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Multiple estimates of effective population size for monitoring a long-lived vertebrate: an application to Yellowstone grizzly bears

Pauline L. Kamath^{1*}, Mark A. Haroldson¹, Gordon Luikart², David Paetkau³, Craig Whitman¹, Frank T. van Manen¹

¹U.S. Geological Survey, Northern Rocky Mountain Science Center, 2327 University Way, Bozeman, Suite 2, MT 59715, USA

²Wildlife Genetics International, Box 274, Nelson, British Columbia V1L 5P9, Canada

³Flathead Lake Biological Station, Division of Biological Sciences, University of Montana, Missoula, MT 59812, USA

***Corresponding author:** E-mail: pkamath@usgs.gov, Tel: +1 406-994-7456

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Abstract

Effective population size (N_e) is a key parameter for monitoring the genetic health of threatened populations because it reflects a population's evolutionary potential and risk of extinction due to genetic stochasticity. However, its application to wildlife monitoring has been limited because it is difficult to measure in natural populations. The isolated and well-studied population of grizzly bears (*Ursus arctos*) in the Greater Yellowstone Ecosystem provides a rare opportunity to examine the usefulness of different N_e estimators for monitoring. We genotyped 729 Yellowstone grizzly bears using 20 microsatellites and applied three single-sample estimators to examine contemporary trends in generation interval (GI), effective number of breeders (N_b), and N_e during 1982–2007. We also used multi-sample methods to estimate variance (N_{ev}) and inbreeding N_e (N_{ei}). Single-sample estimates revealed positive trajectories, with over a 4-fold increase in N_e (≈ 100 to 450) and near doubling of the GI (≈ 8 to 14) from the 1980s to 2000s. N_{ev} (240–319) and N_{ei} (256) were comparable with the harmonic mean single-sample N_e (213) over the time period. Reanalyzing historical data, we found N_{ev} increased from ≈ 80 in the 1910s–1960s to ≈ 280 in the contemporary population. The ratio of effective to total census size (N_e/N_c) was stable and high (0.42–0.66) compared to previous brown bear studies. These results support independent demographic evidence for Yellowstone grizzly bear population growth since the 1980s. They further demonstrate how genetic monitoring of N_e can complement demographic-based monitoring of N_c and vital rates, providing a valuable tool for wildlife managers.

Introduction

The examination of long-term trends in population size is vital to evaluating the status of wildlife populations of conservation concern. As a population declines in size, the risk of extinction increases as a result of demographic and genetic stochasticity (e.g., loss of alleles, increased frequency of deleterious alleles, and inbreeding depression) (Lynch & Gabriel 1990). Effective population size (N_e) is the size of an “ideal population” (e.g., random mating, random variance in reproductive success, even sex ratio) that would lose genetic diversity at the same rate of the observed population (Fisher 1930; Wright 1931). The N_e of natural (observed) populations is usually far lower than for ideal populations (in which N_e equals the census size, N_c), due to high variance in reproductive success among individuals. It is a key parameter for quantifying the rate at which genetic diversity will be lost due to random genetic drift, and is influenced by factors such as variance in fecundity and survival, and mating system. Therefore, unlike census size, N_e captures the effects of both demographic and genetic processes and also relates to a population’s evolutionary potential (i.e., ability to respond to selection or environmental change), making it an important parameter for population monitoring in both evolutionary ecology (Charlesworth 2009) and conservation biology (Frankham 2005).

Both direct (ecological) and indirect (genetic) estimation of N_e in natural populations is challenging, particularly for iteroparous species with overlapping generations. Multiple genetic estimation approaches have been developed that are based on theoretically different concepts of N_e (e.g. variance N_e , inbreeding N_e and eigenvalue N_e ; Crow & Denniston 1988; Ewens 1982). These approaches not only differ in the time frame they apply to (Waples 2005), but can also be

affected differently by population-level processes, such as gene flow, genetic substructure, changes in population size and life history parameters. Therefore, in natural populations that are typically under non-equilibrium conditions, distinguishing between and comparing estimates based on the various N_e concepts is critical (Crandall *et al.* 1999; Serbezov *et al.* 2012; Wang 2005), particularly when applied within a monitoring framework.

The most frequently used approach for estimating contemporary N_e (corresponding to the past few generations or less; Luikart *et al.* 2010) has been the temporal method (Nei & Tajima 1981), also called variance N_e (N_{ev}). This method is based on the assumption that changes in allele frequencies over time due to genetic drift (i.e., the effects of mutation, migration, and selection are negligible) are inversely related to N_e . A disadvantage of this approach is that it assumes discrete, non-overlapping generations when, in contrast, most species are age-structured. Violation of this assumption may result in biased N_e estimates. These biases may be overcome, if the time period between samples is large and, thus, the drift signal is strong (Nei & Tajima 1981; Waples & Yokota 2007). Jorde and Ryman (1995) modified the standard temporal method, correcting for biases resulting from population age structure, but this unbiased estimator has not been widely implemented because it requires information on age-specific survival and fecundity from cohorts sampled in successive years. A recent update to their technique allows for cohort sampling any number of generations apart (Jorde 2012). A similar concept is inbreeding N_e (N_{ei}) which is based on the rate of increase in inbreeding (as measured by the loss of heterozygosity over time) as opposed to the rate of allele

frequency change due to genetic drift (i.e., N_{eV}). N_{eI} and N_{eV} are theoretically expected to yield similar values under the assumption of constant population size (Crow & Denniston 1988).

Despite advancements in the temporal (variance) method, and its extensive use for estimating N_e , sampling remains difficult for most studies. This is due to the requirement of either multiple samples separated by an interval of one or more generations, or samples from cohorts accompanied by detailed demographic information. Thus, the development and implementation of single-sample N_e estimators has increased substantially. Single-sample estimators are generally based on patterns that result from processes acting in the parental generation (e.g., heterozygosity, gametic disequilibrium, individual relatedness) and are thus expected to be measures of inbreeding N_{eI} (Laurie-Ahlberg & Weir 1979). These estimators include the linkage disequilibrium (LD) (Hill 1981; Laurie-Ahlberg & Weir 1979; Waples 2006a), estimator by parentage assignments (EPA) (Wang *et al.* 2010), and sibship assignment (SA) (Wang 2009) methods.

The LD approach is based on the principle that gamete disequilibrium among independent loci is generated by genetic drift, the strength of which is determined by N_e per generation and the effective number of breeders (N_b) per year (Hill 1981; Waples *et al.* 2014). The SA and EPA methods use frequencies of related individuals in a random sample (Wang *et al.* 2010); SA estimates the frequencies of full and half siblings, whereas EPA infers parentage using a likelihood approach. The LD and SA methods also assume discrete generations, but in age-structured populations they can be used to estimate the N_b that produced a given cohort. Although the relationship between N_b and N_e is complex, N_b can be a comparable index of

genetic health and, when measured in cohorts over time, is particularly useful for population monitoring (Waples 2005).

In the contiguous ('lower') 48 states of the United States of America, grizzly bears (*Ursus arctos*) are a "conservation-reliant" species (Scott *et al.* 2005). Their abundance and distribution declined dramatically through the 1900s, with bears disappearing from approximately 98% of their historic range (Mattson *et al.* 1995). Due in part to concerns regarding the future of Yellowstone bears, grizzly bear populations in the contiguous United States were listed as threatened in 1975 under the U.S. Endangered Species Act (ESA) (US Fish and Wildlife Service 1993). Even before listing under the ESA, intensive research efforts were focused on this population, resulting in the availability of long-term, comprehensive datasets that include detailed capture histories. Since a likely nadir in the early 1980s, multiple indices used to estimate trends suggest that the GYE population has experienced positive growth for the past three decades (Boyce *et al.* 2001; Eberhardt 1995; Eberhardt *et al.* 1994; Harris *et al.* 2006; Harris *et al.* 2007; Interagency Grizzly Bear Study Team 2012).

However, the GYE grizzly bear population has experienced isolation for over a century (Mattson & Merrill 2002) and genetic data have confirmed that it is genetically distinct (Proctor *et al.* 2012), with no evidence of recent immigration from the nearest populations in the Northern Continental Divide Ecosystem (NCDE), Cabinet-Yaak, or southern Selkirk Mountains (Haroldson *et al.* 2010). The GYE population likely was also subjected to loss of genetic diversity following historical reductions in size due to anthropogenic influences (e.g., human-related mortality, garbage dump closures). Consequently, the genetic diversity of GYE bears is among

the lowest reported for continental brown bear populations in North America (Paetkau *et al.* 1998b; Proctor *et al.* 2012). Despite this, using museum specimens from GYE bears, Miller and Waits (2003) detected only a gradual decline in diversity over the past century. Using a temporal-based estimator, they estimated an N_e of ≈ 80 throughout the 20th century, with N_e approaching 100 during the late 1990s. Recent trends in both genetic diversity and N_e , however, have not been examined in this ecosystem.

Conditions that form an optimal research framework for estimation of N_e in wildlife populations are difficult to obtain. Most notably, few studies meet the assumption of genetic isolation and also have long-term demographic and genetic samples representative of individuals from all age classes (but see Palm *et al.* 2003; Serbezov *et al.* 2012). As an isolated and comprehensively studied population, the GYE grizzly bear population presents a rare opportunity to examine and compare the utility of various N_e estimation techniques as a potential tool for population monitoring. Our objectives were to 1) examine trends in N_b and N_e of GYE grizzly bears over recent decades using both single- and multi-sample temporal N_e estimation methods; 2) contrast estimates based on the different methods; and 3) assess whether trends in N_e estimates, independent of demographic-based estimators of population size (N_c), corroborate or refute observed population trends.

Materials and Methods

Study area

The study area encompassed the GYE, which includes Yellowstone National Park, Grand Teton National Park, all or portions of 5 national forests, other federal lands, plus state, tribal, and private lands in Wyoming, Montana, and Idaho (Fig. S1). Geographically, the GYE includes headwaters of the Missouri–Mississippi, Snake–Columbia, and Green–Colorado river systems, the Yellowstone Plateau, and 14 surrounding mountain ranges (Marston and Anderson 1991). The grizzly bear population in the GYE (centered at latitude 44.64° N, 110.52° W) has been expanding its range over the past several decades and currently occupies approximately 50,000 km² (Bjornlie *et al.* 2014). More detailed descriptions of the study area can be found in Blanchard and Knight (1991), Mattson *et al.* (1991), and Schwartz *et al.* (2006).

Sampling, DNA extraction and genotyping

Tissue, blood, and hair samples ($n = 729$) were collected by the IGBST from grizzly bears born between 1962 and 2010 (Fig. S1). These samples were obtained through live captures and mortalities. Detailed capture methods are described in Schwartz *et al.* (2006) and sample preservation protocols can be found in Haroldson *et al.* (2010). Genomic DNA was extracted using DNeasy tissue kits, following manufacturer's instructions (Qiagen Inc.), at the Wildlife Genetics International laboratory (Nelson, British Columbia, Canada). Bears were genotyped at 20 microsatellite loci: REN145P07, REN144A06, CPH9, CXX20, CXX110, G1A, G10B, G10C, G1D, G10H, G10J, G10L, G10M, G10P, G10U, G10X, MU23, MU50, MU51, MU59 (Breen *et al.* 2001; Fredholm & Wintero 1995; Ostrander *et al.* 1993; Paetkau *et al.* 1995; Paetkau *et al.* 1998a; Proctor *et al.* 2002; Taberlet *et al.* 1997). Altered primer sequences for REN145P07, REN144A06,

MU23, and MU51 are reported in Table S1. Genotyping protocols are described in Paetkau *et al.* (1998a).

In a previous study involving samples analyzed in the same genetics laboratory, Kendall *et al.* (2009) estimated genotyping error rates (i.e., combination of scoring errors, allelic dropout, and false amplifications) for 16 of the 20 loci we used. Error rates were low (0.002 per loci/sample) based on >4,000 hair samples from more than 500 individual grizzly bears within the NCDE. Because their study involved non-invasive samples, these genotyping error rates are conservative (i.e., overestimates) when applied to our GYE sample from higher-quality materials (i.e., primarily tissue samples). We further estimated per-locus null allele frequencies using the expected maximum algorithm implemented in FreeNA (Chapuis & Estoup 2007). We accounted for modeled estimates of genotyping error due to null alleles in methods based on making relatedness inferences (SA, EPA), as described below (see *Single sample estimation of effective population size and numbers of breeders*).

Marker tests and genetic diversity indices

We performed exact tests to test for departures from Hardy-Weinberg (H-W) proportions and genotypic linkage equilibrium. We tested for departures in population samples spaced by 5-year intervals spanning the time period (1985 to 2010) using a Markov chain algorithm (Guo & Thompson 1992) (dememorization steps = 1,000; batches = 100; Markov chain iterations/batch = 1,000) as implemented in GENEPOP v4.2 (Raymond & Rousset 1995). We corrected for multiple comparisons by applying the sequential Holm-Bonferroni procedure

(Holm 1979) to adjust the critical P -value ($\alpha = 0.05$). Bear population genetic diversity was examined across temporally spaced population samples (from 1985 to 2010) by estimating the observed heterozygosity (H_O) and expected heterozygosity (H_E) in Arlequin v3.5 (Excoffier & Lischer 2010). We also measured allelic richness (A) after correcting for unequal sample sizes by rarefaction in HP-RARE (Kalinowski 2005). We performed paired t -tests, comparing allelic richness over the time period for each of the 20 loci, to test the null hypothesis of no change in diversity over time. Furthermore, to compare diversity estimates with previously reported results in Miller and Waits (2003), we recalculated diversity indices using the 8 microsatellites common to both studies: G1A, G10B, G10C, G1D, G10L, G10M, G10P, and G10X.

Single-sample estimation of effective population size and number of breeders

We used three approaches based on single samples to estimate effective population size (N_e) over time: (1) estimator by parentage assignments (EPA, Wang *et al.* 2010); (2) linkage disequilibrium (LD) estimator (LDNe, Waples 2006a; Waples & Do 2008); and (3) the sibship assignment estimator (SA, Wang 2009). The EPA method offers a direct estimate of N_e and the generation interval (GI) by incorporating individual sex, age, and genotype data from a population (sample) with overlapping generations. The remaining two methods provide an estimate of N_b in a single cohort (i.e., a discrete age class).

In applying the EPA method, we organized known-aged bears (born between 1970 and 2007) into annual samples of animals alive during 1982–2007, based on birth and mortality (or oldest known age) data. Data were truncated at 2007 due to a potential sampling bias because

data obtained from a cohort or age class increases as individuals are captured and aged into the future. Thus, this sampling approach may affect the probability of offspring presence in the data, and consequently, N_e estimates for the most recent sampling years. Age-specific sample sizes per year for the period are reported in Table S2. We used Age-struct (Wang *et al.* 2010) and incorporated age, sex, and multi-locus genotypes to assign parentage within the random population sample, fitting them to a genetic model (Johnson 1977) from which we estimated N_e and GI. We applied modeled estimates of null allele frequencies, and assumed a minimum breeding age of 3 years, 95% reliability of parentage assignments, and 0.5 prior probability of including a parent in the sample. We conducted 1,000 bootstrap replicates to obtain 95% confidence intervals on estimates.

In contrast to the EPA method, LDN_e and SA methods generally assume discrete, non-overlapping generations and cannot estimate N_e directly (but see Waples *et al.* 2014). Instead, when applied to a random sample from a single cohort in a population with overlapping generations, these methods can provide an adequate estimate of N_b (the effective number of breeders that produced the cohort) (Robinson & Moyer 2013). Thus, we applied these methods to estimate N_b for individual cohorts. To obtain acceptable sample sizes and account for intermittent breeding, we organized bear samples into 3-year running (pooled) ‘cohorts’ from 1983 to 2008 based on birth year, which assumes a minimum reproductive age of 3 years to ensure no parent-offspring pairs exist within a ‘cohort’. The LDNe approach uses Weir’s 1979 (Weir 1979) unbiased estimator of Burrow’s Δ to estimate LD, with a correction for sample size

bias (Waples 2006a). We applied a random mating model and upheld a critical threshold for the lowest allele frequency (P_{crit}) based on the criterion that $P_{\text{crit}} > 1/(2n)$ (Waples & Do 2010).

Next, we applied the SA method to the same cohorts using program Colony 2 (Jones & Wang 2010), which simultaneously infers parentage and sibship dyad assignments to estimate N_b from frequencies of full- and half-sibling pairs (Wang 2009). We used full likelihood inference and assumed a polygamous breeding system without inbreeding. When estimating relatedness, we accounted for genotyping error due to null alleles using the model estimated error rates. We improved sibship inference through parentage assignments by defining a pool of candidate parents for each bear, including all individuals that were ≥ 3 years old in the bear's birth year.

Multi-sample *estimation of variance and inbreeding effective population size*

We applied the multi-sample temporal method (Krimbas & Tsakas 1971; Nei & Tajima 1981; Waples 1989) to estimate variance N_e (N_{ev}). This method is based on the Wright-Fisher model (Fisher 1930; Wright 1931) and assumes temporal changes in allele frequencies are caused by genetic drift alone. We calculated the standardized variance in allele frequency with two moment-based F -statistic estimators, F_S (Jorde & Ryman 2007) and F_C (Nei & Tajima 1981), as implemented in NeEstimator V2 (Do *et al.* 2014). We performed this analysis using two temporally-spaced random population samples (including adults and cubs), separated by 25 years (1982 vs. 2007), which is equivalent to 2.5 generations under an assumed 10-year GI. We conservatively repeated the analysis with a GI of 12 years (~ 2 generations), which is expected to

228 yield lower N_e estimates because more drift (allele frequency change) can occur if more years
229 pass per generation.

230 We set the starting population census size to 200 in 1982, based on demographic
231 estimates (Eberhardt & Knight 1996). We applied sampling plan I, which is a correction for
232 temporal estimates when taking samples with replacement non-lethally before reproduction
233 (see Waples 2005), and a minimum allele frequency of 0.01. In addition, we implemented the
234 modified temporal approach of Jorde and Ryman (2007) in GONe (Coombs *et al.* 2012) using 26
235 consecutive birth year cohorts (from 1982 to 2007) and a starting cohort size of 50, while
236 incorporating estimates of age-specific survival and fecundity (data from Waples *et al.* 2013).
237 We further used a likelihood-based temporal approach, calculating a pseudo-maximum
238 likelihood estimator of effective size in MLNe (Wang 2001; Wang & Whitlock 2003). With this
239 approach, we assumed a closed and isolated population, with no migration, substructure or
240 selection, and a prior upper bound for N_e equal to 5,000.

241 To contrast our results to historical estimates, we obtained genotype data representing
242 three past time periods (1910s, 1960s, 1990s) from which N_{ev} was previously estimated using
243 both maximum likelihood and moment-based estimators (see Miller & Waits 2003). With these
244 data, we reevaluated N_{ev} for the 1910–1960, 1960–1990, and 1910–1990 time frames using the
245 same variance estimators (F_S , F_C , and MLNe) applied to the current data, as described previously.
246 For these historical data, we assumed a GI of 10 years and a starting census population size of
247 350 in 1910, as in Miller and Waits (2003).

Finally, we applied another multi-sample approach to estimate inbreeding N_e (N_{ei}) based on loss of expected heterozygosity. This approach applied the following equation: $H_t = H_0 (1 - 1/2N_e)^t$, where t is the number of generations, H_0 is the expected heterozygosity at time zero, and H_t is the expected heterozygosity at generation t (Nei *et al.* 1975). In contrast to N_{ev} , this approach generally yields a longer-term estimate of N_e corresponding more closely to the parental generation. N_{ei} is less sensitive to population declines than N_{ev} because of the delay in mating between close relatives (Allendorf *et al.* 2013; Kimura & Crow 1963). Again, we assumed approximately 2.5 generations and used H_E estimates from 1985 versus 2010.

Evaluating sensitivity of N_e estimates to biases in sampling and model parameters

We evaluated bias due to small sample sizes at earlier time periods for all single sample N_b and N_e estimation approaches by randomly subsampling to equal sample sizes on select data points over the monitoring period (every 5 years from 1985 to 2005). We randomly selected 10 data subsets of equal sample size (EPA: $n = 100$, SA/LD: $n = 50$) and conducted independent replicate runs to estimate the mean and 95% confidence intervals of the N_e point estimates. We further investigated the sensitivity of EPA-based N_e estimates to the reliability threshold for parentage assignments specified in the underlying genetic model. We assumed a low probability of parentage assignment threshold of 0.80, and compared this with our previous estimates for which we assumed a threshold of 0.95.

Comparisons with demographic-based estimates

We used N_e estimates and demographic-based estimates of total population size (i.e., census size estimates, or N_c) to calculate the N_e to N_c ratio and determine if population trends differ between the demographic- and genetic-based techniques. We used two demographic-based population estimators for these comparisons: (1) Chao2 estimator based on counts of females with cubs-of-the-year (Cherry *et al.* 2007; Keating *et al.* 2002), and (2) a mark-resight (M-R) estimator based on systematic observation flights of marked and unmarked females with cubs-of-the-year (Higgs *et al.* 2013). The N_e/N_c ratio was calculated for each year using the EPA-derived N_e point estimates in the numerator and 3-year running means of the demographic estimates (Chao2, M-R) in the denominator. These two techniques provided estimates of the number of females with cubs-of-the-year, which we used to derive estimates of total population size (Interagency Grizzly Bear Study Team 2012). Chao2 estimates covered the time period from 1984 to 2007, whereas M-R estimates were from 2001 to 2007. We chose the latter period for the M-R technique because it coincided with a period over which this estimator showed little change [29] and thus provided a useful period to assess the N_e/N_c ratio. Estimates of N_c included all bears (dependent young, subadults, and adults) in the population. We used harmonic means over the entire time period to calculate the final N_e/N_c ratios.

Results

Null allele frequency, H-W equilibrium and genetic diversity indices

We observed low null allele frequencies across all our microsatellite markers (average frequency = 0.006, SD = 0.008; Table S3). In each population sample assessed, we detected no

evidence of departures from H-W expectations and no LD among loci pairs across all 6 temporally spaced population samples. However, in 4 out of 6 samples tested, the following loci pairs were in significant LD: G1D/G10P, CPH9/G10U. Significant loci associations may represent a signal of population substructure or, alternatively, represent a random statistical artifact with only ~3% of tests below the significance threshold value of 5%.

Paired comparison tests showed no statistical support ($P_{1985-2010} = 0.23$) for a decline in mean allelic richness (number of alleles/ locus) from 1985 to 2010 ($A_{1985} = 4.65$, $A_{2010} = 4.52$; Table 1). Expected heterozygosity of the GYE grizzly bear population remained stable over the past several decades and paired comparisons similarly suggested no change over time ($P_{1985-2010} = 0.56$). When limiting the analyses of genetic diversity (allelic richness) to the same eight microsatellite loci examined by Miller and Waits (2003), and correcting for differences in sample sizes across studies, the 1990s estimates were similar across studies (Table S4). When using the eight markers in common, we again observed stability in diversity indices from 1985 to 2010.

Single-sample estimates of generation interval, N_b , and N_e

Annual sample sizes, used in EPA-based estimate of N_e , ranged from 52 to 379 bears (Table S2, Table S5), whereas sample sizes by cohort used in LDNe and SA analyses ranged from 22 to 108 (Tables S5 and S6). EPA-derived estimates of N_e indicated an increasing trend, with over a 4-fold increase in N_e from 102 (95% CI: 64–207) in 1982 to 469 (95% CI: 284–772) in 2007 (Fig. 1a, Table S5). These results were accompanied by an increase in the generation interval over this time period (Fig. 1b, Table S5). Results from SA analyses indicated a similar increase in

N_b from 44 in 1984 to 145 in 2007 (Fig. 1c; Table S6), whereas LD-based analyses indicated an increase from a low of 46 in the mid-1980s to 92 in 2007 (Fig. 1c; Table S7).

Temporal-based estimates of N_e

Temporal estimates of N_e ranged from 240 to 319 for the time period 1982–2007 when assuming a GI of 10 years (Fig. 2). Estimates were slightly lower (range = 202–268) when assuming a longer GI of 12 years. These estimates generally agreed with N_e estimates from the EPA single-sample method over this time period (mean = 274, harmonic mean = 213; Table S5). Using the unbiased approach of Jorde and Ryman (2007) (based on successive birth years and life history information), N_e was 259 (95% CI = 180–353) for the time period 1982–2007. The maximum likelihood-based method for estimating N_e (in MLNe) did not perform well. Estimates were large and reached the upper prior limit of 5,000 (Fig. 2), indicating that this approach had low precision or reliability when estimating N_e with our dataset. Finally, the loss of heterozygosity from 0.615 to 0.612 in 1985 to 2010, respectively (Table 1), suggested a 0.2% rate of inbreeding over the time period, with an estimated harmonic mean N_{ei} of 256.

Historical estimates of N_{eV} were similar to those previously reported (Miller & Waits 2003) (Fig. 2). The 1910–1960 estimates ranged from 69 to 89, whereas estimates were lower and more variable (range = 35–95) for the 1960–1990 time period. For the entire time frame, 1910–1990, N_e was slightly higher and in some cases exceeded 100 (range = 82–133).

Evaluating sensitivity in N_e and N_b estimation approaches

We evaluated potential sensitivity in EPA-based N_e estimates due to the reliability of parentage assignment threshold specified. Estimates of N_e were slightly lower and more precise when relaxing this threshold from 0.95 to 0.80 (Fig. S3a). However, confidence intervals overlapped and a similarly increasing population trend was observed, regardless of the specified probability of parentage assignment. Estimates of GI from 1985 to 1995 were similar for the 0.80 and 0.95 thresholds. For 2000 and 2005, when applying a strict (0.95) parentage assignment threshold, we observed an increase in GI; whereas, when we relaxed this threshold (0.80), GI remained relatively constant (Fig. S3b).

We examined sensitivity to sample size in single-sample estimates and found that even when applying a consistent sample size ($n = 100$), EPA-derived N_e increased over time, albeit at a lower rate (Fig. S4a). Estimates were increasingly depressed as sample size relative to N_e decreased over time. Estimates of GI were similar for 1985, 1990, and 1995, but low in more recent years (2000 and 2005; Fig. S4b). The EPA method is particularly sensitive to sampling proportion (i.e. the percentage of the population sampled) because the probability of capturing parent-offspring pairs decreases at lower sampling proportions, thereby affecting the accuracy of GI and N_e estimates (Wang *et al.* 2010). The LD-derived N_b estimate did not appear to be sensitive to sample size when the appropriate critical threshold for lowest allele frequency was applied (Fig. S5a). In contrast, SA-based N_b estimates were lower with smaller sample size relative to N_e (Fig. S5b). Again, similar to the EPA method, these results confirmed that an increase in N_b over time remains apparent even when sample size is held constant.

N_e/N_c ratio

Harmonic mean estimates of the N_e/N_c ratio based on EPA-derived N_e over the period from 1984 to 2007 were 0.66 and 0.42 when using the Chao2 and M-R derived estimates of population size, respectively. The N_e/N_c ratio generally remained stable throughout the study time period (Fig. S2).

Discussion

Grizzly bears in the lower 48 states are threatened and receive considerable conservation interest. Monitoring of genetic diversity and effective population size helps to inform management decisions regarding their recovery and protective status under the United States Endangered Species Act. Our results from the EPA (estimator by parentage assignments) N_e estimator suggested over a 4-fold increase from 1982 to 2007. The single-sample N_b estimators (LD, SA) also increased over this time period, with SA increasing more rapidly than LD estimates. This observation is consistent with increasing N_e because LD estimates can be affected by the previous one to few generations due to residual disequilibria that have not yet broken down through recombination (Hill & Robertson 1968; Sved 1971; Waples 2005), whereas SA estimates are not. In addition, the harmonic mean EPA N_e estimate over the entirety of the study period ($N_e = 213$; Tables S4) corresponded to N_{ev} temporal estimators (range = 240–319; Fig. 2). Furthermore, N_{el} as estimated from the loss of heterozygosity over the time period, fell within the same range ($N_{el} = 256$) and suggested a low rate of inbreeding

(0.2%) since 1985. The comparisons among multiple independent estimation procedures substantiate the reliability of these methods and strengthen confidence in our findings.

We also contrasted current N_{eV} and N_{eI} relative to estimates using genotype data obtained from museum specimens of Yellowstone bears. Based on these historical data, Miller and Waits (2003) reported an N_e of ≈ 85 during the 1910–1960s and ≈ 75 –89 during the 1960–1990s. Assuming the GYE population was as diverse as the NCDE in the early part of the 20th century, their heterozygosity-based estimate was very low ($N_e = 22$) relative to the current N_{eI} estimate of 256. Here, we reanalyzed their genotype data using the same temporal estimators implemented in this study (including the unbiased Jorde-Ryman approach; Jorde & Ryman 2007), and confirmed that N_{eV} increased roughly 3- to 4-fold, from ≈ 80 in the 1910–1960s to ≈ 280 in the contemporary population (Fig. 2).

Yellowstone grizzly bears have been isolated from other populations for over a century, and the prospect for restoring natural connectivity with northern populations (e.g., the NCDE, 100 km north) has improved as both populations have increased in numbers and expanded their range (Haroldson *et al.* 2010; Proctor *et al.* 2012). Reflecting this isolation, genetic diversity displayed an initial decline in both allelic richness (A) and expected heterozygosity (H_E) in the early half of the 20th century (Miller & Waits 2003). Our data for 1985–2010 indicate genetic diversity remained stable over the span of this study (Table 1). When limiting our contemporary data to the same eight microsatellites used in Miller and Waits (2003), patterns of diversity over time were similarly stable (Table S4). Relative to most North American (Paetkau *et al.* 1998b; Proctor *et al.* 2012) and European (De Barba *et al.* 2010; Skrbínsek *et al.* 2012a; Waits *et al.*

2000) brown bear populations, genetic diversity in Yellowstone bears is moderately low, with several isolated populations exhibiting diversity estimates that are approximately half those of Yellowstone bears (summarized in Skrbínsek *et al.* 2012b). Given that this is an isolated population, there will inevitably be loss of genetic variation without immigrants or transplants. Increasing G_I and N_e will slow the loss in genetic variation over the long term (100–200 years) (e.g., Perez-Figueroa *et al.* 2012).

Technical Considerations

The accuracy of N_e and N_b estimates is affected by the amount of marker information (number and variability of genetic loci) and sample sizes used in calculations. Bias will decrease as the number of genetic loci available increases. The SA and EPA methods are particularly sensitive to this because they rely heavily on the accuracy of assigning genetic relationships among individuals. Wang *et al.* (2010) showed that a minimum of 8 microsatellites are needed to accurately estimate N_e with the EPA approach, even with low sampling effort (16% of each age class). Another study revealed the SA method, when applied to polygamous species, essentially becomes unbiased with 20 loci, but this is also contingent on having a sufficient sample size relative to N_e (Wang 2009). Simulations indicated that SA-based estimates are biased low (Wang 2009) and EPA-based estimates display a bimodal distribution as the sampling proportion decreases (Wang *et al.* 2010).

Here, using empirical data, we confirmed that N_e estimates derived from both relatedness-based methods might be sensitive to small sample sizes (Figs. S4, S5b). For example,

with an approximate 50% reduction in sample size, estimates were 18% and 28% lower for the EPA and SA methods, respectively. Also, sensitivity appears to increase as sample sizes decrease (Fig. S6). The potential nature and magnitude of sample size sensitivity should be considered when assessing overall trends in N_e . For example, if sample size in any given year/cohort is small, the true N_e may be higher than what is reported. Alternatively, the observation that relatedness-based estimates showed greater sensitivity to sampling in more recent years may be more directly related to a decrease in the sampling proportion, imposed by holding sample size constant and given that the true N_e has increased over time.

In contrast, LD-based estimates appeared to be less sensitive to decreased sample size ($n = 50$) when correcting for the lowest allele frequency applied (Fig. S5). For the LD method, a minimum cohort sample size threshold of $n = 30$ animals/cohort is recommended, based on evidence from simulations suggesting that estimates are reasonably unbiased when $n > 30$ and $n/N_e > 0.1$, given that N_e is not very large ($N_e < 300$ – 500) (Waples 2006a; Waples & Do 2010). The 1984 (i.e., 1983–1985) through 1986 (i.e., 1985–1987) cohorts had sample sizes < 30 , which is reflected in the low precision of N_e estimates for these years (Fig. 1c). Sampling proportions were likely lower in the 1980s and, thus, if such bias exists it may only apply to estimates from earlier years. Despite these possible biases, when we accounted for differences in sample size across yearly or cohort samples, we consistently observed an increasing trend in N_b and N_e for the GYE grizzly bear population (Figs. S4 and S5).

All N_e estimators can be influenced by the sampling scheme (Waples & Yokota 2007), with most methods assuming random sampling from a single discrete generation or cohort in

the population. If sampling is nonrandom, N_e might be underestimated because the frequency of related individuals is likely to be higher than the population as a whole. In this study, yearly sampling was conducted broadly throughout the whole ecosystem (Fig. S1) and the probability of nonrandom sampling was low. The assumption of discrete, non-overlapping generations is a major drawback, as it is often violated in long-lived species such as grizzly bears. In fact, there are very few species that would satisfy this requirement, and many studies have applied these approaches to age-structured populations (e.g., Baalsrud *et al.* 2014; Jansson *et al.* 2012; Skrbínsek *et al.* 2012a; Wright *et al.* 2012).

In heterogeneous samples with individuals from multiple age classes, allele frequency differences among age classes may cause biases in N_e estimates that cannot be overcome by increasing sample size. In large mammals in particular, estimates may be biased high (Luikart *et al.* 2010; Waples 2010; Waples & Yokota 2007). The temporal method of Jorde and Ryman (2007) addresses this problem by measuring allele frequency changes over consecutive homogeneous cohorts. It has been demonstrated as being nearly unbiased and therefore the best option if demographic data are available (Waples & Yokota 2007). Of the single-sample approaches, only the EPA (Wang *et al.* 2010) method is regarded as unbiased and applicable to age-structured populations. Nonetheless, here we show coincident estimates and trajectories for N_e and N_b in GYE grizzly bears using a range of both temporal and single-sample approaches that vary in the assumption of non-overlapping generations.

Simulations have shown that the standard temporal method is biased when violating the assumption of discrete generations, but can reasonably approximate N_e in populations with

457 overlapping generations when the time interval between samples (t) is large ($t \gg G_I$) and the
458 population size is small (Nei & Tajima 1981; Waples 2010; Waples & Yokota 2007). The
459 separation of samples many generations apart maximizes the drift signal to overcome noise due
460 to sampling variance and internal age-sex structures (Jorde & Ryman 1995; Waples & Yokota
461 2007). Whereas the maximum likelihood approach (MLNe) for estimating N_{ev} has been shown
462 to be more precise than other temporal methods using F -statistics, due to the presence of rare
463 alleles (Wang 2001), this MLNe method did not appear to perform well with our dataset. Point
464 estimates of N_e using MLNe were imprecise and unrealistically high, approaching the *a priori*
465 assumed upper threshold of 5,000 (Fig. 2). This result may be due to a low signal to noise ratio,
466 potentially resulting from small t and large population size. As population size increases, the
467 drift signal becomes weak and, thus, larger t may be required to detect an adequate signal for
468 N_e estimation. Also, in our data, sample size in 1982 was small ($n = 52$), and sampling variance
469 possibly contributed to the noise. We repeated the analysis using a larger sample, but more
470 closely separated in time (i.e., 1987: $n = 74$, 2 generations apart), and results were similar (data
471 not shown). We conclude that the maximum number of generations between samples applied
472 (2.5 generations) was probably not sufficient and that the available samples and study time
473 frame limited our ability to estimate N_e with the MLNe approach. We also note that in contrast
474 to the other moment-based temporal estimators of Jorde and Ryman (2007) and Nei and Tajima
475 (1981), the MLNe method incorrectly assumes sampling without replacement, which may
476 introduce another source of error.

When violating the assumption of non-overlapping generations, estimates from the LD and SA approaches yield estimates of N_b (number of breeders that produced the cohort) rather than N_e , as described previously. Here, in order to estimate N_b using these approaches, we pooled cohorts over three successive years, to increase sample sizes, while also accounting for intermittent breeding and excluding the possibility of parent-offspring pairs within the 3-year interval. Both Waples *et al.* (2014) and Robinson and Moyer (2013) showed that LD estimates are downwardly biased when using random samples from adults of all age classes. The effects of pooling cohorts varies across species, depending on the ratio of N_b to N_e , with a slight upward bias in grizzly bears when three cohorts are pooled and when $N_b/N_e \approx 1$ (see Figure S7 in Waples *et al.* 2014). However, negative bias due to skip breeding might counterbalance this positive bias due to pooling cohorts. The power of the SA approach, in particular, should not be compromised by the inclusion of multiple cohorts as long as individuals can be partitioned into offspring and candidate parents (Wang 2009), which we were able to do with considerably detailed information on individuals' sex and age due to long-term monitoring of this population.

The relationship between N_b and N_e is complicated and, in previous studies (e.g., (Skrbinsek *et al.* 2012a) has been generalized from semelparous species that show N_b multiplied by the GI is approximately equivalent to N_e (i.e., $N_e \approx GI \times N_b$) (Waples 2006b). In reality N_e may fall somewhere between N_b and $GI \times N_b$ because the number of breeders each year consists of adults from multiple age classes that represent several generations (Luikart *et al.* 2010; Wang 2009). A recent study by Waples *et al.* (2013) demonstrated that the ratio of N_b/N_e is a function of a few key life-history traits and varies much more than previously expected in species with

overlapping generations. Using previously published life history parameters, their study also estimated the N_b/N_e ratio in grizzly bears to be close to 1. However, this estimate would be reduced if lifetime variance in reproductive success among individuals in a cohort (V_k) is greater than the mean number of offspring produced by a parent over its lifetime ($\bar{K}$).

It is not clear how the N_b/N_e ratio may be affected by changes in demographic parameters (e.g., sex-specific survival, sex ratio, GI) through time and our data suggest it is probable that these parameters have not remained constant in Yellowstone bears. For example, GI increased over our study period from approximately 8 years in the 1980s to 10 years in the 1990s and 14 years in the 2000s (Fig. 1b). This finding is supported by demographic evidence of an increase in GI based on population projections (1983–2001: GI = 10.6–10.8, 2002–2011: GI = 12.9–13.6; IGBST, unpublished data). In addition, a shift in the female to male sex ratio from 3:2 to 1:1 has been detected, as male survivorship has increased (Interagency Grizzly Bear Study Team 2012). Intermittent breeding may also decrease N_b and increase N_e , which together would reduce the N_b/N_e ratio, although this reduction has been theoretically shown to be small (7–16%) for species with Type I survival, such as grizzly bears (Waples & Antao 2014).

Given the recent work of Waples *et al.* (2013), N_{el} could be potentially much closer to N_b in grizzly bears and other species. Also, in theory, heterozygosity-based estimates of N_e are expected to be lower than variance-based estimates in a growing population (Allendorf *et al.* 2013; Crow & Denniston 1988). In this study, neither of these expectations was met, although N_{el} did fall on the lower end of the range of N_{eV} values.

When comparing N_e and N_b estimates from single- and multiple-sample temporal approaches, it is important to note that the time frame to which the estimates apply may differ (Waples 2005). The LD- and SA-based N_b estimates are expected to apply to the 3-year time period during which a cohort was sampled, representing the number of breeders producing that cohort (e.g. Beebee 2009; Skrbinek *et al.* 2012a). In non-equilibrium populations undergoing fluctuations in effective size, however, the LD approach is affected by previous generations that are theoretically expected to introduce transient biases in N_e due to residual linkage disequilibria (Waples 2005). In contrast, N_e estimated using the EPA approach is expected to represent the generation (i.e., approximately 10 years) prior to when the population sample was collected (Wang *et al.* 2010). Temporal estimates do not provide information on N_e relevant to the most recent generation sampled, but instead reflect the harmonic mean of N_e over the entire time interval analyzed (Waples 2005).

Ratio of effective to census population size

The N_e/N_c ratio in our study ranged between 0.42 and 0.66, which is considerably higher than other published estimates for this population that are based on historical genetic data ($N_e/N_c = 0.27$; Miller & Waits 2003) and demographic simulations accounting for a broad range in the variance of reproductive success ($N_e/N_c = 0.24$ – 0.32 ; Harris & Allendorf 1989). Our estimated ratio was also much higher than brown bear populations in Alaska ($N_e/N_c = 0.04$ – 0.19 ; Paetkau *et al.* 1998b) and southern Sweden ($N_e/N_c = 0.06$ – 0.14 ; Tallmon *et al.* 2004), but comparable to a population in the Pasvik Valley of Northern Europe ($N_e/N_c \sim 0.6$; Schregel *et al.*

2012). Results from a meta-analysis indicated that the N_e/N_c ratio is often higher in small populations (Palstra & Ruzzante 2008), which may have contributed to the ratios we observed.

A high ratio of N_e/N_c may also be caused by inflated N_e estimates or conservative estimates of N_c (i.e., demographic estimates that are biased low). Our sensitivity analyses indicated a tendency to underestimate N_e at low sampling proportions, so we do not regard that as a likely explanation. However, an underestimation bias has been documented for the Chao2 estimator (Cherry *et al.* 2007; Schwartz *et al.* 2008), which produced the largest N_e/N_c ratios. Indeed, the N_e/N_c ratios were lower when using the M-R estimates, which appear to be unbiased (Higgs *et al.* 2013). If in fact the N_e/N_c ratio corresponds more closely with previous estimates, these findings suggest the M-R estimates are much closer to the true population size than the Chao2 estimates. This interpretation is consistent with the study of Higgs *et al.* (2013), who concluded based on simulations that M-R estimates were centered on the true value, although variability was large. Inferring N_c from N_e may only be appropriate if the N_e/N_c ratio remains stable over time, but is generally not advisable if variance in reproductive success is high (reviewed in Luikart *et al.* 2010). We observed relatively stable values for the N_e/N_c ratio for the duration of the study period (Fig. S2) and reproductive parameters tend to have low variance among Yellowstone grizzly bears (e.g., low standard error for fecundity; Schwartz *et al.* (2006), IGBST 2012:33). This implies that if we could accurately measure this ratio, estimation of N_e could provide an additional tool for evaluating N_c .

Conclusions

Our results suggest that the Yellowstone grizzly bear population has increased substantially over the past three decades, supporting demographic evidence that conservation measures implemented under the ESA have thus far aided the recovery of this population. Specifically, our findings suggest that genetic diversity has shown no decline in recent decades, and contemporary N_e substantially exceeds the inbreeding avoidance goal ($N_e > 50$) and may eventually approach the long-term viable population criterion ($N_e > 500$) defined by Franklin and Frankham (1998) (Fig. 1a). Thus, our study suggests that current effective population sizes are sufficiently large ($N_e \gg 50$) to avoid substantial accumulation of inbreeding depression, reducing concerns regarding genetic factors affecting the viability of Yellowstone grizzly bears. Nonetheless, the historically small N_e , relatively low diversity, and isolation over many generations suggest the grizzly population could benefit from increased fitness following the restoration of gene flow (e.g., Hogg *et al.* 2006; Tallmon *et al.* 2004), particularly given the unpredictability of future climate and habitat changes.

Our study shows that genetic monitoring of N_e can be a valuable tool for animal conservation and management. Effective population size summarizes the effects of many demographic factors (e.g., N_c , sex ratio, variance in fecundity and survival) on the strength of inbreeding and genetic drift processes and can provide valuable information regarding a population's long-term evolutionary potential and susceptibility to short-term inbreeding depression. Although N_c is positively related to N_e , two populations with the same N_c may differ in N_e and thus follow different evolutionary (and viability) trajectories. Therefore, monitoring of N_c alone may not be sufficient for making informed decisions regarding long-term population

health. This study demonstrates how multiple independent estimators of the genetically effective population size can be used to complement traditional ecological estimators of abundance to improve understanding of eco-evolutionary processes and population monitoring for conservation and management.

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Data Accessibility

Microsatellite genotype data, associated bear life history data (sex, birth and death years), and input files used in analyses can be accessed through a Dryad repository, doi:10.5061/dryad.s6764.

Author Contributions

Conceived and designed the study: PK, MH, FvM. Collected and managed the data: CW, MH, FvM. Performed the genotyping: DP. Analyzed the data: PK. Wrote the paper: PK, MH, GL, FvM.

Tables and Figures

Table 1. Genetic diversity of the Greater Yellowstone Ecosystem grizzly bear population over time. We evaluated population genetic diversity at 5-year intervals (1985–2010) based on mean allelic richness (A), observed heterozygosity (H_O), and expected heterozygosity (H_E) across 20 microsatellite loci. Standard errors (se) of estimates are reported.

Year	No. gene copies	A^1 (se)	H_O (se)	H_E (se)
1985	132	4.65 (0.31)	0.611 (0.042)	0.615 (0.038)
1990	228	4.60 (0.30)	0.618 (0.040)	0.609 (0.037)
1995	422	4.59 (0.30)	0.621 (0.038)	0.616 (0.036)
2000	654	4.55 (0.30)	0.617 (0.036)	0.614 (0.036)
2005	740	4.56 (0.29)	0.610 (0.037)	0.612 (0.036)
2010	576	4.52 (0.29)	0.610 (0.038)	0.612 (0.037)

¹Estimates of A corrected for sample size through rarefaction in HP-RARE (Kalinowski 2005) using smallest number of gene copies (132, or 68 diploid individuals).

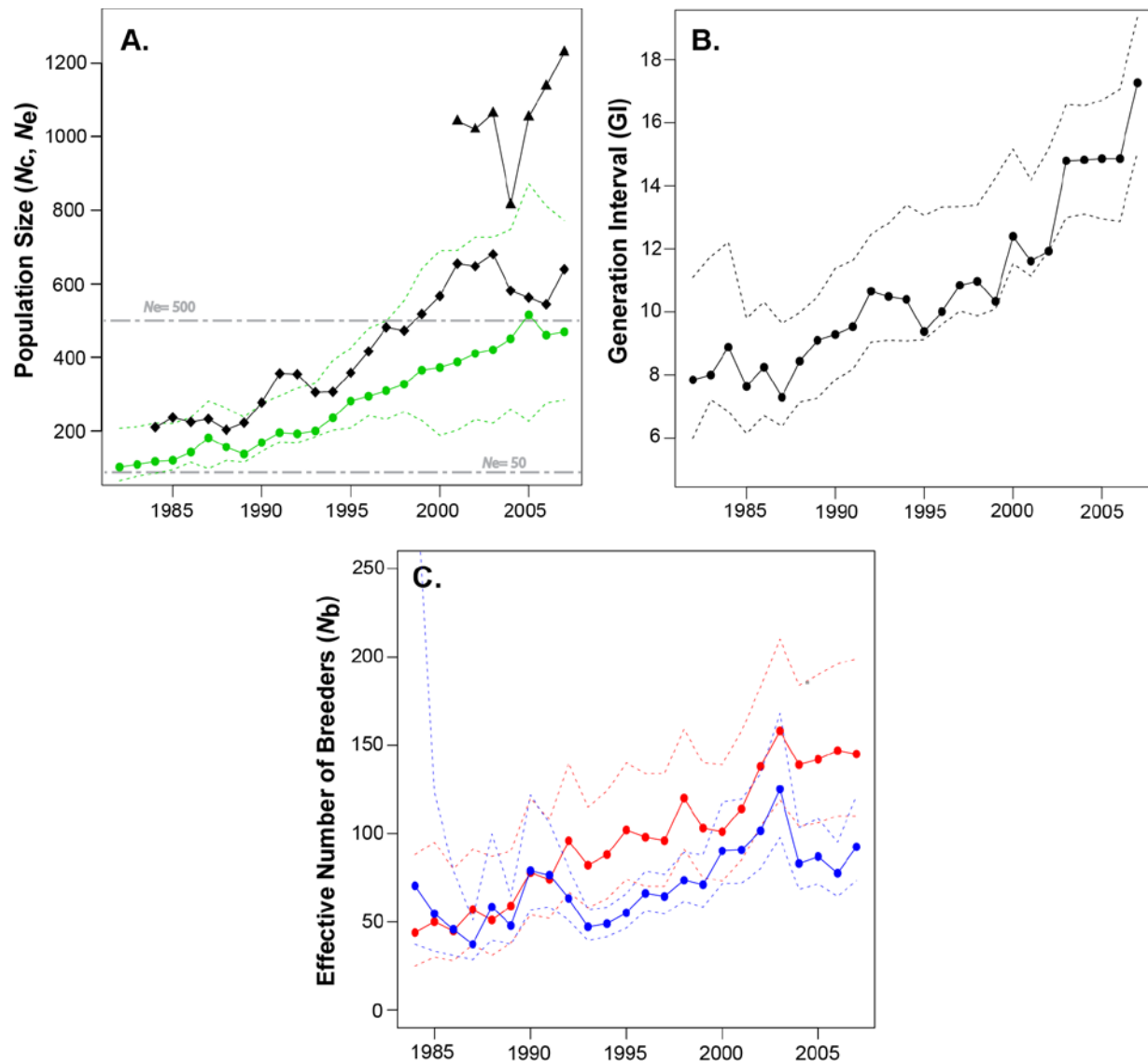


Figure 1. Population size indices of grizzly bears in the Greater Yellowstone Ecosystem, 1982–2007. (A) Estimates census population size (N_c), including bears of all ages, based on Chao2 (black diamonds; 1984–2007) and Mark-Resight (black triangles; 2001–2007) methods are shown with respect to EPA (Estimator by Parentage) estimates (green) of effective population size (N_e). Gray dash-dotted lines indicate the N_e thresholds for short-term ($N_e = 50$) and long-term ($N_e = 500$) conservation as defined by Franklin and Frankham (1998); (B) Generation interval (GI); (C) Cohort-based estimates of the effective number of breeders (N_b) using the LD (blue; Waples 2006a) and SA (red; Wang 2009) methods. Point estimates for cohort-based approaches represent the middle year of the 3-year period (e.g., 1984 estimate was determined using the 1983–1985 cohort); For all N_e and N_b indices, annual point estimates and 95% confidence intervals are indicated by solid and dotted lines, respectively.

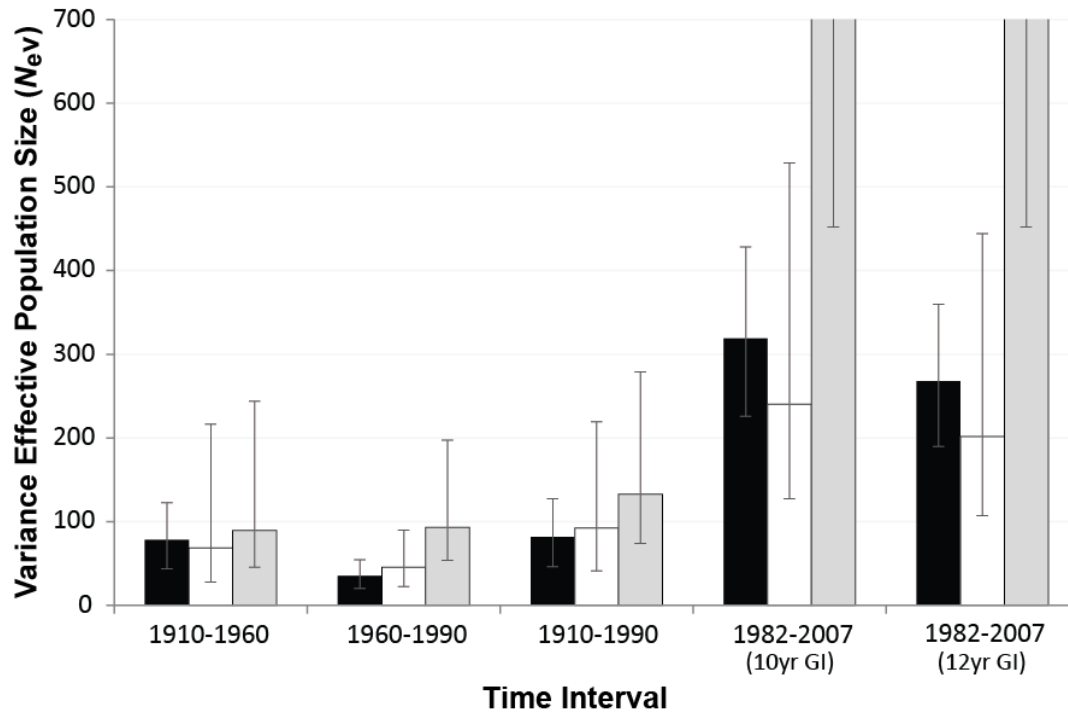


Figure 2. Variance effective population size estimates of grizzly bears in the Greater Yellowstone Ecosystem for four time intervals (1910–1960, 1960–1990, 1910–1990, 1982–2007) and three temporal effective population size (N_e) estimators: Jorde-Ryman (black), Nei-Tajima (white), and maximum likelihood (gray). Vertical bars represent 95% confidence intervals. Genotype data for 1910–1960, 1960–1990, and 1910–1990 were taken from Miller and Waits (2003) and used to recalculate N_e with the methods used on the contemporary data (1982–2007). Estimates were computed assuming both a 10- and 12-year GI for the 1982–2007 time period. Upper limits of 95% confidence intervals for some estimates exceeded the upper limit (i.e., >5,000) and are not shown.