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W. Chris Funk[1], Eric D. Forsman[2], Thomas D. Mullins[1] and Susan M. Haig[1]

Abstract

The northern spotted owl (*Strix occidentalis caurina*) is one of the most controversial threatened subspecies ever listed under the U.S. Endangered Species Act. Because of concern for persistence of the subspecies, logging on Federal lands in the U.S. Pacific Northwest was dramatically reduced under the Northwest Forest Plan in 1994. Despite protection of its remaining forest habitat, recent field studies show continued demographic declines of northern spotted owls. One potential threat to northern spotted owls that has not yet been shown is loss of genetic variation from population bottlenecks that can increase inbreeding depression and decrease adaptive potential. Here, we show recent genetic bottlenecks in northern spotted owls using a large genetic dataset (352 individuals from across the subspecies' range and 11 microsatellite loci). The signature of bottlenecks was strongest in Washington State, in agreement with field data. Interestingly, we also found a genetic signature of Pleistocene expansion in the same study areas where recent bottlenecks were shown. Our results provide independent evidence that northern spotted owls have recently declined, and suggest that loss of genetic variation is an emerging threat to the subspecies' persistence. Reduced effective population size (N_e), shown here in addition to field evidence for demographic decline, highlights the increasing vulnerability of this bird to extinction.

Introduction

The debate over northern spotted owl conservation and logging is one of the most famous chapters in conservation history. Declines stemming from harvest of the northern spotted owl's old-forest habitat in the U.S. Pacific Northwest led to listing of the subspecies as threatened under the U.S. Endangered Species Act in 1990 (U.S. Fish and Wildlife Service, 1990). In addition, the percentage of federal forest land in the range of spotted owls allocated to reserves was increased to 77 percent under the Northwest Forest Plan in 1994, dramatically reducing timber harvest (Stokstad, 2005; Noon and Blakesley, 2006). Nonetheless, recent field studies indicate that northern spotted owls have continued to decline at an average rate of 3.7 percent per year, and that declines are most severe in Washington State (Anthony and others, 2006). Anecdotal evidence also suggests severe declines in British Columbia. One possible cause for continued decline is competition and hybridization with invasive barred owls (*Strix varia*), which have rapidly expanded into the range of northern spotted owls from their historic range in eastern North America (Kelly and others, 2003; Haig and others, 2004; Kelly and Forsman, 2004; Olson and others, 2005; Anthony and others, 2006; Funk and others, 2007). An additional potential threat that has not yet been shown for northern spotted owls is loss of genetic variation when effective population size (N_e) decreases, known as a genetic bottleneck. Loss of genetic variation is expected to increase inbreeding depression (Crow and Kimura, 1970), which can reduce survival and reproductive rates, in turn decreasing population growth rates and increasing extinction probabilities (Saccheri and others, 1998). Decreased genetic variation also reduces adaptive potential (Bürger and Lynch, 1995). Thus, determining whether N_e of northern spotted owls are decreasing is essential for fully understanding the threats to the subspecies and possible reasons for its continued decline.

New genetic methods allow detection of population bottlenecks as well as population growth or expansion. Although these methods have tremendous potential for detecting changes in N_e, their power and limitations in natural populations are still being explored. One potential limitation is that the genetic signal of historic expansions may swamp the signal of recent declines, or vice versa, since expansions and declines have opposite effects on patterns of genetic variation. This problem may be particularly acute in temperate species such as northern spotted owls that are currently declining, but that likely expanded during Pleistocene interglacial cycles as has been shown for many temperate species (Milá and others, 2000; Lessa and others, 2003). A possible solution to this problem is to use different methods for detecting changes in N_e that perform best over different time scales. Another potential limitation of genetic methods for detecting changes in N_e is

[1]U.S. Geological Survey, Forest and Rangeland Ecosystem Science Center, 3200 SW Jefferson Way, Corvallis, Oregon 97331, USA

[2]USDA Forest Service, Pacific Northwest Research Station, 3200 SW Jefferson Way, Corvallis, Oregon 97331, USA

that they may not have the power to detect relatively slow, steady rates of population decline, as seen in northern spotted owls. There are several examples of the utility of genetic bottleneck tests for detecting catastrophic population crashes (Cornuet and Luikart, 1996; Goossens and others, 2006), but their capacity to detect slower rates of decline is less clear. Nonetheless, detection of slower declines will be important scientific information to obtain before populations decrease to the point where recovery is unlikely.

We genotyped 352 northern spotted owls across the subspecies' range at 11 variable microsatellite loci to test for recent bottlenecks and Pleistocene expansion. Our three primary questions were: (1) is there a genetic signature of population decline in northern spotted owls despite relatively slow, steady declines; (2) if declines are detected, does the geographic pattern of decline identified with genetic data match the pattern observed in the field; and (3) is the genetic signature of Pleistocene expansion preserved in study areas in which recent declines are detected?

Materials and Methods

Sampling

We collected blood samples from 352 northern spotted owls from 16 study areas across the subspecies' range from 1990 to 2006 following the American Ornithologists' Union protocol (Gaunt and Oring, 1997; fig. 1). Ninety-four percent of these were collected from 1994 to 2006. Study areas were bounded by landscape features such as mountain ridges, rivers, and non-forested habitat. No known close relatives (parent-offspring or siblings) were included.

Microsatellite Data

DNA extraction, PCR, and fragment analysis were performed as described previously (Funk and others, 2007). All owls were genotyped at 11 variable microsatellite loci (table S1 in appendix 1) developed for Mexican spotted owls (*S. o. lucida*, Thode and others, 2002), Lanyu scops owls (*Otus elegans botelensis*, Hsu and others, 2003, 2006), and ferruginous pygmy-owls (*Glaucidium brasilianum*, Proudfoot and others, 2005). One of these loci (Oe128) and an additional microsatellite marker (Bb126) are diagnostic of spotted versus barred owls (Funk and others, 2007) and were genotyped to assure that no barred owls or spotted owl-barred owl hybrids were included. One barred owl was detected and removed from subsequent analyses.

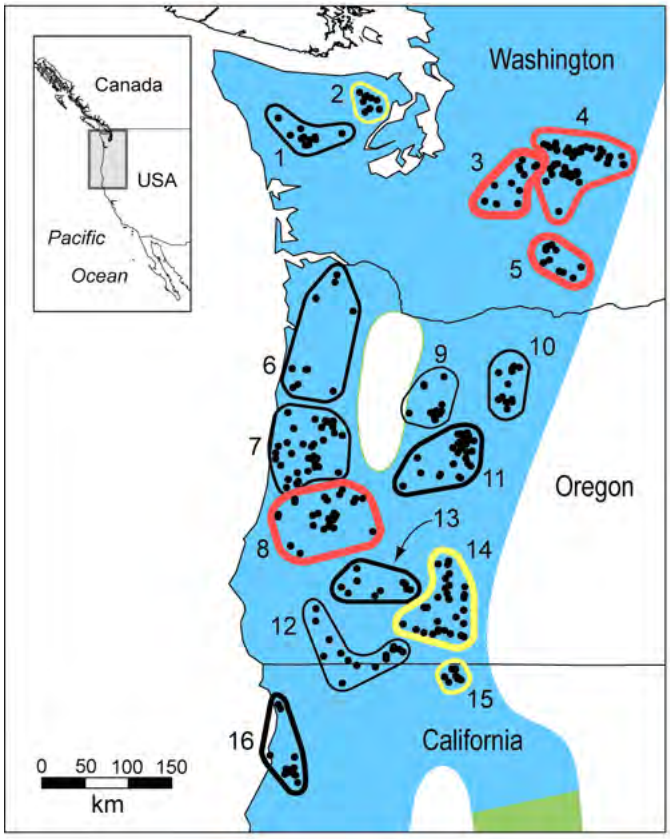

Figure 1. Recent population bottlenecks in northern spotted owls. Points represent the 352 individual owls included in the analysis which are grouped into 16 study areas. The thickness of study area boundaries is proportional to heterozygosity excess which reflects the magnitude of bottlenecks. Study areas with statistically significant bottlenecks are shown in red (p =0.01 critical level) and yellow (p =0.05) under a 10% multi-step mutation model. Bottlenecks are most severe in the Washington Cascades and southern Oregon Coast Range. Blue shading shows the range of northern spotted owls and green the range of California spotted owls *(S. o. occidentalis).*

Standard population genetic analyses were performed using Genepop 3.4 (Raymond and Rousset, 1995). There were no significant departures from Hardy-Weinberg (HW) proportions within any study area, suggesting little or no genetic structure within study areas. Out of 869 tests for linkage disequilibrium (LD), fewer were significant (41 tests) than expected by chance (43.5) and there was no evidence for consistent LD between any two loci, indicating that loci were independent. FST across all study areas was 0.023 and pairwise FSTs ranged from 0.0002 to 0.062.

Cornuet and Luikart Method

We tested for recent population bottlenecks in all 16 study areas using an analysis developed by Cornuet and Luikart (1996). This method is based on the loss of rare alleles predicted in recently bottlenecked populations. It uses a single population sample to test whether there has been a recent reduction in allelic variation. Simulations (Cornuet and Luikart, 1996; Williamson-Natesan, 2005), theory (Garza and Williamson, 2001), and case studies (Cornuet and Luikart, 1996; Goossens and others, 2006; Beebee and Rowe, 2001; Spear and others, 2006) all show that this is the best method available for detecting recent, low-magnitude declines in N_e. Simulation studies (Cornuet and Luikart, 1996;, Williamson-Natesan, 2005) also demonstrate that this method has low type I error rates (that is, falsely detecting a bottleneck when there is not one). We used program Bottleneck 1.2.02 (Piry and others, 1999) to implement this analysis. The two-phase mutation model (TPM) was used to generate null distributions under mutation-drift equilibrium, as the TPM is considered to be most appropriate for microsatellites (Garza and Williamson, 2001; Di Rienzo and others, 1994). A wide range of values was used for the percent of multi-step mutations (5, 10, 20, and 30 percent, table 1). The Wilcoxon signed rank test was used to determine significance of heterozygosity excess.

Bottleneck tests assume random mating and population closure (no gene flow). Non-random mating can produce genealogies that resemble bottlenecks, whereas gene flow may resemble recent expansion by introducing rare alleles (Cornuet and Luikart, 1996; Goossens and others, 2006). Agreement with HW proportions above supported random mating within study areas (that is, lack of genetic structure), but low FST values suggested gene flow among areas. Yet despite gene flow that can mimic recent expansion, we found a consistent signature of bottlenecks, providing even stronger evidence for recent reductions in N_e. Bottlenecks in northern spotted owls may therefore actually be more severe than they appear in our analysis.

At one locus, 1C6, extreme heterozygosity deficiency was observed while at the other 10 loci, heterozygosity excess was observed, a pattern suggesting strong positive selection at 1C6 or a linked locus (Maynard Smith and Haigh, 1974; Watterson, 1978) or a different mutation model. For example, mean heterozygosity excess (H_e, Hardy-Weinberg heterozygosity, minus H_{eq}, equilibrium heterozygosity) across all study areas was 0.036 at the other 10 loci, but was -0.269 at 1C6 under the 10 percent multi-step mutation model. H_e was also significantly lower than expected under mutation-drift equilibrium at 1C6 in 13 out of 16 study areas under this model. 1C6 was the only trinucleotide repeat microsatellite analyzed (see table S1) which may explain the divergent pattern in heterozygosity observed at this locus. For example, this locus could be an expanded repeat of an amino acid (TAT which encodes tyrosine) that is under selection.

Table 1. Mean heterozygosity excess and *P*-values for northern spotted owls.

[Het exc, mean heterozygosity excess across loci; *P*, probability of observed heterozygosity excess under mutation-drift equilibrium and the given two-phase mutation model. Significant *P*-values ($p = 0.05$) are shown in **bold**]

Study area	*n*	Percent multi-stepmutations (under two-phase mutation model)							
		5		10		20		30	
		Het exc	*P*	Het exc	*P*	Het exc	*P*	Het exc	*P*
1	13	0.015	0.216	0.024	0.188	0.032	0.138	0.039	0.065
2	9	0.022	0.053	0.026	**0.042**	0.035	**0.042**	0.042	**0.042**
3	13	0.055	**0.003**	0.063	**0.001**	0.074	**0.001**	0.081	**0.001**
4	51	0.033	**0.016**	0.047	**0.002**	0.067	**0.0005**	0.082	**0.0005**
5	18	0.048	**0.042**	0.057	**0.005**	0.070	**0.002**	0.082	**0.002**
6	12	0.020	0.216	0.026	0.188	0.041	0.161	0.046	0.138
7	47	0.009	0.348	0.023	0.188	0.042	0.080	0.056	**0.012**
8	31	0.044	**0.012**	0.058	**0.007**	0.074	**0.003**	0.085	**0.001**
9	15	0.007	0.246	0.014	0.188	0.025	0.053	0.032	**0.042**
10	14	0.014	0.080	0.021	0.053	0.033	**0.042**	0.041	**0.042**
11	28	0.022	0.313	0.035	0.080	0.049	0.053	0.061	**0.009**
12	17	0.008	0.313	0.017	0.246	0.027	0.246	0.038	0.216
13	10	0.027	0.313	0.032	0.216	0.041	0.097	0.047	0.080
14	32	0.042	0.065	0.052	**0.012**	0.068	**0.003**	0.079	**0.002**
15	14	0.027	0.097	0.034	**0.042**	0.046	**0.016**	0.053	**0.009**
16	28	0.028	0.138	0.040	0.080	0.054	0.065	0.068	**0.009**

Since the pattern of genetic variation at 1C6 appeared to reflect selection rather than demographic history, it was excluded from further analysis. Nonetheless, inclusion of 1C6 did not qualitatively affect evidence for population bottlenecks. For example, even with 1C6, mean heterozygosity excess was still greater than zero in study areas 3–5, 8, and 14–16 regardless of mutation model and was significant for study area 3 under the 10 percent multi-step model and study areas 3–5 and 15 under the 20 percent multi-step model. Detection of bottlenecks despite inclusion of a locus with a strong signal of positive selection reinforces evidence for declines.

Mean heterozygosity excess was not correlated with mean or median year of sample collection across study areas (p=0.320 and p=0.410, respectively). Thus, there was no evidence for a sampling-year effect.

Bayesian Methods

We next used two Bayesian coalescent-based methods to test whether the signature of Pleistocene expansion was preserved in three study areas in which recent declines were detected, study areas 4 (n=51), 8 (n=31), and 14 (n=32). Only three study areas were chosen for the Bayesian analyses because these analyses are extremely computationally intensive and time consuming. These methods use the full allelic distribution to infer past changes in N_e. The Beaumont (1999) method estimates the posterior distribution of r, the ratio of current population size (N_0) to ancestral population size (N_1), while the Storz and Beaumont (2002) method estimates the posterior distributions of N_0, N_1, and x_a, the time in years since population growth or decline. Although these methods are capable of detecting recent declines (Goossens and others, 2006), they are predicted to be best at detecting long-term, gradual changes in N_e (Williamson-Natesan, 2005).

The Beaumont and Storz and Beaumont methods were implemented using the msvar programs (Storz and Beaumont, 2002). The Beaumont method assumes rectangular prior distributions. Wide bounds were used for priors (table S2 in appendix 1) so that posterior distributions would be minimally affected. For each study area, at least four independent runs were performed using different starting values and thinning intervals (table S2). All runs had at least 10^9 iterations and thinning intervals of 10^5–10^6. Posterior distributions of log(r) were very similar among different runs (fig. S1). The last half of each run was combined to generate a final posterior distribution for each study area (shown in fig. 2).

The Storz and Beaumont method assumes log-normal priors, and broad priors were used once again (table S3). Three independent runs were performed for each study area using different prior distributions of log(x_a). All runs had at least 109 iterations and thinning intervals of 10^5. A mean generation time of 8.8 years was calculated from parents of known age in 2003 (n=43 parents), 2004 (n=102), and 2005 (n=67). Changing generation time to lower or higher values (for example, 5 or 12 years) did not alter the conclusions. Posterior distributions were similar among runs for log(N_0), log(N_1), and log(x_a) (figs. S2 and S3).

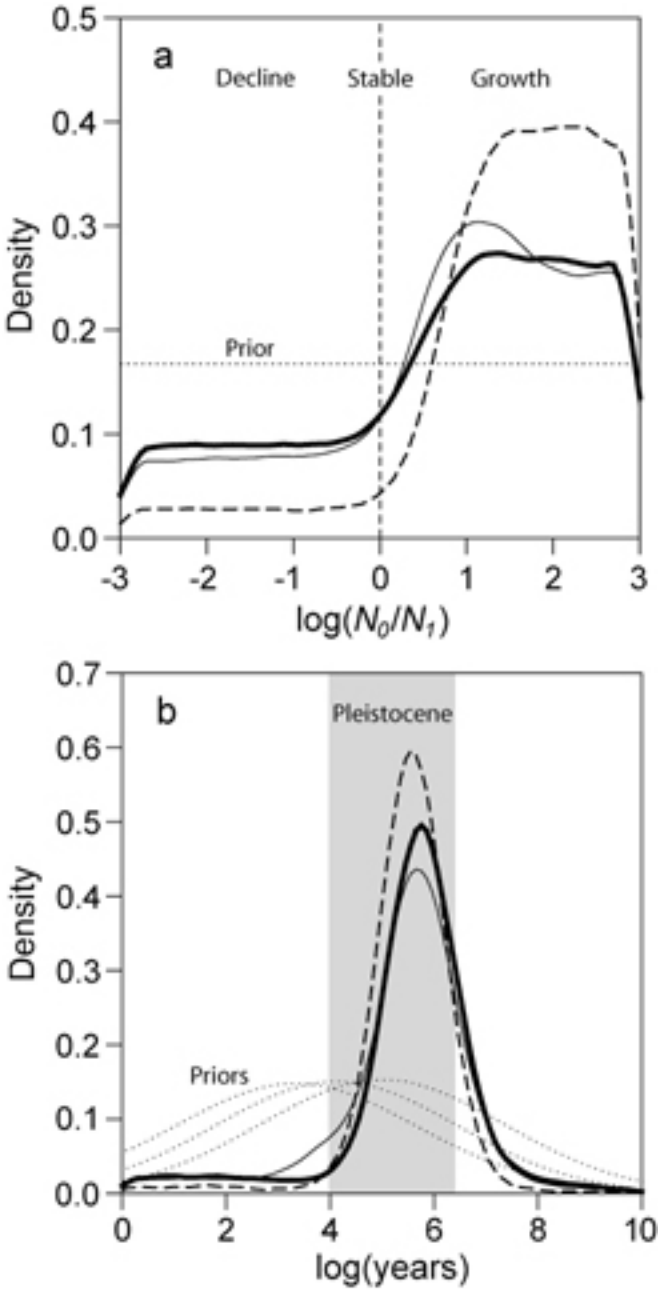

Figure 2. Pleistocene expansion in northern spotted owls. (a) Posterior probability distributions of r, the ratio of current (N_0) to ancestral (N_1) population size, in study areas 4 (dashed line), 8 (thin line), and 14 (thick line). Support was much stronger for population growth than decline, particularly for study area 4. The dotted line shows the rectangular prior distribution for r. (b) Posterior distributions of x_a, the time in years since population growth for the same three study areas. Support was greatest for expansion during the Pleistocene, especially in study area 4. Dotted curves show prior distributions for x_a.

Results

Recent Bottlenecks

We found a significant signature of population decline using the Cornuet and Luikart method for several study areas, regardless of the mutation model assumed (fig. 1, table 1). The signature of declines was strongest for study areas 3–5 in the Washington Cascades and area 8 in the southern Oregon Coast Range. Declines were also significant when study areas were lumped into regions in the Washington Cascades (study areas 3–5; p=0.002 under a 10 percent multi-step model), the Oregon Coast Range (areas 6–8; p=0.016), and the Klamath Mountains (areas 12–15; p=0.005), demonstrating that detection of declines was not sensitive to grouping method. In addition, mean heterozygosity excess across loci was greater than zero in all 16 study areas regardless of mutation model (table 1), significantly more areas than the 50 percent expected to show heterozygosity excess by chance (exact binomial probability; p=0.00003). This suggests that declines have occurred throughout the subspecies' range. Thus, the Cornuet and Luikart method detected recent bottlenecks despite relatively slow, steady declines. The geographic pattern of decline detected with the genetic data (strongest signal of declines in Washington) also matches the large-scale pattern observed in the field (Anthony and others, 2006). In addition, bottleneck tests were able to detect genetic declines in study areas 8 and 14 not detected with field data.

Pleistocene Expansion

Bayesian analysis revealed a genetic signature of population growth during the Pleistocene in all three study areas examined (fig. 2, see fig. S4 in appendix 1). Evidence for Pleistocene expansion was greatest for the most northerly study area (study area 4, see fig. 1) which is closest to Pleistocene ice sheets (Mann and Hamilton, 1995) and, therefore was likely most directly affected by glacial cycles. The posterior odds for a growing versus declining population were 10.6, 3.2, and 2.7 for study areas 4, 8, and 14, respectively. Posterior odds for a Pleistocene versus non-Pleistocene timing of growth were 4.1, 1.7, and 1.8 for these same three study areas. Median values of x_a were 3.49×10^5, 3.82×10^5, and 4.75×10^5 years, respectively, all in the mid-Pleistocene. Thus, a genetic signature of Pleistocene expansion was preserved in study areas in which recent declines were also detected.

Discussion

These results provide strong evidence for recent declines and Pleistocene expansion in northern spotted owls. Although the Cornuet and Luikart method does not provide an estimate of the timing of decline as does the Storz and Beaumont method, two findings strongly suggest that the genetic signal of decline reflects recent decreases in N_e. First, the geographic pattern of decline detected with the Cornuet and Luikart method matches the overall pattern of demographic declines observed in a field study from 1985 to 2003 (Anthony and others, 2006). Second, the Bayesian analysis provides little support for older, historic declines. Additionally, as mentioned above, previous work (Cornuet and Luikart, 1996; Beebee and Rowe, 2001; Garza and Williamson, 2001; Williamson-Natesan, 2005; Goossens and others, 2006; Spear and others, 2006) demonstrates that the Cornuet and Luikart method is most effective at detecting recent changes in N_e. Previous mitochondrial DNA (mtDNA) studies of northern spotted owls did not find a statistically significant signature of bottlenecks (Barrowclough and others, 1999, 2005; Haig and others, 2004), likely due to lower power resulting from much smaller sample sizes and use of mtDNA which is inherited as a single locus (compared to 11 microsatellite loci used here).

Importantly, evidence for genetic declines detected here with bottleneck tests indicates reduction in N_e and genetic variation rather than demographic population size. This means that in addition to habitat loss and invasive barred owls, northern spotted owls may become increasingly threatened by genetic factors such as inbreeding depression and loss of adaptive genetic variation. Inbreeding depression may reduce stage-specific survival and reproductive rates, causing an increase in the rate of decline in a process termed an extinction vortex (Soulé and Mills, 1998). A large-scale field study of northern spotted owls (Anthony and others, 2006) found evidence for declines in survival and fecundity in some study areas, which could be caused by the loss of genetic variation detected here. Thus, it is possible that northern spotted owls are already caught in an extinction vortex. At this point, however, it is not possible to determine whether loss of genetic variation is causing vital rate reductions or vice versa. Regardless, future efforts to conserve northern spotted owl populations will require greater consideration of genetic threats to persistence.

Our results demonstrate the potential of genetic methods for monitoring changes in N_e of threatened species (Schwartz and others, 2007). First, it was possible to detect current declines in northern spotted owls despite historic expansion by using a combination of methods with different temporal resolution. This demonstrates that genetic methods have the capacity to detect reductions in N_e even in populations with complex demographic and biogeographic histories. Additionally, declines were detected in spite of the relatively slow, steady rate of decline in northern spotted owls. For example, it is not surprising that the Cornuet and Luikart method was able to detect declines in orangutans (*Pongo pygmaeus*) which have experienced declines of up to 33 percent in a single year (Goossens and others, 2006). In contrast, the average rate of decline in northern spotted owls from 1985 to 2003 across its range was only 3.7 percent, with a maximum average rate of 10.4 percent for a single study area (Anthony and others, 2006). This agrees with simulation studies showing that the Cornuet and Luikart method is able to detect declines of relatively low magnitude (Williamson-Natesan, 2005). Thus, genetic methods should become increasingly useful for population monitoring, especially as it becomes easier and less expensive to acquire large numbers of molecular markers, which will provide greater power.

Acknowledgments

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Appendix 1

Table S1. Microsatellite loci used to test for population decline and growth in northern spotted owls.

[H_e, Hardy-Weinberg heterozygosity. Primer sequences and annealing temperatures can be found in original references. 1C6 was excluded from bottleneck tests because of evidence for strong positive selection. See Methods and Materials section for details]

Locus	Repeat	Size range (bp)	No. alleles	H_e	Reference no.
6H8	GATA	86–114	8	0.793	23
15A6	GATA	142–166	7	0.656	23
1C6	ATT	105–138	11	0.392	23
13D8	GATA	167–195	8	0.709	23
4E10.2	ATTTT	196–246	11	0.827	23
Oe3-7	GATA	118–138	6	0.763	24
Oe053	GATA	200–224	7	0.722	24
Oe128	GATA	315–327	4	0.639	24
Oe129	GATA	253–285	9	0.726	25
Oe149	GATA	236–276	10	0.736	25
FEPO5	AGAT	258–286	8	0.795	26

Table S2. Parameters used for Beaumont method runs.

[Exp, exponential; lin, linear; $\theta = 2N_0 \mu$ (the product of 2, current population size, and mutation rate); $r = N_0/N_1$ (current population size divided by ancestral population size); $t_f = T_a/N_0$ (number of generations since population growth or decline divided by current population size). Starting values are shown in columns 3–5 and the range of rectangular prior distributions in columns 6–8 (see Beaumont 1999 for details). Thinning is the number of update steps between successive lines of output. Parameters which were changed from run 1 are underlined. Runs 2 and 3 were only used for study area 8, but all other runs were used for all three study areas analyzed (4, 8, and 14)]

Run	Model	θ	r	t_f	log(θ) range	log(r) range	log(t_f) range	Thinning
1	Exp	0.1	1	10	-5–3	-3–3	-4–6	10^5
2	<u>Lin</u>	0.1	1	10	-5–3	-3–3	-4–6	10^5
3	Exp	0.1	1	10	-5–3	-3–3	-4–6	<u>10^6</u>
4	Exp	0.1	<u>100</u>	10	-5–3	-3–3	-4–6	10^5
5	Exp	0.1	<u>0.01</u>	10	-5–3	-3–3	-4–6	10^5
6	Exp	<u>10</u>	1	<u>1000</u>	-5–3	-3–3	-4–6	10^5

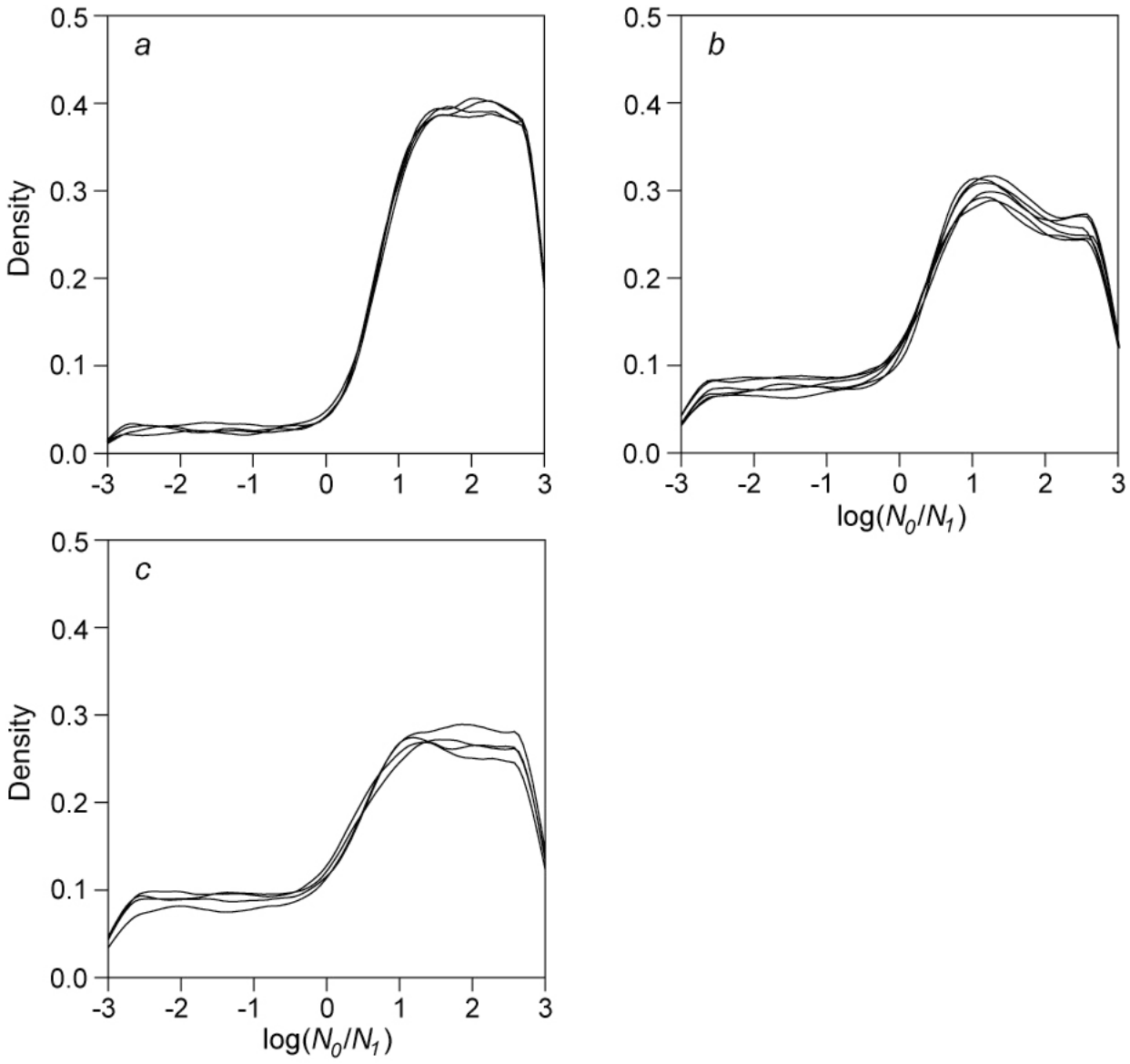

Figure S1. Posterior distributions of population growth ($r = N_0/N_1$) for independent runs. Posterior distributions are shown for study areas 4 (*a*), 8 (*b*), and 14 (*c*). Independent runs gave similar posterior distributions despite different starting values and thinning intervals.

Table S3. Parameters used for Storz and Beaumont method runs.

[N_0, current population size; N_1, ancestral population size; μ , mutation rate; x_a, time in years since population growth or decline. In each starting value column, the figures are the starting mean and variance of the corresponding parameters (see Storz and Beaumont 2002 for details). In each hyperprior column, the first two figures are the mean and variance of a normal distribution from which the mean was drawn for the prior log-normal distribution. The second two figures are the mean and variance of a truncated normal distribution from which the variance was drawn for the prior log-normal distribution. Parameters which were changed from run 1 are underlined. The three parameter combinations were implemented in all three study areas analyzed (4, 8, and 14)]

Run	Starting values				Hyperpriors			
	log(N_0)	log(N_1)	log(μ)	log(x_a)	log(N_0)	log(N_1)	log(μ)	log(x_a)
1	2.5 0.5	2.5 0.5	-4 0.5	4 0.5	2.5 2 0 0.5	2.5 2 0 0.5	-4 0.5 0 0.5	4 3 0 0.5
2	2.5 0.5	2.5 0.5	-4 0.5	4 0.5	2.5 2 0 0.5	2.5 2 0 0.5	-4 0.5 0 0.5	<u>5 3 0 0.5</u>
3	2.5 0.5	2.5 0.5	-4 0.5	4 0.5	2.5 2 0 0.5	2.5 2 0 0.5	-4 0.5 0 0.5	3 3 0 0.5

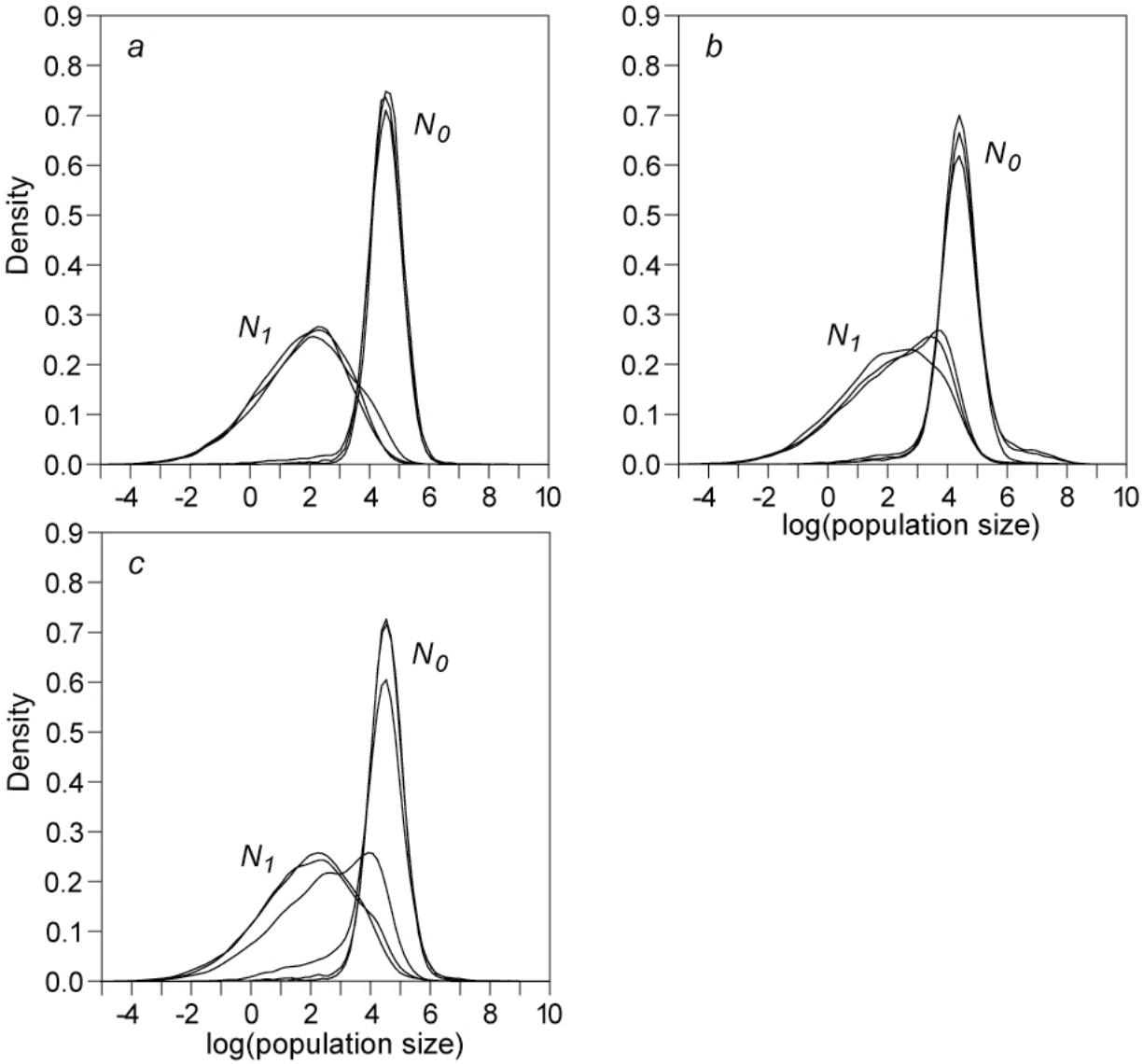

Figure S2. Posterior distributions of current (N_0) and ancestral (N_1) population sizes for independent runs. Posterior distributions are shown for study areas 4 (*a*), 8 (*b*), and 14 (*c*). Independent runs gave similar posterior distributions.

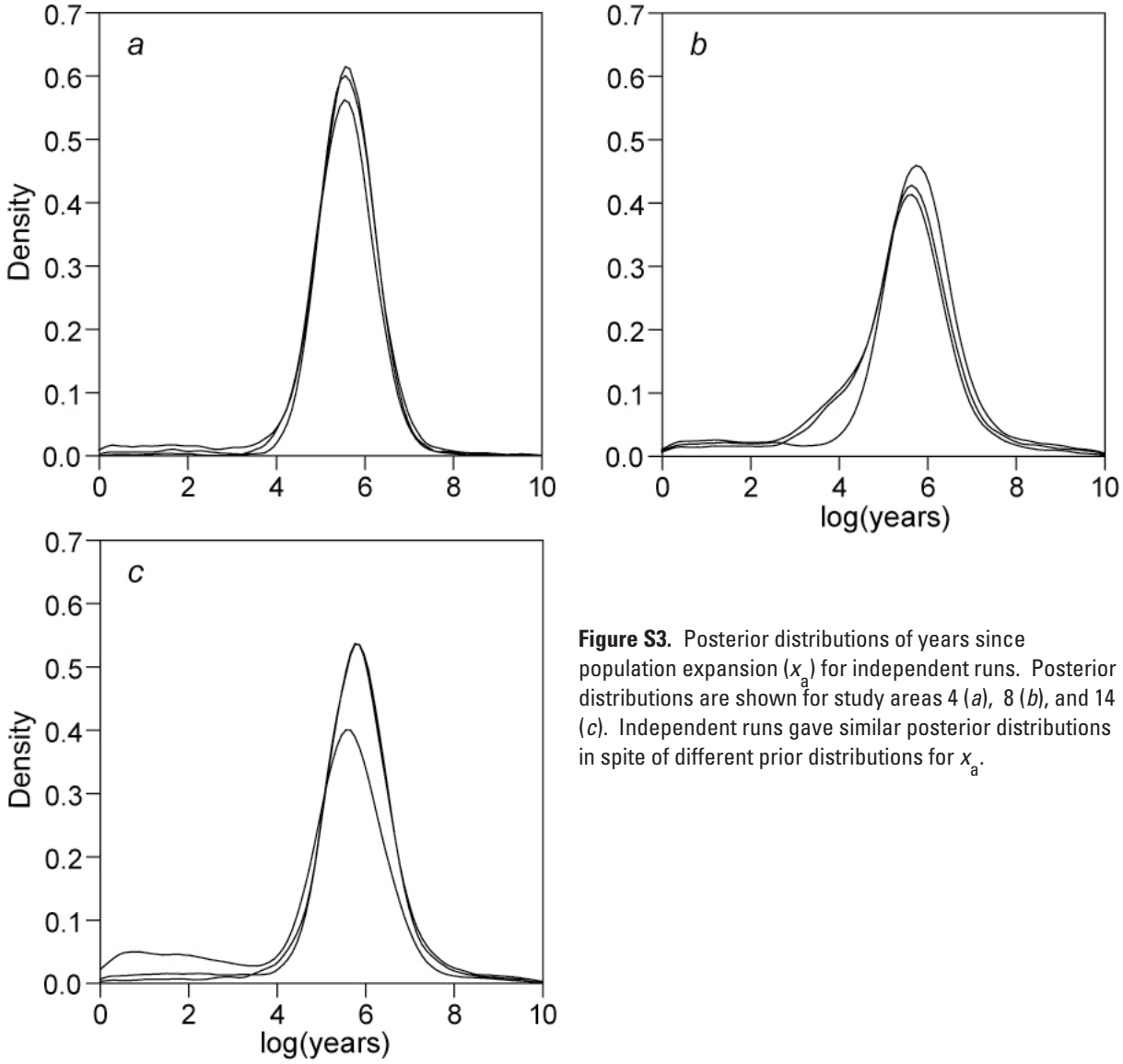

Figure S3. Posterior distributions of years since population expansion (x_a) for independent runs. Posterior distributions are shown for study areas 4 (*a*), 8 (*b*), and 14 (*c*). Independent runs gave similar posterior distributions in spite of different prior distributions for x_a.

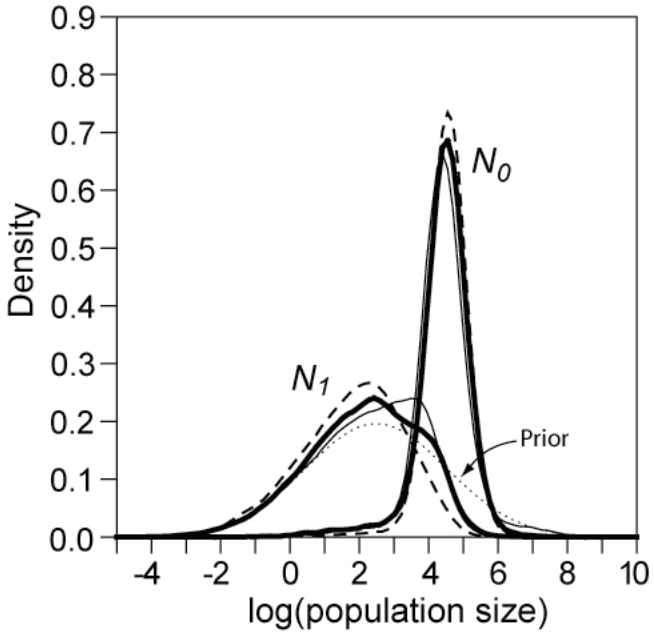

Figure S4. Current and ancestral population sizes in northern spotted owls. Posterior probability distributions of current (N_0) and ancestral (N_1) population sizes in study areas 4 (dashed line), 8 (thin line), and 14 (thick line) estimated using the Storz and Beaumont method. Current population sizes were larger than ancestral population sizes in all study areas, indicating population growth (as found with the Beaumont method). The dotted curve shows the prior distribution used for N_0 and N_1.

For more information concerning the research in this report, contact the
Director, Forest and Rangeland Ecosystem Science Center
U.S. Geological Survey, 777 NW 9th Street, Suite 400
Corvalis, OR 97330
http://fresc.usgs.gov/