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## DNA-based approach to aging martens (*Martes americana* and *M. caurina*)

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Demographic structure is central to understanding the dynamics of animal populations. However, determining the age of free-ranging mammals is difficult, and currently impossible when sampling with noninvasive, genetic-based approaches. We present a method to estimate age class by combining measures of telomere lengths with other biologically meaningful covariates in a Bayesian network. We applied this approach to American and Pacific martens (*Martes americana* and *M. caurina*) and compared predicted age with that obtained from counts of cementum annuli. Using telomere length and the covariates sex, species, and estimates of population density obtained from commercial trapping records, we assigned martens to juvenile (<1 year) or adult ( $\geq 1$  year) classes with 75–88% accuracy. In our analysis for live-captured martens, for which information on body size and whether animals were juveniles or adults would be available, we achieved 90–93% accuracy when assigning individuals to 5 discrete age classes (0–4+ years). This general approach could be extended to other species for noninvasive estimation of age class, or in place of invasive aging methods, and enable demographically based population analyses that have heretofore been impossible.

Key words: cementum annuli, mesocarnivore, Q-PCR, senescence, telomeres

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Aging, or senescence, is the result of processes that are progressive, irreversible, and unavoidable. Although the exact physiologic and evolutionary mechanisms underlying senescence remain unclear (Williams 1999), the impacts of aging on individual fitness are well understood. Age alters an individual's physiological state and ability to respond to environmental conditions; aging reduces cognitive ability, physical stamina (MacNulty et al. 2009), and immunocompetence (Clark 2004). Because population growth is a function of age-specific fecundity and survival, accounting for age structure of sampled populations is essential. However, determining the age of free-ranging mammals is difficult. Traditionally, field biologists have used several approaches to approximate the age of animals: weighing eye lenses, measuring skeletal and cranial characteristics, and counting cementum annuli of teeth (Schroeder and Robb 2005). Although useful, these techniques are highly invasive, some requiring the sacrifice of animals, which is particularly problematic when studying threatened or endangered species.

Further, such traditional approaches are unviable for the growing number of biologists using noninvasive sampling on the basis of deoxyribonucleic acid (DNA) analyses of fur, feather, skin, or scat samples. Lacking an aging technique, noninvasive studies thus far have been limited by the inability to quantify age-specific fecundity, survival or dispersal, or demographic structure.

Biomedical scientists have identified a relationship between the length of telomeres—repetitive, highly conserved DNA sequences ( $[(T_2AG_3)_n]$ ) that cap the ends of eukaryotic chromosomes—and senescent processes in individuals (Frenck et al. 1998). Past work indicates that telomeres can be useful not only in understanding senescence but also influence longevity (Vaziri et al. 1994), survival (Bize et al. 2009), and reproductive success (Pauliny et al. 2006). During



**TABLE 1.**—Location, sample size, and age (median, minimum–maximum) of 399 martens (*Martes* spp.) used to quantify telomere length.

State	Counties	<i>n</i>	Age (years)
Alaska <sup>a</sup>	Ketchikan Gateway, Prince of Wales, Skagway–Hoonah–Angoon	274 <sup>b,c</sup>	0, 0–10
Wyoming	Albany, Fremont	37 <sup>c</sup>	0, 0–3
Michigan	Alger, Baraga, Chippewa, Iron, Luce, Mackinac, Marquette, Ontonagon, Schoolcraft	33 <sup>b</sup>	1, 0–6
Minnesota	Cook, Itasca, Koochiching, Lake, St. Louis	27 <sup>b</sup>	0, 0–4
British Columbia <sup>a</sup>	Skeena–Queen Charlotte (Queen Charlotte Islands)	21 <sup>c</sup>	1, 0–7
Wisconsin	Forest, Vilas	5 <sup>b</sup>	1, 1–3
Colorado	Gunnison	2 <sup>c</sup>	1–4

<sup>a</sup> Alaska and British Columbia are organized by boroughs and regional districts, respectively.

<sup>b</sup> *M. americana* sampled from each location. Within Alaska 226 of our samples were from *M. americana*.

<sup>c</sup> *M. caurina* sampled from each location. Within Alaska 48 of our samples were from *M. caurina*.

each cell cycle telomere sequences are lost because DNA polymerase is incapable of fully replicating the 3' end of DNA (Watson 1972); thus, telomeres progressively shorten with each cell division. Past research has demonstrated age-related attrition of telomeres in a variety of animals (Hausmann et al. 2003a), although using telomere length to predict individual age has not yet been successful (Nakagawa et al. 2004). In addition to aging, telomeric loss is accelerated by physiological stress (Epel et al. 2004; Kotrschal et al. 2007), chronic disease (Ilmonen et al. 2008), and poor condition (Terry et al. 2008). Thus, telomeres reflect cumulative biological stress rather than strictly chronological age (Hausmann and Mauck 2007; Salomons et al. 2009). No study to date has evaluated the potential of using telomere length to predict age while accounting for these important covariates.

To develop a reliable method for estimating the age of wild mammals noninvasively we quantified telomere length for a large sample of American (*Martes americana*) and Pacific martens (*M. caurina*). These North American mesocarnivores have a maximum life span of 13 years (Powell et al. 2004), providing a sufficiently broad age distribution to assess changes in telomere length; can be aged reliably with dental techniques (Poole et al. 1994); and are trapped for fur, so large numbers of tissue samples are available from many locations across their geographic range. We quantified telomeric loss in relation to chronological age in these 2 species of North American martens and evaluated covariates hypothesized to influence telomere attrition, including species, sex, body condition, parasite load, habitat quality, and geographic location. We then generated a series of Bayesian network (BN) models to predict age from telomere length and relevant covariates.

## MATERIALS AND METHODS

**Sample preparation.**—We obtained carcasses of martens that were trapped for fur across North America (Table 1) from commercial trappers and state agencies. We followed the taxonomy used by previous authors (MacDonald and Cook 2009), recognizing 2 morphologically (Anderson 1970) and genetically discrete (Carr and Hicks 1997) species of marten in North America (*M. americana* and *M. caurina*). From each carcass we determined the species and sex and removed 20 mg

of intact muscle tissue. Tissue samples were stored at  $-40^{\circ}\text{C}$  until we extracted DNA with standard procedures (QIAGEN DNeasy tissue extraction kit; QIAGEN Inc., Valencia, California). Teeth (canine and second premolar) recovered from each carcass were used to determine animal age by counting the cementum annuli (Matson Laboratory, Milltown, Montana). Sampling procedures were in accordance with the guidelines established by the American Society of Mammalogists (Gannon et al. 2007).

**Telomere amplification.**—We quantified the relative length of telomeres with a real-time quantitative polymerase chain reaction (Q-PCR). This approach measures relative telomere lengths by comparing quantities of telomeric DNA (T) with those of an arbitrary reference single-copy gene (S) derived from the same sample. The T/S ratio of one individual relative to the T/S for another reflects relative differences in telomere length between individuals (Cawthon 2002). Q-PCR is highly accurate (Cawthon 2002) for quantifying relative telomere length and particularly for differentiating telomere length among individuals within a species (Nakagawa et al. 2004). We chose the single-copy gene, 36B4, originally used to quantify telomere length in humans (Cawthon 2002) and more recently applied to rodents, bovids, and birds (Bize et al. 2009; Callicott and Womack 2006; Kotrschal et al. 2007). Using genome data for 8 species (carnivores, primates, birds, amphibians, ungulates, and rodents; accessible at <http://www.ncbi.nlm.nih.gov/>) and the computer program ClustalX (version 1.81—Thompson et al. 1997), we conducted a sequence alignment and determined that the 36B4 gene is highly conserved across vertebrate taxa and is a suitable internal standard for a wide range of species, including martens.

We conducted telomere PCR and single-copy gene PCR on different 96-well plates; preparation of telomere and single-copy plates was identical except for the primers. We diluted extracted DNA with distilled water to 3 ng/ $\mu\text{l}$ . For each animal we added 10  $\mu\text{l}$  of diluted DNA to 2 adjacent wells. To generate a standard curve we diluted DNA from an arbitrarily chosen animal to 1 ng/ $\mu\text{l}$ , 2.5 ng/ $\mu\text{l}$ , 4 ng/ $\mu\text{l}$ , and 6 ng/ $\mu\text{l}$  and added 10  $\mu\text{l}$  of each concentration to 3 adjacent wells. Between rows of samples distilled water without template DNA was added to 2–4 wells as negative controls. Plates were sealed, centrifuged briefly, and heated in a thermocycler at  $96^{\circ}\text{C}$  for 10 min. After cooling the plate for 10 min, we added

2.25  $\mu\text{l}$  of distilled water and 12.5  $\mu\text{l}$  of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, California). The final primer concentrations were tel 1b, 100 nM; tel 2b, 900 nM; 36B4u, 300 nM; and 36B4d, 500 nM (primer sequences in Callicott and Womack 2006). Additionally, using standard PCR techniques and gel electrophoresis (1.5% agarose with  $5\times$  loading buffer and a 100-base-pair [bp] Molecular Ruler; Bio-Rad Laboratories, Hercules, California), we evaluated the specificity of the telomere primer. Standard PCR was conducted with no genomic template present, and with DNA from *Escherichia coli* (which lacks telomeres) to determine whether primer-dimers were produced; *M. americana* DNA also was included. We expected that most of the product would be approximately 79 bp in length, the sum of the lengths of the 2 primers, with a smear of reflectance for larger base-pair sizes.

To perform the Q-PCR reactions we used an automated thermocycler (7500 Real-Time PCR System; Applied Biosystems). For telomeres the reaction profile began with a 94°C incubation for 1 min, followed by 40 repetitions of 1 s of denaturing at 96°C, then 1 min of annealing–extending at 54°C. For the single-copy PCR the incubation lasted 10 min at 95°C, followed by 35 repetitions of 95°C for 15 s and 58°C for 1 min. Standard curves were constructed following automated curve estimation procedures in Applied Biosystems software. From each standard curve the amount of telomere and single-copy gene was calculated for each sample. Relative telomere length, T/S, was calculated from these quantities (Callicott and Womack 2006; Cawthon 2002, 2009). We performed Q-PCR in duplicate for each sample and used the average in subsequent statistical analyses. Although this Q-PCR technique quantifies both terminal and interstitial ( $\text{T}_2\text{AG}_3$ )<sub>n</sub> repeats, previous work has demonstrated that relative telomere length is correlated strongly with terminal-restriction, fragment-based analyses for a wide range of taxa (Bize et al. 2009).

*Data analysis.*—We explored the contribution of each variable to telomeric attrition and to predict the age of martens. Individual-level covariates included species (*M. americana* and *M. caurina*), sex, and zygomatic width (a metric for structural size). Additionally, we hypothesized that habitat quality and population density could affect rates of telomeric attrition. Therefore, we calculated net primary productivity (NPP) and indices of marten density for each location where carcasses were collected. We obtained multiyear (2000–2006) estimates of NPP ( $\text{g C}/\text{km}^2 \text{ year}^{-1}$ ) at 1-km spatial resolution from the MOD17 data set from the Numerical Terradynamic Simulation Group (University of Montana, Missoula, Montana). For each sampling location we identified the management unit and quantified an average NPP for that area. We obtained estimates of marten density 1 year before sample collection for each location using state harvest records. From these trapping records we estimated catch per unit effort, where we divided the number of martens trapped within the area by the number of active trappers and the length of the trapping season (days). Additionally, for a subset of

individuals for which entire carcasses were available, we documented the number of nematode parasites within the stomach ( $n = 206$ ) and overall body condition ( $n = 233$ ) with the dry mass (g) of greater omental fat (Thomas et al. 2008).

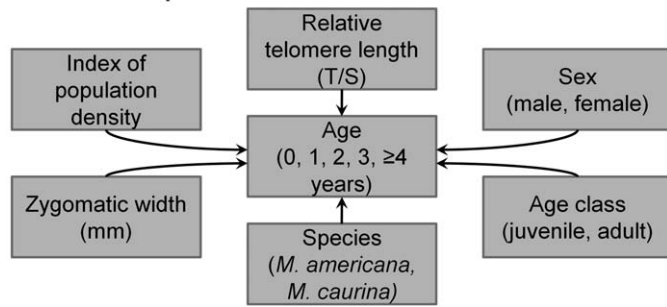
We explored relationships among covariates with simple bivariate Pearson correlations and detected a significant correlation of marten density with NPP ( $r = 0.49$ ,  $n = 369$ ,  $P < 0.001$ ). We tested for differences in telomere length between the 2 species, sexes, and sampling location (islands of the Pacific Northwest versus continental sites) with 2-sample *t*-tests assuming equal variance. We assessed the effect of continuous variables on telomere length directly with regression analyses, linearizing the predictor variables body condition and number of parasites with a natural log transformation (Zar 1999).

We used the BN modeling shell Netica v. 4.11 (Norsys Software Corp., Vancouver, Canada) to develop and test 27 models with a variety of covariates. Telomere length was included as a covariate in every model. BN modeling allowed us to express outcomes (individual ages in years or age classes) in probabilistic terms (Marcot et al. 2006) and to use cases with missing data to induce the model structure and test model performance (Ramoni and Sebastiani 1997). We constructed alternative model structures (i.e., to link selected covariates to the selected response variables) and incorporated the case data to induce the probability structure by using the expectation maximization algorithm (Dempster et al. 1977; Marcot et al. 2006). This resulted in fully parameterized Bayesian models that calculated posterior probabilities of marten age classes from covariate values (Fig. 1).

We calculated complexity of each model as number of covariates and as number of conditional probability values in each model. The latter is equal to the product of the number of discrete states in all covariates and response variables in each model (Marcot et al. 2006). These 2 measures pertain to model parsimony; equally performing models that have fewer numbers of covariates or conditional probabilities are simpler and thus preferred, although greater number of states in the variables provides increased precision. We conducted sensitivity analyses of the best-selected models by calculating variance reduction (Marcot et al. 2006) to determine the relative contribution of each covariate to the posterior probability values of the response variables (Castillo et al. 1998). We also conducted a maximum entropy analysis of relative telomere length using the Maxent software package v. 3.2.19 (Phillips et al. 2006), on the basis of all 399 samples, to assess the importance of relative telomere length in distinguishing age classes.

We compared the most probable prediction of individual age from each Bayesian model to the age or age class obtained from cementum annuli. We calculated spherical payoff (an index of model classification performance, ranging from 0 to 1—Marcot et al. 2006) and Schwarz's Bayesian information criterion (Castillo et al. 1998; Schwarz 1978) for each model. Models with greatest values of spherical payoff performed well in correctly predicting age or age classes. We tested the

A. Live capture



B. Noninvasive

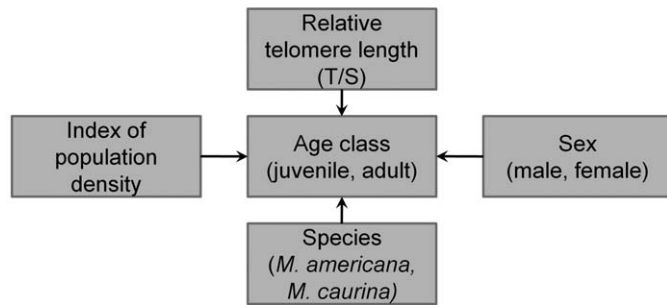


FIG. 1.—Bayesian network models to predict age class of martens (*Martes americana* and *M. caurina*) with covariates obtainable through A) live-capture or B) noninvasive sampling (i.e., no capture). Covariates are described in Table 2.

predictive performance of each model by calculating confusion error (percentage of cases incorrectly classified to age classes) of the most probable predicted outcome by testing each model against the full data set used to parameterize its probability structure. Overall, we used measures of spherical payoff and confusion error to identify the best live-capture and noninvasive sampling models, for both the all-data and island or continent versions. We further tested the classification performance of the best-selected models by conducting 10-fold cross-validation with the case data (Boyce et al. 2002).

RESULTS

*Telomere amplification and relationship to covariates.*—Via gel electrophoresis and standard PCR we detected telomere product for marten DNA samples within the expected size range, whereas we did not detect any product in the 2 negative controls (i.e., those lacking template or having *E. coli* [a prokaryote that lacks telomeric repeats] DNA; Fig. 2). Telomere length declined with increasing age ( $r^2 = 0.018$ ,  $F_{1,367} = 6.74$ ,  $P = 0.010$ ), although telomere lengths varied considerably for each age class (Fig. 3). We detected a strong species effect ( $t_{367} = 3.99$ ,  $P < 0.001$ ), with *M. americana* possessing longer telomeres than *M. caurina* (Fig. 4). Telomeres were also longer for martens captured on islands of the Pacific Northwest compared with those captured at continental sites ( $t_{367} = 2.82$ ,  $P = 0.005$ ; Fig. 4). Telomere length also exhibited a weak positive relationship with marten density in the previous year ( $r^2 = 0.024$ ,  $F_{1,367} = 9.21$ ,  $P =$

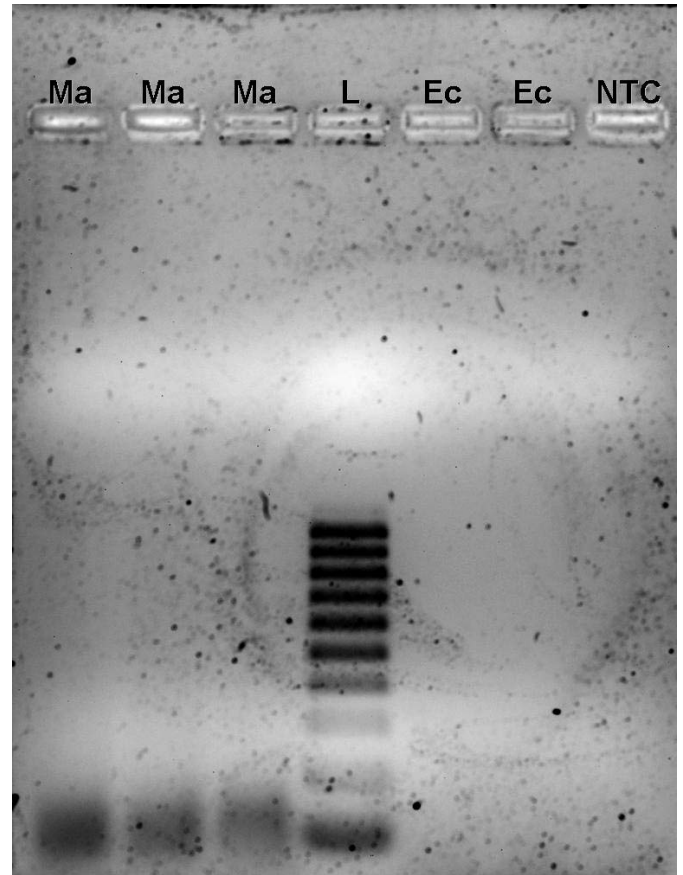


FIG. 2.—Agarose gel electrophoresis following standard PCR reaction with telomere primers (Ma = *Martes americana*, L = size standard, Ec = *Escherichia coli*, NTC = no template control). Because neither *E. coli* nor NTC contains telomeric repeats, they were used as negative controls. When mammalian template DNA was present, the majority of products migrated to the expected 79-bp region.

0.003; Fig. 4). We did not detect a relationship between telomere length and parasite load ( $F_{1,205} = 1.22$ ,  $P = 0.27$ ), body condition ( $F_{1,232} = 0.88$ ,  $P = 0.35$ ), zygomatic width ( $F_{1,272} = 1.17$ ,  $P = 0.28$ ), or NPP ( $F_{1,367} = 1.54$ ,  $P = 0.22$ ),

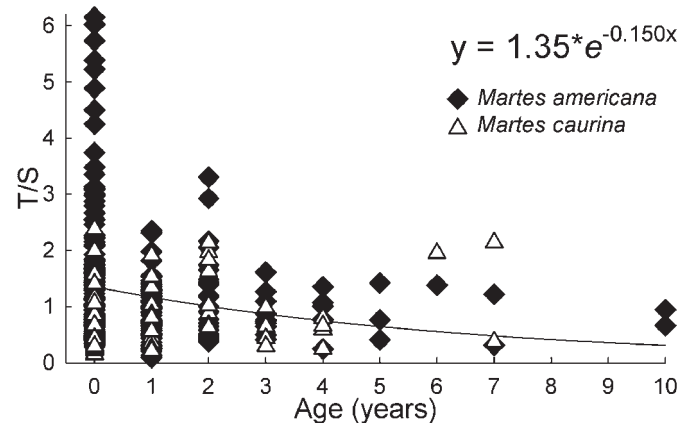


FIG. 3.—Plot of relative telomere length (T/S) against age for American (*Martes americana*) and Pacific (*M. caurina*) martens. Ages were obtained from counts of cementum annuli. T/S were quantified via real-time polymerase chain reaction.

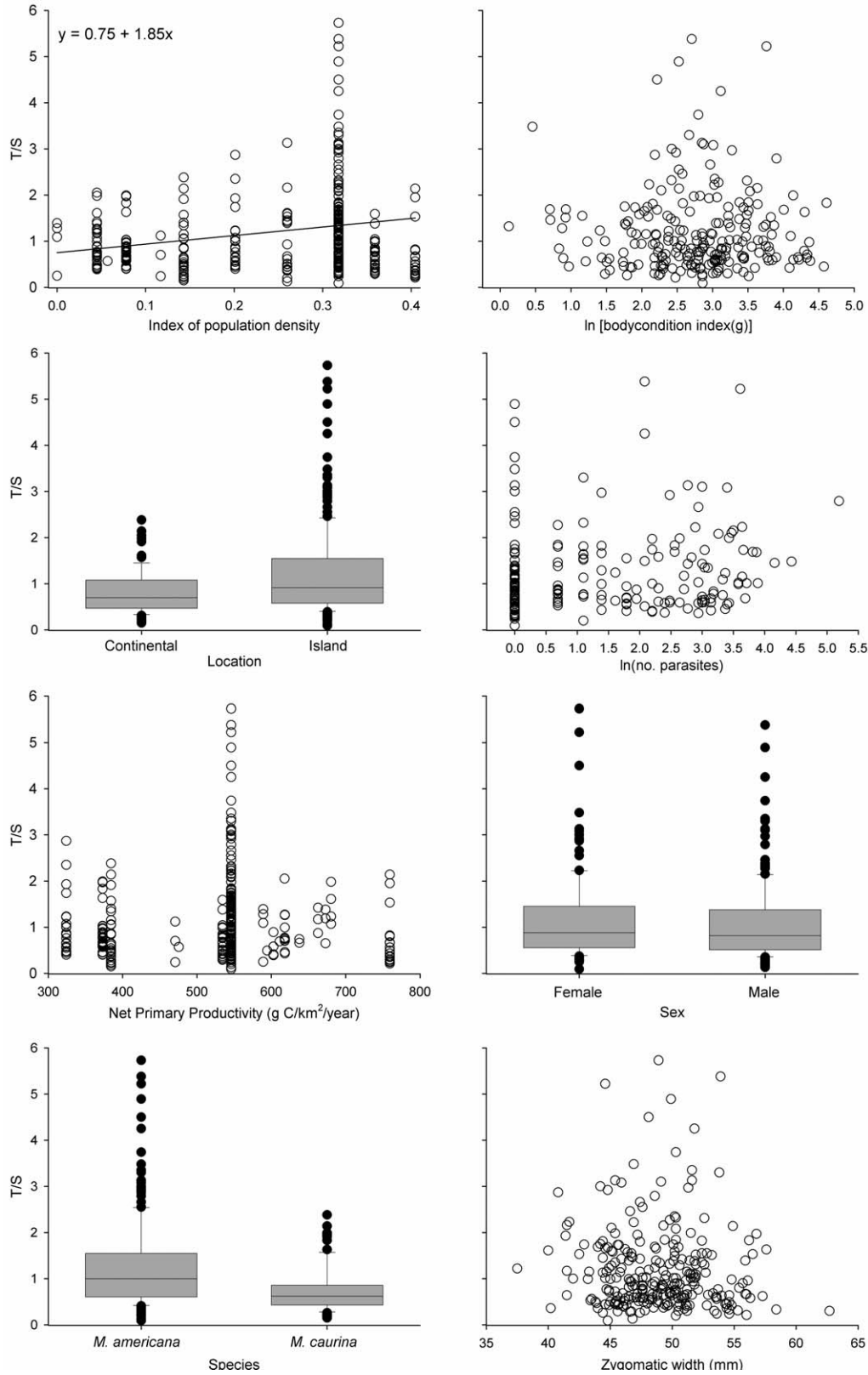


FIG. 4.—Relationship of relative telomere length (T/S) with a series of covariates that we hypothesized would influence telomere lengths in martens (*Martes americana* and *M. caurina*).

**TABLE 2.**—Model complexity and classification performance of the 5 best-performing Bayesian models. These were selected from a set of 27 Bayesian network models, predicting marten age class as a function of telomere length and other covariates.

Model	Data set	Spherical payoff	Model complexity		Model confusion error rates, % (n)		
			No. covariates	No. conditional probabilities	Juvenile	Adult	Total
Live capture <sup>a</sup>							
1	All	0.936	6	56,266			9.5 (399)
2	Island	0.958	6	56,266			6.5 (294)
Noninvasive <sup>b</sup>							
3	All	0.838	4	1,051	14.0 (207)	29.7 (192)	21.5 (399)
4	Island	0.816	4	1,051	14.9 (161)	36.8 (133)	24.8 (294)
5	Continent	0.907	4	1,051	15.2 (46)	10.2 (59)	12.4 (105)

<sup>a</sup> Models intended for captured animals to identify age class (0–4+ years) from the following covariates: telomere length, marten density, zygomatic width, species, sex, age class (juvenile or adult). Because we lacked data on zygomatic widths from martens collected from the continent, we did not present the results of that model. Confusion error rates by age class can be found in Fig. 6.

<sup>b</sup> Models intended for animals sampled noninvasively (i.e., without capture) to identify juveniles (<1 year) from adults (≥1 year) with the following covariates: telomere length, marten density, species, sex.

or a difference between sexes ( $t_{367} = 1.34$ ,  $P = 0.18$ ; Fig. 4). We used BN to predict individual ages (y) and age classes using the predictor variables telomere length, sex, species, and marten density. Although we did not detect differences between male and female martens, we retained this variable in subsequent BN, as sex-based differences have been found in telomere length for other mammal species (Cherif et al. 2003; Ilmonen et al. 2008; Nordfjäll et al. 2005).

*BNs to predict individual age.*—We developed 2 groups of BN models. The first presumed live capture of martens, where juveniles and adults can be inferred from their overall appearance (e.g., tooth sharpness and discoloration, sagittal crest size), if captured during the postbreeding period (S.W. Buskirk, University of Wyoming, pers. comm.) and when measurements of zygomatic width are obtainable. The second model set presumed that animals are sampled noninvasively, without capture (e.g., plucked hair), where such information would be lacking (hereafter referred to as live-capture and noninvasive models, respectively; Fig. 1). For the live-capture models we predicted martens to 5 age classes (ages 0, 1, 2, 3, and 4+ years—individual age [y]). For the noninvasive models we discriminated between 2 age classes (juveniles [<1 year] and adults [≥1 year]). We developed such models on the basis of the entire case data and of data from only islands of the Pacific Northwest or from only continental sites because initial data evaluations suggested that telomere length of martens from island or continental populations could have significantly different covariate relationships.

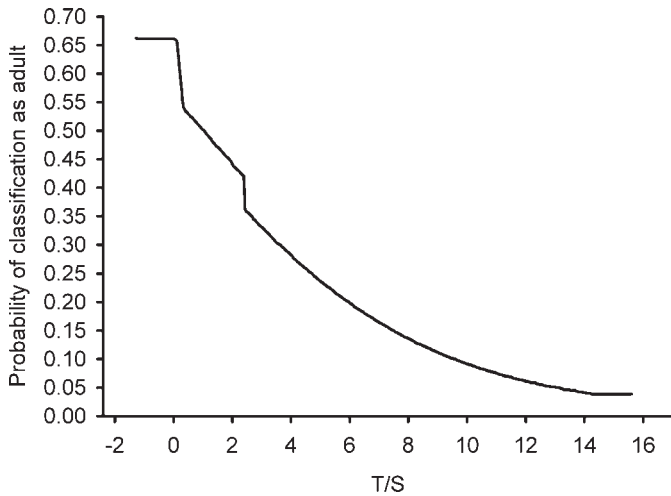
We developed 27 BN models using several covariates and response variables that resulted in 1,051 to 7,223,165 conditional probabilities (Appendices I and II). We selected 5 best-performing live-capture and noninvasive BN models for all data and island- or continental-only data (Table 2). The final live-capture models included 6 covariates and 52,266 conditional probabilities, and the final noninvasive models included 4 covariates and 1,051 conditional probabilities (Table 2). In general, the selected models were more parsimonious than the other models. We do not present results for the continental-only data set for live captures because that

data set had a large number of missing values for zygomatic width, an important predictor variable in the model.

The 5 selected models varied in their sensitivity structure (Table 3). Among all initial 27 models, spherical payoff values ranged from 0.703 to 0.999, and total confusion error ranged from 0 to 38.6 (Appendices I and II). The 5 models presented represent the best balance between model parsimony and performance. Although telomere length occurred in all 5 models as a covariate, it had less influence on predictability of individual ages (y) than other covariates in the live-capture models. However, telomere length alone contributed to differentiating adult from juvenile age classes (Fig. 5). Marten density most consistently had high influence on determining individual ages and age classes, although without telomere length it had no predictive power. The live-capture models had total confusion error rates <10% (Table 2; Fig. 6), particularly for the island-only data set. Species-specific error rates (model 1: *M. americana* = 7.7%, *M. caurina* = 14.1%; model 2: *M. americana* = 3.2%, *M. caurina* = 16.9%) were comparable with overall levels (Table 2; Fig. 6), with slightly more error being observed in *M. caurina*. The noninvasive models were less accurate, but all had <25% total confusion error and spherical payoff >0.80. Most models predicted juvenile age classes more accurately than adult age classes; the

**TABLE 3.**—Sensitivity structure of the 5 best-performing Bayesian models (see Table 2 for details of model number and covariates). NA, not applicable.

Covariate	Variance reduction				
	Live-capture models		Noninvasive models		
	Model 1	Model 2	Model 3	Model 4	Model 5
Marten density	0.0682	0.0915	0.0243	0.0074	0.0367
Age (juvenile versus adult)	0.0480	0.0463	NA	NA	NA
Species	0.0438	0.0755	0.0184	0.0085	0.0011
Zygomatic width	0.0301	0.0436	NA	NA	NA
Sex	0.0123	0.0190	0.0079	0.0012	0.0014
Telomere length	0.0045	0.0031	0.0033	0.0052	0.0015



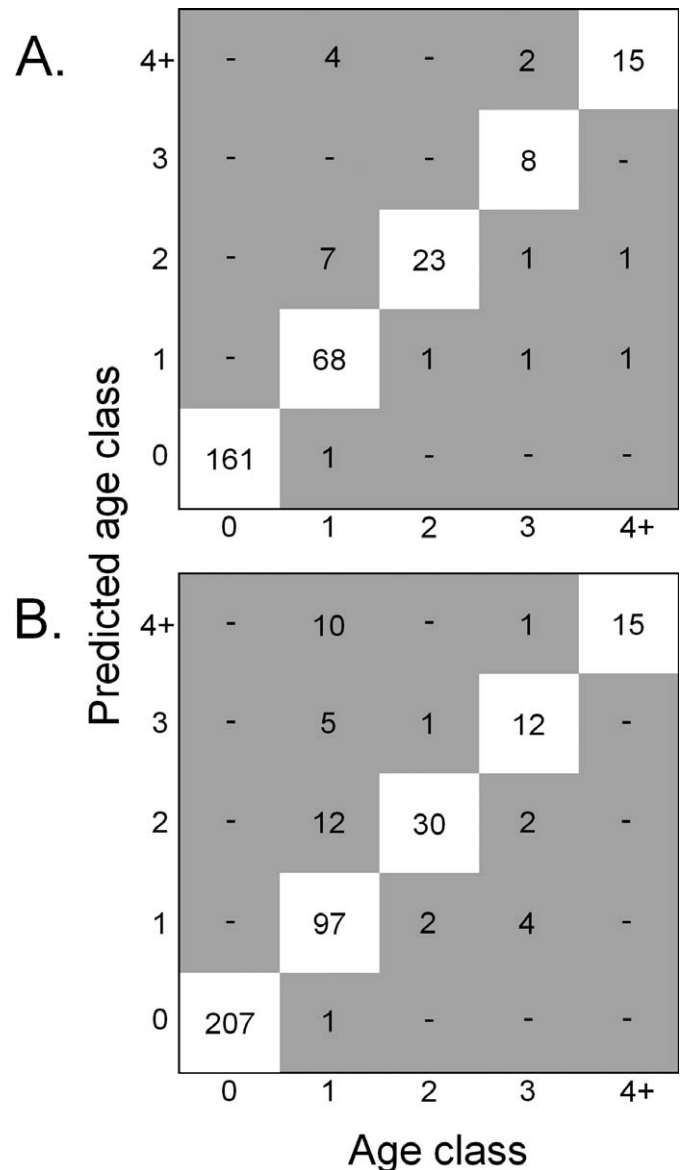
**FIG. 5.**—Marginal response curve from a maximum entropy analysis, illustrating the relationship between relative telomere length (T/S) and the probability that an individual marten falls within the adult age class. Relative telomere length (taken either in combination with other covariates or excluding all other covariates) strongly contributed to differentiating adult from juvenile martens (*Martes americana* and *M. caurina*).

live-capture models had confusion error rates of <1% for juvenile age classes. Results of 10-fold cross-validation (Table 4) also suggest relatively low error rates in classifying juveniles, particularly for the live-capture models (<2% error rate), but far higher error in classifying adults than when the entire data sets were used to both generate and test each model (Table 2; Fig. 6). The higher total and adult classification error rates were not unexpected, given the relatively small sample size of cases used to generate each model.

**DISCUSSION**

By integrating estimates of telomere length with relevant covariates that are also obtainable via noninvasive sampling (e.g., species and sex), we developed a noninvasive approach to assign free-ranging animals to biologically relevant age classes (juvenile versus adult) with reasonable accuracy. Additionally, the accuracy of our live-capture models (90–93%) in assigning animals to individual ages (0, 1, 2, 3, 4+) was comparable with that of “the gold standard” of counting cementum annuli (Poole et al. 1994).

The high variability that we observed in telomere lengths relative to chronological age could have several sources. First, telomere quantification is sensitive to contamination, and T/S ratios are derived from standard curves, so inconsistent PCR can result in considerable measurement error. We are confident that contamination had little effect on our results because we did not detect product in negative controls, reproducibility of T/S was reasonable within individuals (coefficient of variation = 14.6%), and dissociation curves (i.e., the melting temperature of the amplicon) were sharp and unimodal, indicating that our amplifications were specific (Cawthon 2002). Second, telomere length estimation can be



**FIG. 6.**—Confusion matrix of assigning martens (*Martes americana* and *M. caurina*) to the age classes obtained from counts of cementum annuli (0–4+ years) on the basis of covariates that would be available during live capture: telomere length, marten density, zygomatic width, species, sex, age class (juvenile or adult) for samples collected from A) island and B) all sites. Numbers represent sample size. Diagonal values (white boxes) denote correct age classification.

confounded by the presence of (T<sub>2</sub>AG<sub>3</sub>)<sub>n</sub> repeats at the centromeres or interstitial regions along the chromosome arm (Meyne et al. 1990). Such sequences away from the chromosomal termini can add variability to telomere estimation via Q-PCR (Salomons et al. 2009), although relative telomere length is highly correlated with terminal restriction fragment-based analyses for numerous taxa (Bize et al. 2009). Finally, telomeres are influenced by a variety of endogenous and exogenous factors, which can lead to high variation within age groups (Juola et al. 2005).

Early research predicting age from telomere length showed promise, as telomere loss appeared linearly related to age in



**TABLE 4.**—Results of 10-fold cross-validation of the 5 best-performing Bayesian models (see Table 2 for details of model number and covariates).

Model no.	Model confusion error rates (mean %)		
	Total	Juvenile	Adult
1	34.9	0.4	71.9
2	31.9	1.9	73.9
3	32.3	24.9	40.2
4	31.0	21.7	39.4
5	25.0	25.0	19.6

humans (Tsuji et al. 2002) and terns (*Sterna hirundo*; Haussmann et al. 2003b). Thus, it was anticipated that telomeres could provide a direct method for noninvasive estimation of animal age. However, subsequent work demonstrated that for many species of vertebrates, including reptiles (Hatase et al. 2008; Scott et al. 2006), birds (Bize et al. 2009; Pauliny et al. 2006; Salomons et al. 2009), and mammals (Frenck et al. 1998), telomere length and age exhibit a negative but nonlinear relationship (see Haussmann et al. 2003a for a case of telomere elongation with age), making age estimation, particularly for older individuals, problematic. In 2 species of seabirds (*Phalacrocorax aristotelis* and *Diomedea exulans*) the greatest rate of telomeric attrition occurred between chick and adult stages (Hall et al. 2004). Similarly, Frenck et al. (1998) found high rates of telomeric loss among young children ( $\leq 5$  years), stasis during young adulthood, and moderate attrition resuming among older adults. Potentially because of similar telomere dynamics in martens, most of our BN models predicted juvenile age classes with greater accuracy than adult age classes. Researchers or managers using these, or similar models, to predict age should be aware of this potential bias. High rates of telomeric loss early in life appear to be primarily a consequence of rapid cell and energy turnover (Sidorov et al. 2004) but can be elevated by stressors related to maturation into adulthood (Hall et al. 2004), particularly because longer telomeres are more vulnerable to oxidative damage (Salomons et al. 2009). Our success in assigning individuals to relevant age classes, by accounting for biological covariates that confound the estimation of chronological age, indicates that similar opportunities could exist for age estimation in other species.

Our findings highlight the importance of identifying and incorporating relevant biological factors when attempting to predict animal age from telomere length. Without the inclusion of marten density, sex, and phylogeny, telomere length alone was a poor predictor of age. Although marten population size and age structure fluctuate widely (Powell et al. 2004), marten density might reflect overall habitat quality. NPP, the rate at which carbon is converted into plant biomass ( $\text{g C}/\text{km}^2 \text{ year}^{-1}$ ; 31) is an index of food availability for predators (Yom-Tov et al. 2008); we found that estimates of NPP and marten density were correlated. If marten density is an indicator of habitat quality, our data suggest that animals inhabiting poorer habitats exhibit increased rates of telomeric loss. Hall et al. (2004) found that seabird chicks born later in

the season had greater rates of telomere attrition and attributed this to poorer nutritional state resulting from less parental care. Marten density could reflect more than just habitat quality, however. When marten populations are increasing they often have a high proportion of juveniles (Powell et al. 2004), and crowding effects become important. Thus, marten density likely integrates density-dependent effects, age structure, and habitat quality, which might explain why estimates of marten density were an important covariate when predicting age with our BN models. Although we found that NPP was not as good a covariate as marten density in our BN models, future users of this approach might consider using estimates of NPP when measures of animal density are unavailable.

We found that male and female martens possessed similar telomere lengths and that including sex as a covariate only slightly improved our predictive model. Sex-based differences in telomere loss were not found in seabirds (Hall et al. 2004) but have been detected in humans (Nordfjäll et al. 2005), mice (Ilmonen et al. 2008), and rats (Cherif et al. 2003). Female martens are the sole caregivers to kits and will increase their levels of activity while rearing offspring (Powell et al. 2004). Increased nutritional demands and foraging might lead to physiological stress, thereby increasing rates of telomeric attrition. In humans stress among mothers caring for chronically ill children caused greater rates of telomeric attrition (Epel et al. 2004); reproductive activity among female mice also resulted in greater reduction in telomeres compared with nonreproducing females and reproductively active males (Kotrschal et al. 2007). In contrast, estrogen promotes telomerase activity and directly mitigates telomeric loss by reducing the negative effects of reactive oxygen species (Aviv 2002). If telomerase is active in somatic cells of martens (Seluanov et al. 2007), our results suggest that the stress of reproductive demands is counterbalanced by the attenuating effects of estrogen, leading to similar rates of telomeric loss between males and females.

Even among closely related species both telomerase levels (Seluanov et al. 2007) and rates of telomeric attrition (Haussmann et al. 2003a) can differ substantially. We found that the 2 species we evaluated, *M. americana* and *M. caurina*, had different telomere dynamics. The 2 species of martens differentiated roughly 1 million years ago, although hybridization occurs at contact zones in the Rocky Mountains and southeastern Alaska (Stone et al. 2002). Our findings reinforce the importance of accounting for even moderate phylogenetic differences when using telomeres as an aging method.

In contrast to previous work, we found no effect of levels of parasitic infection on rates of telomeric loss. Ilmonen et al. (2008) infected wild-derived mice with the bacterium *Salmonella enterica*, which caused clinical morbidity and resulted in increased loss of telomeres. However, the nematode parasite we evaluated, *Soboliphyme baturini*, reaches extraordinary levels of infection in free-ranging martens, with little consequence to body condition of the host (Thomas et al. 2008). The lack of relationship between telomere length and parasite number corroborates these

previous findings—namely, that infection by *S. baturini* has little effect on physiological state of martens. Somewhat surprisingly, we found no relationship between indices of body condition and telomeric attrition. Martens are slender carnivores that do not accumulate circannual fat stores. Rather, fat is accumulated and catabolized rapidly, within only a few days (Buskirk and Harlow 1989). Therefore, fat stores appear to be a poor predictor of long-term nutritional condition for martens and, therefore, unrelated to telomere length. Other physiological metrics, like the size of the adrenal gland or levels of stress hormones, might be more accurate indicators of chronic stress and, thus, better predictors of telomere loss.

Although biologists interested in obtaining noninvasive age estimates will need to develop species-specific models, many meaningful covariates are available from publicly accessible databases. Further, telomere analysis requires tissue amounts commonly collected in the course of animal capture or available via noninvasive methods, and Q-PCR equipment is increasingly accessible. We encourage the identification of other potentially important covariates that could refine and improve age estimation via telomeres. Length of telomeres is heritable in both mammals (Nordfjäll et al. 2005) and birds (Hausmann et al. 2005). Studies using noninvasive and DNA-based approaches for free-ranging wildlife presumably will have measures of relatedness via other molecular markers and, thus, would have additional covariates potentially to improve age estimation. Additionally, field biologists who have previously quantified age structure of the population with other techniques can use known-age distributions as another informative covariate in BN that might improve accuracy of aging. Finally, studies have shown that in wild passerine birds telomere length and attrition were better predictors of survival than chronological age (Bize et al. 2009; Hausmann et al. 2005). Evaluation of a telomere–survivorship relationship in free-ranging mammals and the potential inclusion of survival estimates in models of telomeres and age merit further inquiry.

We show that by including relevant covariates the biological age recorded by telomeres can be converted to chronological age or age class, reducing the need for invasive or destructive procedures and long-term data on marked animals. For captured animals this approach will greatly reduce the stress and risk associated with traditional methods for aging. For the growing number of researchers using noninvasive DNA-based approaches, the approach herein can complement DNA fingerprinting methods to quantify age-specific vital rates for free-ranging populations. We urge the application and refinement of this approach, particularly for rare or elusive species for which demographic analyses currently are limited.

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APPENDIX I

Model structure and selection criteria (Schwarz' Bayesian information criterion; BIC), complexity, and classification performance for Bayesian models constructed to predict marten age class as a function of telomere length and other covariates. See text of article for details on quantification of variables.

Covariates <sup>a</sup>	No. of age classes <sup>b</sup>	BN model version <sup>c</sup>	Data sets <sup>d</sup>	Model error rates (%)			No. of conditional probabilities	Model performance indices		
				Adult	Juvenile	Total		Spherical payoff	BIC	ΔBIC
T/S, density, ZW, species, sex	2	Fine	All	22.4	12.6	17.3	34,694	0.879	389.9	312.2
T/S, density, ZW, species, sex	2	Coarse	All	27.6	13.5	20.3	15,228	0.855	389.9	312.2
T/S, NPP, density, ZW, species, sex	2	Coarse	All	28.6	12.1	19.8	151,308	0.858	393.8	316.0
T/S, NPP, density, species, sex, CBL	2	Fine	All	37.0	14.5	25.3	287,414	0.817	511.7	434.0
T/S, NPP, density, species, sex, CBL	2	Coarse	All	37.5	16.4	26.6	75,708	0.802	511.7	434.0
T/S, NPP, density, ZW, species, sex, age	Age	Fine	All	19.9	0.5	9.8	7,223,165	0.933	894.0	816.2
T/S, NPP, density, species, sex, CBL, age	Age	Coarse	All	37.2	0.5	17.8	831,699	0.866	1,168.8	1,091.1
T/S, NPP, density, ZW, species, sex, age	Age	Coarse	All	29.3	0.5	14.3	1,663,299	0.901	894.0	816.2
T/S, NPP, density, ZW, species, sex, insular	2	Coarse	All	28.6	11.1	19.6	302,510	0.861	398.3	320.6
T/S, NPP, density, ZW, sex, US state	2	Coarse	All	28.1	12.1	19.8	529,310	0.858	387.4	309.7
T/S, density, ZW, species, sex, age	5	Final (1)	All	19.4	0.5	9.5	56,266	0.936	643.5	565.7
T/S, density, ZW, species, sex, age	5	Final	Ccontinent	30.5	0.0	17.1	56,266	0.880	na <sup>e</sup>	na <sup>e</sup>
T/S, density, species, sex, age	5	Final	Continent	30.5	0.0	17.1	4,786	0.879	243.1	nr <sup>g</sup>
T/S, density, ZW, species, sex, age	5	Final (2)	Island	13.6	0.6	6.5	56,266	0.958	640.6	nr <sup>g</sup>
T/S, density, ZW, species, sex, age	5	Final	QCI	0.0	0.0	0.0	56,266	1.000	77.7 <sup>f</sup>	nr <sup>g</sup>
T/S, density, ZW, species, sex, age	5	Final	Non-QCI	20.2	0.5	9.8	56,266	0.933	540.9	nr <sup>g</sup>

<sup>a</sup> T/S = relative telomere length; NPP = net primary productivity; density = marten density; ZW = zygomatic width; species = *Martes americana* or *M. caurina*; CBL = condylobasal length average for the population; US state = state where sample was obtained.

<sup>b</sup> Age = actual age (years); 2 = juveniles (<1 year) versus adults (>1 year); 5 = 0, 1, 2, 3, 4+ years.

<sup>c</sup> "Fine" models included a greater number of discrete states of variables T/S and NPP (30 and 19 states, respectively) than did "coarse" models (15 and 10 states, respectively); "final" models differed from "coarse" models only with a further slight reduction in the number of discrete states (to 13) of variable T/S to ensure that all states were represented by at least one case in the "all" data set. Numbers in parentheses correspond to those models presented in Table 1.

<sup>d</sup> Data set used to parameterize the probability structure of the model using the expectation maximization (EM) algorithm, and also to evaluate classification success rates; number of cases in each data set: all = 399, continent = 105, island = 294, QCI (Queen Charlotte Island) = 21, non-QCI = 378.

<sup>e</sup> BIC not calculated because of lack of data on the predictor variable, ZW, for this model.

<sup>f</sup> BIC were calculated from this model excluding the variable "species" because of no variance (all QCI samples are from one species).

<sup>g</sup> ΔBIC not reported because alternate models with the same inputs are not presented.

APPENDIX II

Model structure and selection criteria (Schwarz' Bayesian information criterion; BIC), complexity, and classification performance for Bayesian models constructed to predict marten age class as a function of telomere length and other covariates. See text of article for details on quantification of variables.

Covariates <sup>a</sup>	No. of age classes <sup>b</sup>	BN model version <sup>c</sup>	Data sets <sup>d</sup>	Model error rates (%)			No. of conditional probabilities	Model performance indices		
				Adult	Juvenile	Total		Spherical payoff	BIC	ΔBIC
T/S, NPP, density, species, sex	Age	Fine	All	63.9	7.7	34.6	225,845	0.734	1,350.2	1313.2
T/S, NPP, density, species, sex	Age	Coarse	All	80.6	6.7	38.6	59,499	0.703	1,350.2	1313.2
T/S, NPP, density, species, sex	3	Fine	All	50.0	12.5	32.1	61,695	0.758	118.8	81.8
T/S, NPP, density, species, sex	3	Coarse	All	65.9	48.6	36.1	16,309	0.730	118.8	81.8
T/S, NPP, density, species, sex	2	Fine	All	37.0	14.5	25.3	41,174	0.817	505.8	468.8
T/S, NPP, density, species, sex	2	Coarse	All	37.5	16.4	25.3	10,908	0.802	505.8	468.8
T/S, density, species, sex	2	Final (3)	All	29.7	14.0	21.5	1,051	0.838	513.6	476.6
T/S, density, species, sex	2	Final (5)	Continent	10.2	15.2	12.4	1,051	0.907	113.9	nr <sup>f</sup>
T/S, density, species, sex	2	Final (4)	Island	36.8	14.9	24.8	1,051	0.816	394.6	nr <sup>f</sup>
T/S, density, species, sex	2	Final	QCI	23.1	12.5	19.1	1,051	0.865	37.0 <sup>e</sup>	nr <sup>f</sup>
T/S, density, species, sex	2	Final	Non-QCI	30.2	14.1	21.7	1,051	0.836	454.0	nr <sup>f</sup>

<sup>a</sup> T/S = relative telomere length; NPP = net primary productivity; density = marten density; species = *Martes americana* or *M. caurina*.

<sup>b</sup> Age = actual age (years); 2 = juveniles (<1 year) versus adults (>1 year); 3 = juveniles (<1 years), yearlings (1 year), adults (>1 year).

<sup>c</sup> "Fine" models included a greater number of discrete states of variables T/S and NPP (30 and 19 states, respectively) than did "coarse" models (15 and 10 states, respectively); "final" models differed from "coarse" models only with a further slight reduction in the number of discrete states (to 13) of variable T/S to ensure that all states were represented by at least one case in the "all" data set. Numbers in parentheses correspond to those models presented in Table 1.

<sup>d</sup> Data set used to parameterize the probability structure of the model using the expectation maximization (EM) algorithm, and also to evaluate classification success rates; number of cases in each data set: all = 399, continent = 105, island = 294, QCI (Queen Charlotte Island) = 21, non-QCI = 378.

<sup>e</sup> BIC were calculated from this model excluding the variable "species" because of no variance (all QCI samples are from one species).

<sup>f</sup> ΔBIC not reported because alternate models with the same inputs are not presented.