A Novel Understanding of Global DNA Methylation in Bobcat (*Lynx rufus*)

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Abstract

Epigenetic mechanisms may provide a novel prospective of bobcat *(Lynx rufus)* adaptation to habitat loss/fragmentation. Previous research has focused on bobcat behavior and genetics, but epigenetics has not been studied in bobcat. The aim of this study was to determine the quantity of global DNA methylation in the liver of 30 bobcats. DNA was extracted from liver samples obtained from the Vermont Fish and Wildlife Department. The percent of global DNA methylation was quantified and calculated using the MethylFlash™ Methylated DNA 5-mC Quantification Kit from Epigentek (Farmingdale, NY). Age, sex, and carcass weight data were collected at sampling and analyzed with percent of global DNA methylation. Global DNA methylation was found to range from 0.46% to 2.76%. Age ranged from <1 to 12 years old and weight ranged from 3.18 to 13.61 kg. Further analysis of differential methylation may provide insight into novel means of bobcat conservation within different regions of Vermont. These results reinforce the need for genome-wide epigenetic studies in conservation biology.

Keywords: Conservation, Epigenetics, *Felidae*, Wildlife, Methylation

Introduction

Bobcats are at risk for population decline due to increased vulnerability to habitat fragmentation, road mortality and habitat loss (Donovan et al. 2011; Hunting_and_Trapping Vermont_Fish_and_Wildlife; Team 2015; Woolf et al. 2002). Genetic research in bobcats thus far has been focused on identifying dispersal patterns and levels

47 of genetic diversity within specific populations to understand the genetic effects of habitat
48 loss (Anderson et al. 2015; Diefenbach et al. 2015; Janecka et al. 2016; Janečka et al. 2007).
49 This use of genetic research has proven to be especially informative for conservation
50 management decisions (Allendorf et al. 2010). However, the use of epigenetics can allow
51 for greater comprehension of bobcat adaption to changing environmental conditions
52 (Allendorf et al. 2010).

53 Epigenetic modifications incorporate genetic and environmental interactions that affect
54 phenotypic variation. Epigenetics is described as the heritable change in gene expression
55 that occurs without a change to the nucleotide sequence (Ling and Groop 2009). DNA
56 methylation is a type of epigenetic modification classified as the addition of a methyl group
57 on the fifth position of a cytosine nucleotide resulting in a 5-methylcytosine (5-mC)
58 (Murphy et al. 2013). When DNA methylation is present in a gene promoter, it can inhibit
59 gene expression by preventing transcription factors and polymerases from binding to the
60 promoter (Dayeh et al. 2013). Methylation is variable and can differ between tissues
61 within the same individual as well as the same tissue can differ between individuals.
62 Factors that may influence DNA methylation include developmental stage, tissue type, age,
63 maternal diet, and habitat (Bird 2002).

64 The effect of DNA methylation in relation to weight and age in other species has been previously
65 studied. Takumi et al. (2015) found that feeding mice a methyl-deficient diet decreased mass due
66 to the lack of methionine and choline and promoted DNA demethylation patterns in the liver
67 (Takumi et al. 2015). Studies performed by both Bollati et al. in 2009 and Hannum et al. in 2013
68 determined that DNA methylation in the human genome decreases with age (Bollati et al. 2009;
69 Hannum et al. 2013b). The relationships between epigenetics, weight and age are unknown in

bobcat, but are anticipated to be similar to mammalian epigenetic trends. However, diet and weight has been shown to vary by sex, age, and maturity in bobcats and may contribute to differential methylation between sex, age, and maturity. Litvaitis *et al.* found the diet of mature bobcats and male bobcats consists more of deer than female and immature bobcats. The differences in diet composition in bobcats may cause differential exposure to methyl donors in the body and therefore promote differential DNA methylation between sex, age, weight, and maturity.

Here we determine the percentage of global DNA methylation within the bobcat liver epigenome. The primary objectives for this study are 1) to determine the percentage of global DNA methylation within each bobcat liver genome and 2) examine and correlate the differences in the percentage of global DNA methylation between bobcat age and carcass weight of each bobcat liver sample. This novel study provides the first insight into global DNA methylation in bobcat, which may facilitate a new direction for future conservation research.

Materials and Methods

Sample Collection

Liver samples were collected from 30 bobcats that were harvested by trappers during the 2013/14 furbearer season in the state of Vermont. Carcasses were turned over to Vermont Fish and Wildlife Department as part of the Furbearer project and age and weight were collected (Hunting_and_Trapping Vermont_Fish_and_Wildlife). The samples consisted of 11

males and 19 females with ages ranging from <1 to 12 years old. The carcass weights ranged from 3.18 to 13.61 kg (Supplementary Table 1).

Extraction and Global DNA Methylation Quantification

DNA was extracted from 30 liver samples using a phenol-chloroform extraction process as previously described (Sambrook et al. 1989). The concentration and quality of each DNA sample was obtained using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and the absence of sheared DNA was checked by gel electrophoresis using 50ng of DNA on a 1% agarose gel.

To quantify the global amount of 5-mC within each liver DNA sample, 400ng of DNA for each sample was sent to Epigentek and run on the Methylflash™ Methylated DNA 5-mC Quantification Kit (Colorimetric) (Epigentek, Farmingdale, NY). At Epigentek, the samples were quantified using a picogreen fluorescence method and were otherwise run with standard conditions as described by Epigentek. Briefly, the 30 samples, 6 positive controls (ranging from 0.2ng/µl to 10ng/µl), and a negative control were run in duplicates. For each sample, 50ng of DNA was bound to a plate, and fluorescently labeled for 5-mC presence using various proprietary antibodies. The optical density of each sample was recorded for the plate based on the amount of 5-mC florescence at 450nm. For each positive control, the concentration was plotted by the optical density to create a standard curve. The slope of the standard curve was determined using linear regression and was used to determine the concentration of global 5-mC of each sample. Percent global DNA methylation was determined by dividing the concentration of global 5-mC by 50ng and multiplying by 100.

Statistical Analysis

Statistical analyses were performed in JMP version 13, 2016 (SAS Cary, NC).

The Restricted Maximum Likelihood (REML) method was used to test for correlations between DNA methylation, weight, age, sex, and maturity. A two-way analysis of variance (ANOVA) was used to test for differences in the variation between DNA methylation by sex, maturity, and sex and maturity interactions. The two-way ANOVA was then repeated for age and weight for sex, maturity, and sex and maturity interactions. An analysis of covariance (ANCOVA) for DNA methylation with age and weight as covariates were also used to test for variation in DNA methylation by sex, maturity, and sex and maturity interactions.

Groupings

Maturity of the male and female bobcats was classified as mature for >2 years old for male bobcats and >1 year old for females (Crowe 1975; Fritts and Sealander 1978). One male sample did not have an age score, but was classified as immature based on the light carcass weight (Crowe 1975).

Results

DNA Methylation

Global measures of methylation detected in the bobcat liver ranged from 0.46% to 2.76% with an average methylation of 1.65% (Supplementary Table 1 and Figure 1). The global 5-mC measures ranged from 0.46% to 2.61% in females and 0.82% to 2.76% in males, 0.82% to 2.76% in immature bobcats independent of sex, and 0.46% to 2.61% in mature bobcats

134 independent of sex. The average %5-mC was 1.70% in mature bobcat, 1.60% in immature
135 bobcat, 1.67% in female bobcat, and 1.61% in male bobcat (Table 1). No significant
136 differences were found in DNA methylation between each sex, maturity, or sex and
137 maturity interactions ($p<0.05$) using an ANOVA or ANCOVA. The complete dataset can be
138 found in Supplementary Table 1.
139
140 *Age*
141 The age of the bobcats ranged from 0 to 12 years and averaged 2.52 years (Figure 1). The
142 age of one mature male bobcat was older than the rest of the population (12 years) and
143 classified as an outlier. Male bobcats were significantly older than female bobcats
144 ($p=0.013$) with a range of 0 to 12 years and an average of 2.70 years. Female bobcats
145 ranged from 0 to 6 years and averaged 2.42 years. The average age was 4.27 years in
146 mature bobcats and 0.64 years in immature bobcats (Table 1). Mature bobcats were
147 significantly older than immature bobcats ($p<0.0001$) and male mature bobcats were
148 significantly older than female mature bobcats ($p=0.048$).
149
150 *Weight*
151 Bobcat weight ranged from 3.18 to 13.61 kg and averaged 6.87 kg (Figure 1). The weight of
152 two mature male bobcats was heavier than the rest of the population (12.7 and 13.61 kg)
153 and classified as outliers. Male bobcats were significantly heavier than female bobcats
154 ($p=0.0001$) with a range of 3.4 to 13.61 kg and an average of 7.86 kg. Female bobcats
155 ranged from 3.18 to 7.71 kg and averaged 6.28 kg. The average weight was 7.86 kg in
156 mature bobcats and 5.82 kg in immature bobcats (Table 1). Mature bobcats were

157 significantly heavier than immature bobcats (p<0.0001) and male mature bobcats were
158 significantly heavier than female mature bobcats (p=0.0049).
159
160 *Correlations*
161 Restricted Maximum Likelihood (REML) method was used to determine the correlation
162 between phenotypes. Age and weight had a significant positive correlation (p<0.0001).
163 When outliers were excluded, the correlation remained significant and positive (p<0.007).
164 DNA methylation was not significantly correlated to age or weight (p>0.05). No correlation
165 was found between maturity and age, weight, or DNA methylation.
166

167 Discussion

168 This is the first report on DNA methylation in bobcat. However, DNA methylation has been
169 reported in other Felidae species. Global DNA methylation levels of 0.94% have been
170 reported for the liver methylome of *Panthera uncia* (snow leopard) (Jabbari et al. 1997).
171 These reports are similar to our results which found the bobcat liver epigenome to range
172 from 0.46% to 2.76% 5-mC (Figure 1). A report of the *Felis catus* (domestic house cat)
173 genome reported 10.5% of cytosines to be methylated in whole blood (Tamazian et al.
174 2014). This is also similar to what we found, but reported as percentage of methylation out
175 of total cytosines and our study reports methylation as a percentage of the entire genome.
176 While these previous studies on Felidae DNA methylation are based on a single animal, we
177 have reported DNA methylation in 30 bobcats. Here we show there is some variation in the
178 level of DNA methylation between individual bobcat with a standard deviation of 0.57%

(Table 1). The genetic differences between species, tissue type and environment all can contribute towards the observed variation in DNA methylation between Felidae. Variation of methylation within the same tissue between animals and between tissues within the same animal is highly documented (Bird 2002). Therefore, a portion of the observed differences in global methylation between members of the family Felidae can be attributed to the use of different tissue types in the different studies performed. However, environmental differences of these three species may contribute towards considerable variation in global DNA methylation levels.

Measurements of DNA methylation have been found to decrease with age in a variety of species. Bollati et al. (2008) showed a gradual decrease in DNA methylation in the human Alu transposable repetitive element over a span of eight years (Bollati et al. 2009). Hannum et al. (2013) showed similar patterns in genome-wide DNA methylation and showed methylation as strong biomarker of biological aging in humans (Hannum et al. 2013b). The consistent correlation between age and methylation was also reflected by Teschendorff et al. (2013) who found correlations with the deregulation of DNA methylation to age and correlations to the environmental factors driving methylation deregulation early in life (Teschendorff et al. 2013). The adverse effect of age on DNA methylation levels has been reported in reptiles. In a study performed by Parrott et al. (2014), measures of global DNA methylation in blood was higher in juveniles compared to adult American alligators (*Alligator mississippiensis*) (Parrott et al. 2014). Our results did not find a correlation between methylation and age. However, our findings could be influenced by a relatively small sample size of both the male and female bobcats and unequal representation of age. Regardless, the trend in our findings are similar to those

reported in mice, humans and rats (Unnikrishnan et al. 2018). In fact, the hypothesis that global DNA methylation decreased with age was initially reported in 1988 (Zs-nagy et al. 1988) and advances since then have confirmed that there is an effect of aging on DNA methylation but that effect is more likely to be seen at specific methylated cytosines or at patterns of specific cytosine methylation as opposed to global measures of DNA methylation. Further, changes in specific methylated cytosines have been utilized to generate DNA methylation clocks to determine chronological and biological age of different species. A majority of work regarding DNA methylation clocks has been performed in humans (Hannum et al. 2013a; Horvath 2013; Unnikrishnan et al. 2019). Although, ages of dogs and wolves are able to be estimated to within a year in dogs and wolves, using an epigenetic aging clock (Thompson et al. 2017). As accuracy of these tools improve, it will be interesting to realize the utility of aging clocks in wildlife populations and determine their usefulness as a noninvasive means of determining age.

DNA methylation and weight can both be influenced by diet and home range size. A study performed by Gittleman and Harvey (1982) showed in carnivores that the amount of flesh in the animal's diet has a significant positive correlation to home range size (Gittleman and Harvey 1982). There may be similar relationships in bobcat in which the home range size affects diet composition and potentially the amount of methylation in the diet. Mature male bobcats in Vermont have a home range of 70.9 km^2, enabling them to access different environments and consume different food sources than mature female bobcats, which have the smaller home range of 22.9 km^2 (Donovan et al. 2011). While male bobcats in Vermont have a larger home range size than females, Donovan *et al.* also found that all bobcats regardless of sex, age, or season stayed within 1km of habitats with a greater prey density

and cover (Donovan et al. 2011). The similar behaviors between bobcats of different sexes and ages in regard to food proximity may allow for bobcats in Vermont to overall have a similar level of methylation in their diets. This may explain why we did not find global DNA methylation to differ by sex or age globally, but the resolution of DNA methylation in this study may also not be enough to detect differences.

As the contribution of genetics and genomics towards conservation efforts has been established, the role of epigenetic modifications to conservation efforts is just beginning to be investigated. Loss of genetic diversity (Sheldon et al. 2018), outbreeding depression (Bossdorf et al. 2008) and genotype-by-environmental interactions are among the factors contributing to conservation that are regulated by both genetic and epigenetic mechanisms. Environmental effects that have been studied in wildlife species and may play a role in conservation efforts include mercury associated hypomethylation in polar bear brains (Richard Pilsner et al. 2010) and heritable effects of DNA methylation relative to paternal effects on adaption and anxious offspring in stickleback fish (McGhee and Bell 2014). Integration of genetic and epigenetic data, such as DNA methylation, may provide connections between population genetics and genomics that have not been investigated thus far (Allendorf et al. 2010). In order to eluciate the role of ecological epigenetics with respect to conservation of wildlife species, additional epigenetics research needs to be performed. This study found individual variation in DNA methylation in bobcats and, to the best of our knowledge, is the first study to examine DNA methylation in bobcats.

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Table 1: Descriptive Statistics for DNA Methylation, Age, and Weight in Bobcats

"Obs. No." is the number of observations for each category. "SD" is the standard deviation and "SE" is the standard error. The 95% confidence interval is reported as "CI 95%" with "LL" as the lower limit and "UL" as the upper limit.

Measurement	Group	Obs. No.	Mean	SD	SE	CI 95% LL	CI 95% UL
	Total	30	1.65	0.57	0.1	1.44	1.86
	Female	19	1.67	0.56	0.13	1.4	1.94
Methylation	Male	11	1.61	0.6	0.18	1.21	2.02
(Percentage)	Immature	15	1.6	0.62	0.16	1.26	1.94
	Mature	15	1.7	0.53	0.14	1.4	1.99
	Total	29	2.52	2.65	0.49	1.51	3.53
	Female	19	2.42	2.01	0.46	1.45	3.39
Age (Years)	Male	10	2.7	3.71	1.17	0.04	5.36
	Immature	14	0.64	0.84	0.23	0.16	1.13
	Mature	15	4.27	2.58	0.67	2.84	5.69
	Total	29	6.87	2.45	0.46	5.94	7.81
	Female	18	6.28	1.37	0.32	5.59	6.96
Weight (kg)	Male	11	7.86	3.45	1.04	5.54	10.17
	Immature	14	5.82	2.12	0.57	4.59	7.04
	Mature	15	7.86	2.39	0.62	6.54	9.18

Figure 1: Scatterplot of animals by their weight (x axis) and age (y axis), with their methylation levels (color from red to dark blue) and sex (square size)