and paler stripes in the head and barrel region. At sampling, we identified individuals by their unique stripe patterns and derived their chronological ages from studbook information, in which dates of birth are typically accurate to within one month. One exception is a biopsy sample from a founder that was captured for the project as a young mare and would have been at least 25 years old at sampling. We obtained remote biopsies using an air-powered rifle affixed with a 1 mm wide by 20-25 mm deep biopsy dart and preserved in RNAlater (Qiagen). Veterinarians collected blood opportunistically during activities of the Quagga Project and preserved them in EDTA tubes. All but four samples were collected from different individuals; two individuals were sampled twice at different ages (one

and three years apart, respectively). We stored all samples at -20 °C. After eliminating samples (24 of 120) with < 90% confidence for individual identity or age, we retained 76 blood samples and 20 biopsy samples, totaling 96 plains zebra samples (Table 1).

The collection of 188 whole-blood samples from domestic horses is described in detail in[85]. The Grevy's zebra (n=5) and Somali wild ass (n=7) are samples from zoo-based animals that were opportunistically collected and banked during routine health exams. The DNA methylation profiles from these samples have been reported previously[69].

Ethics approval

We collected plains zebra samples under a protocol approved by the Research Safety and Animal Welfare Administration, University of California Los Angeles: ARC # 2009-090-31, approved initially in 2009.

DNA methylation data

We generated all DNA methylation data (plains zebra, horse, Somali wild ass, Grevy's zebra) using a custom Illumina methylation array (HorvathMammalMethylChip40)[96]. The array contains 36 thousand probes, 31,836 of which mapped uniquely to the horse genome[97,98]. We normalized methylation values from each species (plains zebra, horse, Somali wild ass, and Grevy's zebra) and tissue (blood and biopsy) using SeSAMe[99]. Unsupervised hierarchical clustering revealed that the plains zebra samples clustered by tissue (Supplementary Fig. 6).

Epigenetic aging models

We studied epigenetic aging in plains zebras using both epigenetic clock (EC)[14,100,101] and epigenetic pacemaker (EPM) models[34,49]. For the ECs we fit generalized linear models in glmnet

v.4.0-2 in R v.4.1.0[102,103]. We treated the methylation data from other equids (domestic horse, Grevy's zebra, Somali wild ass) independently and did not use them for zebra clock development. In addition to the LOO cross-validation presented here, we also conducted analyses using 10-fold cross-validation. The two forms of cross-validation did not produce appreciably different results.

We tested the ability of the blood-based EC to predict chronological age for other equids (domestic horse, Grevy's zebra, Somali wild ass) by inputting the DNA methylation profiles of these species into the plains zebra model. We used the mean absolute error of chronological age estimation and the Pearson correlation between predicted ages and known ages per species to assess accuracy.

To construct plains zebra EPMs we used sites in which methylation levels were highly correlated with individual chronological age. The Pearson correlation (*r*) thresholds for entry into the model were absolute values of 0.75 for blood and biopsy, and 0.6 for the combined EPM. The threshold used to select the sites for input into the combined EPM was lower because only one CpG site had *r* higher than 0.75. Epigenetic states were estimated using a leave-one-out cross-validation with EpigeneticPacemaker 0.0.3[48] in Python 3.7.4[104]. Supplementary Data 2 provides all Pearson coefficients for methylation levels against chronological age; and the rate and intercept values per site for blood, biopsy, and combined EPMs.

Association of inbreeding with biological aging

We prepared libraries and conducted 2x150 bp paired-end RAD sequencing as described in[105]. To maximize the number of SNPs ascertained, we sequenced reads produced by two restriction enzymes, SbFI and PacI (74155 and 127429 cut sites in the horse genome, respectively). We

aligned reads to the horse genome EquCab3[97] using BWA[106]. We called genotypes using the haplotype-based callers Freebayes[107]and Sentieon[108], retaining only the intersection of variants called by these callers. We retained only variants within the first read of each paired-end read (a total of ~60.5 Mb). We also removed indels, multiallelic and non-autosomal sites, loci genotyped in < 10% of individuals, and individuals genotyped at < 20% of loci. We followed GATK's basic guidelines for additional filtering (https://gatk.broadinstitute.org/hc/en-us/articles/360035890471-Hard-filtering-germline-short-variants), excluding SNPs with QD < 2, FS > 60, SOR > 3, MQ < 40, MQRankSum < -12.5, and ReadPosRankSum < -8. Finally, we removed SNPs with MAF < 0.01. Our filtering strategy resulted in 56 individuals genotyped at 322542 loci. The number of loci is consistent with levels of heterozygosity observed in inbred wild populations of plains zebras[105].

The 56 samples with RADseq genotypes were used as a reference panel to impute these same 322542 SNPs in 89 individuals sequenced at low coverage. Low coverage libraries were constructed from 200ng genomic DNA using Truseq Nano kit (Illumina), indexed with unique dual indices (Integrated DNA Technologies), and sequenced 24 libraries per lane on a HiSeqX platform (Illumina), generating 6.2±1.4 raw Gbp per sample. We aligned sequences to the horse genome EquCab3[97] using BWA[106]. Genotypes were then imputed using GLIMPSE[38]. As part of the GLIMPSE pipeline, genotype likelihoods were called from the low coverage bams using mpileup in bcftools[109], ignoring indels and duplicate reads, and recalculating base alignment quality on the fly. Thirty-five of the 89 imputed individuals were also present in the RADseq data and were used to assess imputation quality using a leave-one-out-approach.

Forty-two individuals from the RAD-seq data and 28 from the imputed data had associated epigenetic data. The combined sample of 70 individuals spans seven generations of the Quagga Project. With the exception of removing singletons and private doubletons, we did not further MAF prune or LD prune the combined RAD and imputed data, as such pruning can bias the detection of ROH in an inbred population[110]. We used 313,645 autosomal SNPs to estimate the inbreeding coefficient F and to detect runs of homozygosity (ROH). F was estimated in PLINK[111] using methods of moments. We used PLINK's default settings to detect runs of homozygosity (ROH) with the exception that we increased the stringency to require detection of runs in 150 rather than 100 bp windows, and final runs had to be at least 1.5 MB long. In addition, we allowed only two missing SNPs per homozygous window. We converted ROH to the inbreeding coefficient F_{ROH}[112] by dividing the total length of ROH for each individual by the length of the genome over which we screened for ROH[110].

Linear regression models to assess the relationship between inbreeding and age acceleration were fitted with the lmtest v.0.9-38[113] package in R v.4.1.0[103]. We fitted four linear models in total, running separate analyses for the two estimates of inbreeding, F and F_{ROH}, and separate analyses for the two measures of age acceleration calculated based on EPM or EC. Age acceleration was the dependent variable and was calculated as the residuals of chronological age regressed on predicted age. In each analysis the independent variables were sex, chronological age, inbreeding, and the interaction between chronological age and inbreeding. We checked the residuals for normality and adjusted for heteroskedasticity via Huber-White with the package sandwich v.3.0-1[114,115]. We repeated our analyses using only the individuals genotyped directly with RAD-seq data to ensure imputed genotypes did not bias our results.

EWAS and functional analysis of plains zebra tissues

To identify genes potentially associated with aging, we performed EWAS in each tissue separately using the R function "standardScreeningNumericTrait" from the "WGCNA" R package[116]. The results were combined across tissues using Stouffer's meta-analysis method[117]. We estimated the distance of each significant CpG site to the closest transcriptional start site. We retained only the 500 CpGs with the most positive z-scores and the 500 with the most negative z-scores from each EWAS (blood, biopsy, and the combined meta-analysis). Restricting the number of analyzed CpGs did not have a drastic impact on the enriched pathway results. We used these CpGs as the input for GREAT analysis software[42]. The background was the human Hg19 genome, limited to 31,836 CpG sites mapping to the horse genome. The options in the analysis included "Basal plus extension" and a maximum of 50 kb flanking window for the CpGs coordinates.

Statistics and Reproducibility

Statistical analyses were performed for the epigenetic models, inbreeding and age-acceleration, and EWAS and functional analysis. The analyses are described in the corresponding Methods sections, including all parameters used to allow reproducibility.

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Data availability

Methylation data for plains zebras can be downloaded from Gene Expression Omnibus GSE184223. RAD sequencing data is available as fastq files on SRA, BioProject ID:

PRJNA670933. Data and scripts associated with model development and the creation of figures 1 and 2 are published on DRYAD (doi:10.5068/D1W39K).

Code availability

Scripts used to run EWAS and functional analyses are at https://github.com/shorvath/MammalianMethylationConsortium.

Author contributions

BL and SH conceived of the study. BL and GP analyzed data, and BL, GP and SH co-wrote the article. The remaining authors helped with the statistical analysis (AH, JZ, CL, CF, MP), or the data generation (BW, CJF, CK, GB, TR, DM). All authors reviewed and edited the article.

Competing Interests Statement

The authors declare the following competing interests: SH is a founder of the non-profit Epigenetic Clock Development Foundation which plans to license several patents from his

employer UC Regents. These patents list SH as inventor. The other authors declare no competing interests.

References

1 Beissinger, S. R. & Westphal, M. I. On the use of demographic models of population viability in endangered species management. *Journal of Wildlife Management* **62**, 821-841, doi:Doi 10.2307/3802534 (1998).

2 Campana, S. Accuracy, precision and quality control in age determination, including a review of the use and abuse of age validation methods. *Journal of Fish Biology* **59**, 197-242, doi:10.1006/jfbi.2001.1668 (2001).

3 Polanowski, A. M., Robbins, J., Chandler, D. & Jarman, S. N. Epigenetic estimation of age in humpback whales. *Mol Ecol Resour* **14**, 976-987, doi:10.1111/1755-0998.12247 (2014).

4 Jarman, S. N. *et al.* Molecular biomarkers for chronological age in animal ecology. *Mol Ecol* **24**, 4826-4847, doi:10.1111/mec.13357 (2015).

5 Thompson, M. J., vonHoldt, B., Horvath, S. & Pellegrini, M. An epigenetic aging clock for dogs and wolves. *Aging* **9**, 1055-1068, doi:10.18632/aging.101211 (2017).

6 De Paoli-Iseppi, R. *et al.* Measuring Animal Age with DNA Methylation: From Humans to Wild Animals. *Front Genet* **8**, 106, doi:10.3389/fgene.2017.00106 (2017).

7 Bell, C. G. *et al.* DNA methylation aging clocks: challenges and recommendations. *Genome Biol* **20**, 249, doi:10.1186/s13059-019-1824-y (2019).

8 Field, A. E. *et al.* DNA Methylation Clocks in Aging: Categories, Causes, and Consequences. *Mol Cell* **71**, 882-895, doi:10.1016/j.molcel.2018.08.008 (2018).

9 Horvath, S. & Raj, K. DNA methylation-based biomarkers and the epigenetic clock theory of ageing. *Nat Rev Genet* **19**, 371-384, doi:10.1038/s41576-018-0004-3 (2018).

10 Petkovich, D. A. *et al.* Using DNA Methylation Profiling to Evaluate Biological Age and Longevity Interventions. *Cell Metab* **25**, 954-960 e956, doi:10.1016/j.cmet.2017.03.016 (2017).

11 Stubbs, T. M. *et al.* Multi-tissue DNA methylation age predictor in mouse. *Genome Biol* **18**, 68, doi:10.1186/s13059-017-1203-5 (2017).

12 Wang, T. *et al.* Epigenetic aging signatures in mice livers are slowed by dwarfism, calorie restriction and rapamycin treatment. *Genome Biol* **18**, 57, doi:10.1186/s13059-017-1186-2 (2017).

13 Nussey, D. H., Froy, H., Lemaitre, J. F., Gaillard, J. M. & Austad, S. N. Senescence in natural populations of animals: widespread evidence and its implications for bio-gerontology. *Ageing Res Rev* **12**, 214-225, doi:10.1016/j.arr.2012.07.004 (2013).

14 Horvath, S. DNA methylation age of human tissues and cell types. *Genome Biol* **14**, R115 (2013).

15 Voisin, S. *et al.* An epigenetic clock for human skeletal muscle. *J Cachexia Sarcopenia Muscle*, doi:10.1002/jcsm.12556 (2020).

16 De Paoli-Iseppi, R. *et al.* Age estimation in a long-lived seabird (*Ardenna tenuirostris*) using DNA methylation-based biomarkers. *Mol Ecol Resour* **19**, 411-425, doi:10.1111/1755-0998.12981 (2019).

17 Ito, H., Udono, T., Hirata, S. & Inoue-Murayama, M. Estimation of chimpanzee age based on DNA methylation. *Sci Rep* **8**, 9998, doi:10.1038/s41598-018-28318-9 (2018).

18 Chen, B. H. *et al.* DNA methylation-based measures of biological age: meta-analysis predicting time to death. *Aging (Albany NY)* **8**, 1844-1865, doi:10.18632/aging.101020 (2016).

19 Christiansen, L. *et al.* DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell* **15**, 149-154, doi:10.1111/acel.12421 (2016).

20 Horvath, S. *et al.* Decreased epigenetic age of PBMCs from Italian semi supercentenarians and their offspring. *Aging* **7**, 1159-1170 (2018).

21 Marioni, R. E. *et al.* DNA methylation age of blood predicts all-cause mortality in later life. *Genome Biol* **16**, 25, doi:10.1186/s13059-015-0584-6 (2015).

22 Perna, L. *et al.* Epigenetic age acceleration predicts cancer, cardiovascular, and all-cause mortality in a German case cohort. *Clin Epigenetics* **8**, 64, doi:10.1186/s13148-016-0228-z (2016).

23 Mitchell, C., Schneper, L. M. & Notterman, D. A. DNA methylation, early life environment, and health outcomes. *Pediatr Res* **79**, 212-219, doi:10.1038/pr.2015.193 (2016).

24 Pérez, R. F., Santamarina, P., Fernández, A. F. & Fraga, M. F. Epigenetics and Lifestyle: The Impact of Stress, Diet, and Social Habits on Tissue Homeostasis in *Epigenetics and Regeneration* 461-489 (2019).

25 Szyf, M., Tang, Y. Y., Hill, K. G. & Musci, R. The dynamic epigenome and its implications for behavioral interventions: a role for epigenetics to inform disorder prevention and health promotion. *Transl Behav Med* **6**, 55-62, doi:10.1007/s13142-016-0387-7 (2016).

26 Lee, R. S. *et al.* Chronic corticosterone exposure increases expression and decreases deoxyribonucleic acid methylation of Fkbp5 in mice. *Endocrinology* **151**, 4332-4343, doi:10.1210/en.2010-0225 (2010).

27 Zannas, A. S. *et al.* Lifetime stress accelerates epigenetic aging in an urban, African American cohort: relevance of glucocorticoid signaling. *Genome Biol* **16**, 266, doi:10.1186/s13059-015-0828-5 (2015).

28 Biemont, C. Inbreeding effects in the epigenetic era. *Nat Rev Genet* **11**, 234, doi:10.1038/nrg2664-c1 (2010).

29 Venney, C. J., Johansson, M. L. & Heath, D. D. Inbreeding effects on gene-specific DNA methylation among tissues of Chinook salmon. *Mol Ecol* **25**, 4521-4533, doi:10.1111/mec.13777 (2016).

30 Vergeer, P., Wagemaker, N. C. & Ouborg, N. J. Evidence for an epigenetic role in inbreeding depression. *Biol Lett* **8**, 798-801, doi:10.1098/rsbl.2012.0494 (2012).

31 Han, W. *et al.* Genome-wide analysis of the role of DNA methylation in inbreeding depression of reproduction in Langshan chicken. *Genomics* **112**, 2677-2687, doi:10.1016/j.ygeno.2020.02.007 (2020).

32 Thompson, M. J. *et al.* A multi-tissue full lifespan epigenetic clock for mice. *Aging (Albany NY)* **10**, 2832-2854, doi:10.18632/aging.101590 (2018).

33 Zhang, Q. *et al.* Improved precision of epigenetic clock estimates across tissues and its implication for biological ageing. *Genome Med* **11**, 54, doi:10.1186/s13073-019-0667-1 (2019).

34 Snir, S., Farrell, C. & Pellegrini, M. Human epigenetic ageing is logarithmic with time across the entire lifespan. *Epigenetics* **14**, 912-926, doi:10.1080/15592294.2019.1623634 (2019).

35 Pinho, G. M. *et al.* Hibernation slows epigenetic aging in yellow-bellied marmots. *bioRxiv* doi: 10.1101/2021.03.07.434299 (2021).

36 Moehlman, P. D. (2002). *Equids: Zebras, Asses and Horses Status Survey and Conservation Action Plan* (Vol. 37). Gland, Switzerland: IUCN/SSC Equid Specialist Group. 190 pp.

37 Moehlman, P. D. & King, S. R. B. IUCN SSC Equid Specialist Group 2020 Report. https://www.iucn.org/commissions/ssc-groups/mammals/mammals-a-e/equid (2020)

38 Rubinacci, S., Ribeiro, D. M., Hofmeister, R. & Delaneau, O. Efficient phasing and imputation of low-coverage sequencing data using large reference panels. *Nature Genetics* **53**, 120-126, doi: 10.1038/s41588-020-00756-0 (2021).

39 Ceballos, F. C., Hazelhurst, S. & Ramsay, M. Runs of homozygosity in sub-Saharan African populations provide insights into complex demographic histories. *Hum Genet* **138**, 1123-1142, doi:10.1007/s00439-019-02045-1 (2019).

40 Curik, I., Ferenčaković, M. & Sölkner, J. Inbreeding and runs of homozygosity: A possible solution to an old problem. *Livestock Science* **166**, 26-34, doi:10.1016/j.livsci.2014.05.034 (2014).

41 Anderson, J. A. *et al.* The costs of competition: high social status males experience accelerated epigenetic aging in wild baboons.*eLife*, 10:e66128, doi:10.7554/eLife.66128 (2020).

42 McLean, C. Y. *et al.* GREAT improves functional interpretation of cis-regulatory regions. *Nature Biotechnology* **28**, doi:10.1038/nbt.1630 PMID - 20436461 (2010).

43 Gronniger, E. *et al.* Aging and chronic sun exposure cause distinct epigenetic changes in human skin. *PLoS Genet* **6**, e1000971, doi:10.1371/journal.pgen.1000971 (2010).

44 Robeck, T. R. *et al.* Multi-species and multi-tissue methylation clocks for age estimation in toothed whales and dolphins. *Communications Biology* **4**, doi:10.1038/s42003-021-02179-x (2021).

45 Jonsson, H. *et al.* Speciation with gene flow in equids despite extensive chromosomal plasticity. *Proc Natl Acad Sci U S A* **111**, 18655-18660, doi:10.1073/pnas.1412627111 (2014).

46 Vilstrup, J. T. *et al.* Mitochondrial phylogenomics of modern and ancient equids. *PLoS One* **8**, e55950, doi:10.1371/journal.pone.0055950 (2013).

47 Jensen-Seaman, M. I. & Hooper-Boyd, K. A. in *Encyclopedia of Life Sciences (ELS)* (John Wiley & Sons,Ltd. 10.1002/9780470015902.a0020813 (2008).

48 Farrell, C., Snir, S. & Pellegrini, M. The Epigenetic Pacemaker - modeling epigenetic states under an evolutionary framework. *Bioinformatics*, doi:10.1093/bioinformatics/btaa585 (2020).

49 Snir, S. & Pellegrini, M. An epigenetic pacemaker is detected via a fast conditional expectation maximization algorithm. *Epigenomics* **10**, 695-706, doi:10.2217/epi-2017-0130 (2018).

50 Charlesworth, B. & Hughes, K. A. Age-specific inbreeding depression and components of genetic variance in relation to the evolution of senescence. *Proc Natl Acad Sci U S A* **93**, 6140-6145, doi:10.1073/pnas.93.12.6140 (1996).

51 Fox, C. W. Inbreeding depression increases with maternal age. *Evolutionary Ecology Research* **12**, 961–972 (2010).

52 Benton, C. H. *et al.* Inbreeding intensifies sex- and age-dependent disease in a wild mammal. *J Anim Ecol* **87**, 1500-1511, doi:10.1111/1365-2656.12878 (2018).

53 Mayne, B., Berry, O., Davies, C., Farley, J. & Jarman, S. A genomic predictor of lifespan in vertebrates. *Sci Rep* **9**, 17866, doi:10.1038/s41598-019-54447-w (2019).

54 McClain, A. T. & Faulk, C. The evolution of CpG density and lifespan in conserved primate and mammalian promoters. *Aging* **10**, 561-572, doi: 10.18632/aging.101413 (2018).

55 Alpi, A. F., Pace, P. E., Babu, M. M. & Patel, K. J. Mechanistic insight into site-restricted monoubiquitination of FANCD2 by Ube2t, FANCL, and FANCI. *Mol Cell* **32**, 767-777, doi:10.1016/j.molcel.2008.12.003 (2008).

56 Kannan, M. B., Solovieva, V. & Blank, V. The small MAF transcription factors MAFF, MAFG and MAFK: current knowledge and perspectives. *Biochim Biophys Acta* **1823**, 1841-1846, doi:10.1016/j.bbamcr.2012.06.012 (2012).

57 Li, Z. *et al.* PBX3 is an important cofactor of HOXA9 in leukemogenesis. *Blood* **121**, 1422-1431, doi:10.1182/blood-2012-07-442004 (2013).

58 Malecki, M. T. *et al.* Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. *Nat Genet* **23**, 323-328, doi:10.1038/15500 (1999).

59 Ding, Q., Joshi, P. S., Xie, Z. H., Xiang, M. & Gan, L. BARHL2 transcription factor regulates the ipsilateral/contralateral subtype divergence in postmitotic dI1 neurons of the developing spinal cord. *Proc Natl Acad Sci U S A* **109**, 1566-1571, doi:10.1073/pnas.1112392109 (2012).

60 Mo, Z., Li, S., Yang, X. & Xiang, M. Role of the Barhl2 homeobox gene in the specification of glycinergic amacrine cells. *Development* **131**, 1607-1618, doi:10.1242/dev.01071 (2004).

61 Giampietro, C. *et al.* The alternative splicing factor Nova2 regulates vascular development and lumen formation. *Nat Commun* **6**, 8479, doi:10.1038/ncomms9479 (2015).

62 Yano, M., Hayakawa-Yano, Y., Mele, A. & Darnell, R. B. Nova2 regulates neuronal migration through an RNA switch in disabled-1 signaling. *Neuron* **66**, 848-858, doi:10.1016/j.neuron.2010.05.007 (2010).

63 Deneen, B. *et al.* The transcription factor NFIA controls the onset of gliogenesis in the developing spinal cord. *Neuron* **52**, 953-968, doi:10.1016/j.neuron.2006.11.019 (2006).

64 Hiraike, Y. *et al.* NFIA co-localizes with PPARgamma and transcriptionally controls the brown fat gene program. *Nat Cell Biol* **19**, 1081-1092, doi:10.1038/ncb3590 (2017).

65 Caricasole, A., C., S., Roncarati, R., Formenti, E. & Terstappen, G. C. Cloning and characterization of the human phosphoinositide-specific phospholipase C-beta 1 (PLCβ1). *Biochimica et Biophysica Acta* **1517**, 63-72 (2000).

66 McOmish, C. E., Burrows, E. L., Howard, M. & Hannan, A. J. PLC-beta1 knockout mice as a model of disrupted cortical development and plasticity: behavioral endophenotypes and dysregulation of RGS4 gene expression. *Hippocampus* **18**, 824-834, doi:10.1002/hipo.20443 (2008).

67 Mittelstaedt, T., Alvarez-Baron, E. & Schoch, S. RIM proteins and their role in synapse function. *Biol Chem* **391**, 599-606, doi:10.1515/BC.2010.064 (2010).

68 Schoch, S. *et al.* RIM1α forms a protein scaffold for regulating neurotransmitter release at the active zone. *Nature* **415**, 321-326 (2002).

69 Lu, A. T. *et al.* Universal DNA methylation age across mammalian tissues. *biorxiv*, 2021.2001.2018.426733, doi:10.1101/2021.01.18.426733 (2021).

70 Nishikawa, K. *et al.* Maf promotes osteoblast differentiation in mice by mediating the age-related switch in mesenchymal cell differentiation. *J Clin Invest* **120**, 3455-3465, doi:10.1172/JCI42528 (2010).

71 Saidak, Z., Hay, E., Marty, C., Barbara, A. & Marie, P. J. Strontium ranelate rebalances bone marrow adipogenesis and osteoblastogenesis in senescent osteopenic mice through NFATc/Maf and Wnt signaling. *Aging Cell* **11**, 467-474, doi:10.1111/j.1474-9726.2012.00804.x (2012).

72 McClay, J. L. *et al.* A methylome-wide study of aging using massively parallel sequencing of the methyl-CpG-enriched genomic fraction from blood in over 700 subjects. *Hum Mol Genet* **23**, 1175-1185, doi:10.1093/hmg/ddt511 (2014).

73 Ambeskovic, M. *et al.* Ancestral stress programs sex-specific biological aging trajectories and non-communicable disease risk. *Aging (Albany NY)* **12**, 3828-3847, doi:10.18632/aging.102848 (2020).

74 Burger, C., Lopez, M. C., Baker, H. V., Mandel, R. J. & Muzyczka, N. Genome-wide analysis of aging and learning-related genes in the hippocampal dentate gyrus. *Neurobiol Learn Mem* **89**, 379-396, doi:10.1016/j.nlm.2007.11.006 (2008).

75 Horvath, S. *et al.* DNA methylation clocks show slower progression of aging in naked mole-rat queens. *biorxiv*, doi:10.1101/2021.03.15.435536 (2021).

76 Rapoport, S. I., Primiani, C. T., Chen, C. T., Ahn, K. & Ryan, V. H. Coordinated Expression of Phosphoinositide Metabolic Genes during Development and Aging of Human Dorsolateral Prefrontal Cortex. *PLoS One* **10**, e0132675, doi:10.1371/journal.pone.0132675 (2015).

77 Dube, J. B. *et al.* Genetic determinants of "cognitive impairment, no dementia". *J Alzheimers Dis* **33**, 831-840, doi:10.3233/JAD-2012-121477 (2013).

78 Hinney, A. *et al.* Genetic variation at the CELF1 (CUGBP, elav-like family member 1 gene) locus is genome-wide associated with Alzheimer's disease and obesity. *Am J Med Genet B Neuropsychiatr Genet* **165B**, 283-293, doi:10.1002/ajmg.b.32234 (2014).

79 Ntalla, I. *et al.* Replication of established common genetic variants for adult BMI and childhood obesity in Greek adolescents: the TEENAGE study. *Ann Hum Genet* **77**, 268-274, doi:10.1111/ahg.12012 (2013).

80 Speliotes, E. K. *et al.* Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. *Nat Genet* **42**, 937-948, doi:10.1038/ng.686 (2010).

81 Gao, Z. *et al.* Neurod1 is essential for the survival and maturation of adult-born neurons. *Nat Neurosci* **12**, 1090-1092, doi:10.1038/nn.2385 (2009).

82 Badawi, Y. & Nishimune, H. Presynaptic active zones of mammalian neuromuscular junctions: Nanoarchitecture and selective impairments in aging. *Neurosci Res* **127**, 78-88, doi:10.1016/j.neures.2017.11.014 (2018).

83 Tollervey, J. R. *et al.* Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. *Genome Res* **21**, 1572-1582, doi:10.1101/gr.122226.111 (2011).

84 Kim, B. H., Nho, K., Lee, J. M. & Alzheimer's Disease Neuroimaging, I. Genome-wide association study identifies susceptibility loci of brain atrophy to NFIA and ST18 in Alzheimer's disease. *Neurobiol Aging* **102**, 200 e201-200 e211, doi:10.1016/j.neurobiolaging.2021.01.021 (2021).

85 Horvath, S. *et al.* DNA methylation aging and transcriptomic studies in horses. *biorxiv*, doi:10.1101/2021.03.11.435032 (2021).

86 Benayoun, B. A., Pollina, E. A. & Brunet, A. Epigenetic regulation of ageing: linking environmental inputs to genomic stability. *Nat Rev Mol Cell Biol* **16**, 593-610, doi:10.1038/nrm4048 (2015).

87 Quach, A. *et al.* Epigenetic clock analysis of diet, exercise, education, and lifestyle factors. *Aging* **9**, 419-446, doi:10.18632/aging.101168 (2017).

88 Crary-Dooley, F. K. *et al.* A comparison of existing global DNA methylation assays to low-coverage whole-genome bisulfite sequencing for epidemiological studies. *Epigenetics* **12**, 206-214, doi:10.1080/15592294.2016.1276680 (2017).

89 Reed, K., Poulin, M. L., Yan, L. & Parissenti, A. M. Comparison of bisulfite sequencing PCR with pyrosequencing for measuring differences in DNA methylation. *Anal Biochem* **397**, 96-106, doi:10.1016/j.ab.2009.10.021 (2010).

90 Tost, J., Dunker, J. & Gut, I. G. Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *Biotechniques* **35**, 152-156, doi:10.2144/03351md02 (2003).

91 Karesh, W. B. Chapter 13. Biopsy Darting. Pp 298-308 in *Zoo and Wild Animal Medicine: Current Therapy* (eds E. Fowler Murray & R. Eric Miller) (Saunders Elsevier, 2008).

92 Chiou, K. L. & Bergey, C. M. Methylation-based enrichment facilitates low-cost, noninvasive genomic scale sequencing of populations from feces. *Sci Rep* **8**, 1975, doi:10.1038/s41598-018-20427-9 (2018).

93 Orkin, J. D. *et al.* The genomics of ecological flexibility, large brains, and long lives in capuchin monkeys revealed with fecalFACS. *Proc Natl Acad Sci U S A* **118**, doi:10.1073/pnas.2010632118 (2021).

94 Snyder-Mackler, N. *et al.* Efficient Genome-Wide Sequencing and Low-Coverage Pedigree Analysis from Noninvasively Collected Samples. *Genetics* **203**, 699-714, doi:10.1534/genetics.116.187492 (2016).

95 Harley, E. H., Knight, M. H., Lardner, C., Wooding, B. & Gregor, M. The Quagga project: progress over 20 years of selective breeding. **39**, doi:10.3957/056.039.0206 PMID - 290552093570922685 1related:Y7donOV7UigJ (2009).

96 Arneson, A. *et al.* A mammalian methylation array for profiling methylation levels at conserved sequences *Biorxiv*, doi:10.1101/2021.01.07.425637 (2021).

97 Kalbfleisch, T. S. *et al.* Improved reference genome for the domestic horse increases assembly contiguity and composition. *Commun Biol* **1**, 197, doi:10.1038/s42003-018-0199-z (2018).

98 Wade, C. M. *et al.* Genome Sequence, Comparative Analysis, and Population Genetics of the Domestic Horse. *Science* **326**, doi:10.1126/science.1178158 (2009).

99 Zhou, W., Triche, T. J., Jr., Laird, P. W. & Shen, H. SeSAMe: reducing artifactual detection of DNA methylation by Infinium BeadChips in genomic deletions. *Nucleic Acids Res* **46**, e123, doi:10.1093/nar/gky691 (2018).

100 Bocklandt, S. *et al.* Epigenetic predictor of age. *PLoS One* **6**, e14821, doi:10.1371/journal.pone.0014821 (2011).

101 Hannum, G. *et al.* Genome-wide methylation profiles reveal quantitative views of human aging rates. *Mol Cell* **49**, 359-367, doi:10.1016/j.molcel.2012.10.016 (2013).

102 Friedman, J., Hastie, T. & Tibshirani, R. Regularization Paths for Generalized Linear Models via Coordinate Descent. *J Stat Softw* **33**, 1-22 (2010).

103 R: A language and environment for statistical computing (R Foundation for Statistical Computing, Vienna, Austria, 2020).

104 Van Rossum, G. & Drake, F. L. *Python 3 Reference Manual*. (CreateSpace, 2009).

105 Larison, B. *et al.* Population structure, inbreeding and stripe pattern abnormalities in plains zebras. *Mol Ecol* **30**, 379-390, doi:10.1111/mec.15728 (2021).

106 Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics* **25**, 1754-1760, doi:10.1093/bioinformatics/btp324 (2009).

107 Garrison, E. & Marth, G. Haplotype-based variant detection from short-read sequencing. *arXiv:1207:3907 [q-bio.GN]* (2012).

108 Freed, D., Aldana, R., Weber, J. A. & Edwards, J. S. The Sentieon Genomics Tools – A fast and accurate solution to variant calling from next-generation sequence data. *biorxiv*, doi:10.1101/115717 (2017).

109 Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078-2079, doi:10.1093/bioinformatics/btp352 (2009).

110 Meyermans, R., Gorssen, W., Buys, N. & Janssens, S. How to study runs of homozygosity using PLINK? A guide for analyzing medium density SNP data in livestock and pet species. *BMC Genomics* **21**, 94, doi:10.1186/s12864-020-6463-x (2020).

111 Purcell, S. *et al.* PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* **81**, 559-575, doi:10.1086/519795 (2007).

112 McQuillan, R. *et al.* Runs of homozygosity in European populations. *Am J Hum Genet* **83**, 359-372, doi:10.1016/j.ajhg.2008.08.007 (2008).

113 Zeileis, A. & Hothorn, T. Diagnostic Checking in Regression Relationships. **2**, 7-10 (2002).

114 Zeileis, A. Econometric Computing with HC and HAC Covariance Matrix Estimators. *Journal of Statistical Software* **11**, 1-17, doi:10.18637/jss.v011.i10 (2004).

115 Zeileis, A., Köll, S. & Graham, N. Various Versatile Variances: An Object-Oriented Implementation of Clustered Covariances in R. *Journal of Statistical Software* **95**, 1-36, doi:10.18637/jss.v095.i01 (2020).

116 Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network analysis. *BMC Bioinformatics* **9**, 559, doi:10.1186/1471-2105-9-559 (2008).

117 Stouffer, S. A., Suchman, E. A., DeVinney, L. C., Star, S. A. & Williams, R. M. J. *Adjustment During Army Life*. (Princeton University Press, 1949).