

Effective number of breeding adults in Oregon spotted frogs (*Rana pretiosa*): genetic estimates at two life stages

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Abstract We used genetic methods to estimate the effective number of breeders (N_b) in a population of *Rana pretiosa*, an imperiled amphibian in western North America. Microsatellite data was gathered from large samples of adults, eggs, and juveniles collected in 2006. We wished to determine where in the life cycle the greatest reductions in N_b occur, and to compare genetic estimates of N_b to an egg mass count estimate of the number of breeding adults. We predicted that N_b estimated at the metamorph stage would be reduced by increased variance in family size due to egg mass mortality. Contrary to our prediction, estimates of N_b at the egg and metamorph stages were similar. Thus, we found no evidence of inflated variance in family size between the two stages. If our results for this population are typical for *R. pretiosa*, then increased variance in family size during the egg to metamorph stage may not be a strong factor in reducing the effective population sizes (N_e) relative to the census sizes (N) in this species.

Keywords Amphibians · Anura · N_e/N ratio · Temporal method · Linkage disequilibrium · Microsatellites

Introduction

Effective population size (N_e) is a fundamental parameter in the theory and practice of conservation genetics. Related to N_e is the effective number of breeders, N_b , a parameter influenced by most of the same demographic factors as N_e but which applies to only the breeding adults of a population in a single reproductive season. Estimates of N_b or N_e in natural populations are usually much lower than the census population size, N (e.g., Frankham 1995). What causes N_e and N_b to be lower than N is not well understood for many species.

The ongoing loss of global amphibian diversity is a widely recognized ecological crisis (Stuart et al. 2004). Values of N_e/N and N_b/N reported for amphibians range widely, from 0.001 (Easteal 1985) to >0.7 (Brede and Beebee 2006). What features of the life histories of different species might predispose them to have different ratios? For example, there is some intriguing evidence that toads of the genus *Bufo* have N_e/N ratios an order of magnitude lower than those of frogs of the genus *Rana* (Hoffman et al. 2004; Brede and Beebee 2006). Understanding what factors in the life cycle of amphibians are most responsible for reductions in N_b or N_e could be very useful for managing loss of genetic diversity in these taxa.

The two factors thought to most dramatically reduce N_e in animal populations are fluctuating population size and non-random variance in family size (Frankham 1995). Pond-breeding frogs may be particularly susceptible to reductions in N_e by these factors. Populations of frogs in the family Ranidae often go through “boom and bust” cycles from year to year as a result of the environmental instability of their breeding habitats (Berven 1995). In addition, variance in family size for these frogs may be greater than under random (i.e., Poisson distributed)

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expectations due to the loss or survival of whole families during the egg stage of the life cycle (Crow and Morton 1955; Rowe and Beebe 2004). Entire egg masses or portions of egg masses are often lost to desiccation, freezing, predation, or disease (Briggs 1987; McAllister and Leonard 1997). If survival operates at the family level, then the inflation of variance in family size (and reduction in N_b) can be enormous (Crow and Morton 1955). In this study we focus primarily on reduction in N_b incurred during the egg to metamorph stage.

The number of breeding individuals in a given year is often estimated for ranid frog populations by doubling the number of discrete egg masses found in the pond(s) that year (Crouch and Paton 2000). This estimate is sometimes used to estimate N_b (Merrell 1968; Berven and Grudzien 1990; Watson et al. 2000). Estimating N_b this way assumes that each female lays only one egg mass per year, each egg mass is fertilized by a single male, each male breeds with only one female per year, and that family size is poisson distributed. The first three assumptions are likely to hold for ‘explosive breeding’ species, which engage in a single, brief (e.g., 1–3 nights) reproductive bout each year (Wells 1977). The fourth assumption is much more dubious, but how much reduction in N_b results from non-random survival between egg laying and metamorphosis has not been estimated.

Here we used genetic estimates of N_b in a population of the Oregon spotted frog (*Rana pretiosa*) to estimate the reduction in N_b owing to reproductive strategy and to non-random survival among families. We analyzed molecular genetic data from large samples of adults, eggs, and post-metamorphic juveniles collected during a single season (Fig. 1). We estimated N_b at two stages in the life cycle using variances in microsatellite allele frequencies between: (1) adults and eggs; and (2) adults and metamorphs. This is a single-season version of Waples’ (1989) temporal method of N_e estimation, and our approach is similar to that of Scribner et al. (1997).

Given our field observations and the fact that *R. pretiosa* is an explosive breeding species, our a priori expectation was that neither extra-pair fertilization nor multiple mating has a strong influence on N_b in this species. Thus, the estimate of the effective number of breeders (\hat{N}_b) derived from the allele frequency differences between adults and eggs should be similar to the egg mass count estimate of the actual number of breeders ($\hat{N}_{ab} = 2 \times$ number of egg masses) On the other hand, mortality of all or parts of some egg masses is well documented in our and other populations of *R. pretiosa* (Bowerman, personal observation; Licht 1971). Non-random survival among individuals due to egg mass mortality (i.e., family-correlated survival) would reduce \hat{N}_b as measured by allele frequency differences between adults and metamorphs. Therefore, our prediction was that the adult-metamorph \hat{N}_b would be much less than the adult-egg \hat{N}_b .

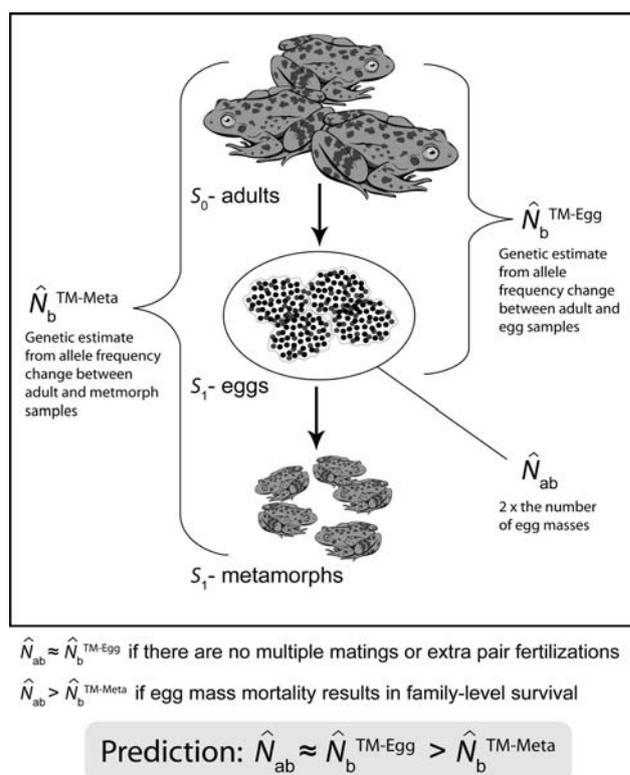


Fig. 1 Sampling scheme for estimating the effective number of breeders (N_b) in a population of *R. pretiosa*. Three samples were collected in 2006: adults, eggs, and metamorphs. For each sample, allele frequencies were calculated for seven microsatellite loci. Two estimates of N_b were derived from this genetic data using the temporal method. The first was based on allele-frequency differences between the adult and egg samples (\hat{N}_b^{TM-Egg}), while the second was based on differences between the adult and metamorph samples ($\hat{N}_b^{TM-Meta}$). An estimate of the actual number of breeding adults was calculated as twice the number of egg masses counted in the pond in 2006 (\hat{N}_{ab}). Our predictions as described in the text are represented by the relationships among \hat{N}_b^{TM-Egg} , $\hat{N}_b^{TM-Meta}$, and \hat{N}_{ab} . Note that the ‘TM’ superscript used here refers to Waples’ (1989) temporal moment method, but this sampling scheme and data were also used for the temporal likelihood (Berthier et al. 2002) analysis. Estimates of N_b were obtained separately for the egg and metamorph samples using the LD method (Hill 1981; Waples and Do 2007)

We also estimated \hat{N}_b from eggs and from metamorphs by the linkage disequilibrium (LD) method (Hill 1981). These estimates should be independent of the temporal method estimates (Waples 1991). Again, we predicted that the N_b estimate from the egg sample would be close to twice the number of egg masses (\hat{N}_{ab}), and that the estimate from the metamorph sample would be substantially less than the estimate from the egg sample.

Finally, we estimated N_e (as opposed to N_b) in the adult population via the LD method and compared it to an estimate of N obtained by intensive mark-recapture sampling. These data provide an additional point estimate of N_e/N for ranid frogs.

Materials and methods

Study organism

Oregon spotted frogs (*R. pretiosa*) live in lakes and ponds in the Pacific Northwest, from southern Oregon in the United States to southern British Columbia in Canada (Hayes 1997; Nussbaum et al. 1983). *R. pretiosa* overwinter in permanent ponds or springs and breeding occurs soon after ice melt in the spring (Licht 1969; Leonard et al. 1997). During the 2–4 week breeding season only mature adults are active at the surface, and the sex ratio is male biased (Watson et al. 2000; personal observations). Breeding is explosive, with most of the egg masses being deposited on one or a few nights (Licht 1969; McAllister and Leonard 1997). Females lay their eggs in communal piles in shallow water, and there may be several of these communal sites per pond. Boundaries between egg masses in a pile are very discrete for a week after laying, which makes counting and sampling individual masses straightforward.

The Oregon spotted frog has been extirpated from 70 to 90% of its original range (Hayes 1997). Fewer than 35 populations remain, and these are mostly small ($N < 1000$), isolated, and restricted to higher elevations (Hayes 1997; Cushman and Pearl 2007). *R. pretiosa* is a candidate for federal listing as endangered by the US Fish and Wildlife Service (2005), is considered “sensitive-critical” by the Oregon Department of Fish and Wildlife, and “endangered” by the state of Washington. It is an endangered species in Canada (Seburn and Seburn 2000). Thus, data on what controls N_e or N_b in this species could be useful for management of the remaining populations.

Sample collections

Sampling took place in a pond located near Sunriver, Oregon (43.85018° N, 121.44768° W). Adult and post-metamorphic juvenile frogs and were captured using underwater funnel traps (Gee’s minnow traps) and dip nets. Adult frogs were individually marked with PIT tags. Metamorphs were not individually marked. Capture-recapture data from marked frogs was collected on 77 occasions from March 6th through December 9th 2006. For genetic sampling, a single toe clip was collected from each adult frog ($n = 208$) and from each metamorph sampled from the 2006 cohort ($n = 401$). Toe-clips were stored in Drierite desiccant (W. A. Hammond Drierite Co., Xenia, OH). During the breeding season (late March through early April), the pond was carefully monitored for the presence of egg masses. About 45 egg masses were deposited on April 6th and were sampled within 48 h. We observed no egg mass mortality prior to taking our egg samples. Approximately ten eggs were sampled from each mass

($n = 452$). The eggs were allowed to develop for several days in the laboratory and then preserved in 70% ethanol. To our knowledge, no additional egg masses were deposited in 2006 and thus our sample of eggs included all families for that year. We excluded from our datasets any individual with missing data for one or more microsatellite loci. This resulted in an adult sample of 176, a total egg sample of 415, and a metamorph sample of 308.

The methods of N_b estimation used in this study assume samples are drawn at random (Hill 1981; Waples 1989). By collecting roughly ten eggs from each egg mass, we may have forced allele frequencies estimated from the egg sample to be more similar to the adult frequencies than if the same number of eggs had been sampled randomly from the entire pool of eggs produced in the pond (Waples, personal communication). This could result in an upward bias of the N_b estimates obtained using the egg sample. To avoid this potential bias, we generated a corrected sample of eggs by drawing a random number of individuals from each egg mass (using a Poisson distribution with $\lambda = 4$; random numbers from this distribution ranged from 0 to 10). We generated five of these corrected samples with replacement (n ranged from 156 to 183), estimated N_b separately for each (see methods below), and then calculated the harmonic mean of \hat{N}_b across the five samples. We report the mean, bias-corrected \hat{N}_b values, though we found that these were very similar to the values obtained using the entire sample of ~ 10 eggs per mass.

Microsatellite genotyping and scoring

Total genomic DNA was extracted from each sample using QIAGEN DNeasy kits (QIAGEN Inc.). Each individual was genotyped at seven microsatellite loci (Table 1). PCR amplifications were run in 20 μ l volumes with the following components: 100–200 ng genomic DNA, 25 mM KCl, 1 mM Tris-HCl pH 9, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 μ M both forward (fluorescently-labeled) and reverse primers, 0.01 units/ μ l of Taq, and water to a final volume of 20 μ l. PCR amplifications were carried out in an MJ Research PTC-200 thermal cycler under the following conditions: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, locus-specific annealing temperature (Table 1) for 30 s, 72°C for 30 s, and a final extension of 72°C for 7 min. Microsatellite PCR products were run on an ABI 3730 automated sequencer, and allele sizes were scored using the program GENOTYPER v. 3.7 (Applied Biosystems). The program GENEPOP (Raymond and Rousset 1995) was used to estimate allele frequencies and to test loci for deviations from Hardy–Weinberg equilibrium. We tested all pairs of loci for linkage disequilibrium using the program FSTAT 2.9.3 (Goudet 2002).

Table 1 Microsatellite primer information

Locus	Primer	Annealing temp (°C)	No. of alleles
RP3 ^a	F: 5'-gaaagcaaaactgggaaagtacata-3' R: 5'-cctgagagccatccaataagtcca-3'	50	3
RP22	F: 5'-accccaccagcagaatacaatga-3' R: 5'-agaccagagccagagcaacc-3'	50	3
RP23 ^a	F: 5'-acatagatacaatagatagatagac-3' R: 5'-cacaggaatgtaaaatctggcttc-3'	45	3
RP193 ^b	F: 5'-ccattttctctctgatgtgt-3' R: 5'-tgaagcagatcactggcaaagc-3'	50	2
RP385	F: 5'-attgaaactgcggctctct-3' R: 5'-ggcatgtgtccacaatgtaa-3'	50	2
RP415	F: 5'-aagttcattaagcagatt-3' R: 5'-ggtatatcttaggttacct-3'	45	2
SFC134 ^{b, a}	F: 5'-tgggaaaagactctgtgt-3' R: 5'-aggaaatgtgtggaagcat-3'	55	3

^a Locus used in Funk et al. (2005)

^b Locus used in Mosen and Blouin (2003)

Estimates of census population size

We had extensive mark-recapture data for the 2006 season, which allowed us to obtain estimates of the adult population size (\hat{N}) using Begon's weighted mean method (Begon 1979) and the program CAPTURE (White et al. 1978). Begon's weighted mean is a modification of the simple Lincoln-Peterson estimate that utilizes capture data from >2 trapping occasions. CAPTURE uses maximum likelihood and a model selection procedure to identify the model that best fits the mark-recapture data from among eight possible models. The eight models differ in what variables they include: effects of time on capture, behavioral effects (e.g., "trap-happy" or "trap-shy" behaviors) on capture, and individual variation in capture probability (White et al. 1978). We averaged the estimated population size from the best-fitting model identified by CAPTURE with the estimate obtained using Begon's weighted mean.

Estimates of N_b and N_e

There are several methods of estimating N_e indirectly using genetic data, and the time frame to which an estimate applies depends on the method used as well as the sampling design (Waples 2005). In any case, N_e applies to one or more generations, whereas N_b is the effective number of breeding adults in a single reproductive season that produce a single cohort of offspring. N_e is difficult to derive from N_b for organisms with overlapping generations because this requires extensive demographic information about the population (Jorde and Ryman 1995; Waples 2005). However, low estimates of N_b are generally expected to reflect low N_e (Waples 2005). N_b can be estimated by the same methods used to estimate the overall effective size of a population.

Although the 13 microsatellite loci we developed for *R. pretiosa* (Blouin, unpublished data) were polymorphic when surveyed across the species' range (Blouin 2002), only seven proved to be polymorphic in the Crosswater population and none had more than three alleles at a locus. This low level of genetic diversity precluded the use of kinship and pedigree methods to accurately match offspring with their parents or siblings, preventing direct, pedigree-based estimation of N_b (e.g., Araki et al. 2007; Blouin 2003). Consequently, we estimated N_b only through indirect genetic methods.

We compared the estimate of the actual number of breeders obtained from an egg mass count (\hat{N}_{ab}) to several \hat{N}_b values obtained from genetic data. The first \hat{N}_b is from a version of the temporal method that uses the differences in allele frequencies between a sample from the adult population and a sample from their offspring (Scribner et al. 1997). We calculated two \hat{N}_b values: one based on allele frequency differences between the adult and egg samples, and another based on allele frequency differences between the adult and metamorph samples. As noted above, each adult-egg estimate of N_b that we report represents the harmonic mean of five random samples generated from the total egg dataset. See Table 2 for notation used.

The first temporal method we used was Waples' (1989) moment-based approach (TM). The standardized variance of allele frequency change for each locus was calculated using Eq. (9) from Waples (1989):

$$\hat{F}_c = \frac{1}{K-1} \sum_{i=1}^K \frac{(x_i - y_i)^2}{(x_i + y_i)/2} \quad (1)$$

where K is the total number alleles at the locus, x_i is the frequency of allele i in the first sample, and y_i is the frequency in the second sample. The mean \hat{F}_c across all seven loci was calculated as:

Table 2 Notation used

N	Actual number of individuals in the population; population census size
N_{ab}	Actual number of breeding adults
N_e	Effective population size
N_b	Effective number of breeding adults in one reproductive season
$\hat{N}, \hat{N}_{ab}, \hat{N}_e, \hat{N}_b$	Estimates of $N, N_{ab}, N_e,$ and N_b
TM	Waples' (1989) temporal moment method of estimating N_e or N_b
TL	Temporal likelihood method of N_e (N_b) estimation from Berthier et al. (2002)
LD	Linkage disequilibrium method of N_e (N_b) estimation
$\hat{N}_b^{\text{TM-Egg}}$	Temporal moment method estimate of N_b , using the adult and egg samples
$\hat{N}_b^{\text{TM-Meta}}$	Temporal moment method estimate of N_b , using the adult and metamorph samples
$\hat{N}_b^{\text{TL-Egg}}$	Temporal likelihood method estimate of N_b , using the adult and egg samples
$\hat{N}_b^{\text{TL-Meta}}$	Temporal likelihood method estimate of N_b , using the adult and metamorph samples
$\hat{N}_b^{\text{LD-Egg}}$	LD method estimate of N_b from LDNE program, using the egg sample
$\hat{N}_b^{\text{LD-Meta}}$	LD method estimate of N_b from LDNE program, using the metamorph sample

$$\text{mean } \hat{F}_c = \sum (K_j - 1) F_{c_j} / \sum (K_j - 1) \tag{2}$$

where K_j is the number of alleles at locus j and F_{c_j} is the estimate of F_c for locus j . Confidence intervals for mean \hat{F}_c were calculated using Eq. (16) from Waples (1989). Because our first sample was collected non-destructively from adults, Waples' (1989) Plan I was the appropriate sampling design. The estimated effective number of breeders was therefore calculated using Eq. (12) from Waples (1989):

$$\hat{N}_b = \frac{t}{2 \left[\text{mean } \hat{F}_c - \frac{1}{2S_o} - \frac{1}{2S_t} + \frac{1}{\hat{N}} \right]} \tag{3}$$

where S_o and S_t are sample sizes for the first and second samples, respectively, t is number of generations between the two samples (1 in this case), and \hat{N} is the census estimate of the total size of the population from which the S_o sample was drawn (see above for how we obtained \hat{N}). We designated the adult-egg and adult-metamorph estimates of N_b from this method as $\hat{N}_b^{\text{TM-Egg}}$ and $\hat{N}_b^{\text{TM-Meta}}$, respectively.

The second temporal approach we used to estimate N_b was the likelihood-based estimator (TL) of Berthier et al. (2002), implemented in the program TM3. The TL method involves the calculation of likelihoods from coalescent-based gene genealogies and Markov chain Monte Carlo sampling to generate a posterior probability distribution of N_e , or in our case, N_b . We designated the adult-egg and adult-metamorph estimates of N_b from this method as $\hat{N}_b^{\text{TL-Egg}}$ and $\hat{N}_b^{\text{TL-Meta}}$, respectively. A maximum possible N_b value is specified as a Bayesian prior in TM3. Although we did not expect maximum \hat{N}_b to be greater than about 90 frogs (based on the egg mass count), we ran several independent TM3 runs using priors of 200, 300, 400, and 1000 for maximum N_b . We set our lowest prior conservatively at

200 to account for the possibility that extra-pair fertilization (i.e., multiple fathers per egg mass) could result in N_b greater than \hat{N}_{ab} . Performing analyses with different priors allowed us to evaluate the sensitivity of $\hat{N}_b^{\text{TL-Egg}}$ and $\hat{N}_b^{\text{TL-Meta}}$ to choice of prior. All TM3 analyses were run with 50,000 iterations.

In addition to the two temporal methods, we used the linkage disequilibrium (LD) method to estimate N_b from single samples of eggs and of metamorphs. We designated the N_b estimates from the LD method as $\hat{N}_b^{\text{LD-Egg}}$ for the egg samples and as $\hat{N}_b^{\text{LD-Meta}}$ for the metamorph sample. Calculations were performed using the program LDNe (Waples and Do 2007). LDNe incorporates a correction for the bias that is introduced when sample size is less than the true effective size and reports confidence intervals obtained via a new jackknife method (Waples 2006). The mating model for this system is equivalent to monogamy and was selected in the LDNe analyses. We report jackknife confidence intervals for \hat{N}_b , with the lowest allele frequency set at 0.05. By excluding alleles with frequencies <0.05, we achieve the most accurate N_b estimate, with an expected tradeoff in precision (Waples and Do 2007). However, even when we ran our analyses with the lowest allele frequency set at 0.01, confidence intervals were very similar to those obtained when the lowest frequency was set at 0.05.

Finally, we used the LD method to estimate N_e (as opposed to N_b) in the adult sample ($n = 176$), under a random mating model in LDNe. We acknowledge that there is some uncertainty about how to interpret LD estimates from mixed-cohort samples from species that have overlapping generations (Waples 1991). However, the LD method has become standard for estimating N_e from such samples (e.g., Aspi et al. 2008; Durrant et al. 2008), so our data should still be useful for comparative purposes.

Results

Genetic diversity

Expected heterozygosity (H_e) for the seven microsatellite loci in this *R. pretiosa* population was 0.40 as calculated from the adult sample. The maximum number of alleles per locus was three. Only one locus in one sample (RP3 in the metamorph sample) was barely out of Hardy–Weinberg equilibrium ($P = 0.0071$; Bonferroni-corrected nominal value of 0.00714). We found one locus pair (RP3 × RP385) with barely significant linkage disequilibrium in the adult sample ($P = 0.00238$; Bonferroni-corrected nominal value of 0.002381). About six pairs of loci in the metamorph sample and three to four pairs in each of the five random egg samples exhibited significant linkage disequilibrium (data not shown).

Estimates of census population size

The two methods of population size estimation yielded very similar results. Begon’s weighted mean method gave \hat{N} of 444 (95% C.I. 343–545). CAPTURE identified the M_t model as the most appropriate for our mark-recapture data. Under this model, each individual has the same probability of capture on a given trapping occasion, but these probabilities are variable across trapping occasions (White et al. 1978). \hat{N} from the CAPTURE analysis was 412 (95% C.I. 343–513). The average of the two \hat{N} values is 428 (95% C.I. 343–529).

Estimates of N_b and N_e

Point estimates of N_b from the TL analysis using the program TM3 were insensitive to the value of Bayesian prior for maximum N_b (Table 4). As one might expect, the upper confidence limit did increase with increasing prior. However, even if extra-pair fertilization was rampant in this population, such that the number of breeding males was more than twice the number of breeding females, maximum N_b should not exceed 200. Thus, using 200 as the upper prior for our reported values (Table 3) probably produced overly liberal upper confidence intervals, even if we are confident in the point estimates.

Estimates of N_b are presented in Table 3. Doubling the number of egg masses found in the 2006 breeding season resulted in an estimate of 90 breeding adults ($\hat{N}_{ab} = 90$). The temporal methods (TM and TL) yielded similar point estimates of N_b for the adult-egg ($\hat{N}_b^{TM-Egg} = 65.0$, $\hat{N}_b^{TL-Egg} = 87.3$) and adult-metamorph comparisons ($\hat{N}_b^{TM-Meta} = 82.5$, $\hat{N}_b^{TL-Meta} = 117.2$). Estimates of N_b from the LD method were also very similar between the two life stages ($\hat{N}_b^{LD-Egg} = 68.5$, $\hat{N}_b^{LD-Meta} = 56.2$). Thus,

Table 3 Estimates of effective number of breeders (N_b) and N_b/N in the CW population

Method		Estimate	95% C.I.	N_b/N
<i>Temporal methods</i>				
<i>Adult-egg</i>				
TM	\hat{N}_b^{TM-Egg}	65.0	18–195	0.15
TL	\hat{N}_b^{TL-Egg}	86.4	20–200	0.20
<i>Adult-metamorph</i>				
TM	$\hat{N}_b^{TM-Meta}$	82.5	23–252	0.19
TL	$\hat{N}_b^{TL-Meta}$	117.2	27–200	0.27
<i>Linkage disequilibrium methods</i>				
<i>Egg</i>				
LD	\hat{N}_b^{LD-Egg}	68.5	30–108	0.16
<i>Metamorph</i>				
LD	$\hat{N}_b^{LD-Meta}$	56.2	26–108	0.13

Estimates are given for two temporal methods: Waples’ (1989) temporal moment (TM) and the temporal likelihood method (TL) of Berthier et al. (2002). Estimates from the linkage disequilibrium method were obtained using LDNE (Waples and Do 2007). Estimates from these various methods are listed along with their 95% confidence intervals (Bayesian credible intervals for the TL estimates) and N_b/N ratios

Table 4 Harmonic means of \hat{N}_b values for four choices of Bayesian prior for maximum N_b

Prior	Harmonic mean	Lower C.L.	Upper C.L.
200	86.35	20	200
300	83.37	17	280
400	91.04	19	352
1000	88.61	15	519

Means were calculated from the five bias-corrected egg samples for each prior

we see (1) point estimates from the egg stage (65.0, 87.3, and 68.5) that are fairly close to the simple estimate of 90 breeding adults, and (2) no indication of a massive drop in N_b in going from the egg to metamorph stage.

The LD estimate of N_e in the adult sample was 36.7 (95% C.I. 19–71.9). Thus, the best point estimate of N_e/N for this population = $36.7/428 = 0.086$.

Discussion

Estimates of N_b/N across the various methods ranged from 0.13 to 0.27 (Table 3). These values are similar to those found for *Rana temporaria* populations in Finland (0.06–0.17; Schmeller and Merila 2007) and Britain (0.333–0.365; Brede and Beebee 2006), but considerably higher than those of toad (*Bufo bufo*) populations in Britain (0.007–0.012 Scribner et al. 1997; 0.034–0.040 Brede and

Beebe 2006). The N_e/N ratio estimated for the adult population was 0.086, which again is in the general range of DNA-based estimates for other ranid frogs (Hoffman et al. 2004; Schmitter and Merila 2007). Thus, our data are consistent with previous suggestions that N_e/N ratios in ranid frogs are in the typical range for vertebrates (e.g., ~ 0.1 – 0.4), while those for bufonids are much lower (Hoffman et al. 2004; Brede and Beebe 2006).

Our main objective was to test a hypothesis about what features of the life cycle of *R. pretiosa* cause N_e to be reduced relative to N . By obtaining separate N_b estimates using egg and metamorph samples we could determine if these N_b estimates differed from each other and from the simple estimate from counting egg masses (\hat{N}_{ab}). To our knowledge, this study is the first to take such an approach. We found that: (1) Estimates for N_b at the egg stage using both temporal methods and the LD method did not differ dramatically from $\hat{N}_{ab} = 90$; and (2) estimates for N_b were similar for eggs and metamorphs (Table 3). The first result is consistent with what we would expect to find if each female produced a single egg mass, each egg mass was fertilized by a single male, and each male bred with only one female. The second result suggests little non-random (family-based) mortality occurred between egg laying and metamorphosis.

The first result was expected because, like many ranid frogs, *R. pretiosa* females are thought to lay one egg mass per season (Olson and Leonard 1997) and explosive breeding reduces the opportunity for males to mate with multiple females (Wells 1977). Indeed, in this year all breeding occurred on a single night. On the other hand, sex ratios in breeding populations are male-biased, which could promote extra-pair fertilization, where some egg masses are fertilized by more than one male. This could occur either passively by free-swimming spermatozoa in communal breeding areas (Laurila and Seppa 1998) or actively by lone ‘pirate’ (or ‘sneaker’) males that fertilize some of the eggs of breeding pairs (Vieites et al. 2004). If extra-pair fertilization was frequent, the resulting decrease in the variance of male reproductive success would increase N_b estimates at the egg stage relative to \hat{N}_{ab} (Sugg and Chesser 1994). In our observations of hundreds of breeding pairs of spotted frogs over multiple years in this population, we have witnessed few instances of behavior that would suggest the occurrence of ‘clutch piracy’. Thus, we interpret our results as consistent with predictions of a basically monogamous mating system in which each female lays a single clutch per year. One practical consequence of these results is that they support the use of egg mass counts as a cost-effective method of population monitoring, in that they probably do give a reasonable estimate of the number of adults that bred in a given year. Whether egg mass counts can consistently provide reliable estimates of N_b

depends on how typical are the results that variance in family size apparently increased little after egg laying.

If our results for this population in 2006 are typical for *R. pretiosa*, then the reduction of N_e relative to N in this species is not owing to the inflation of variance in family size that occurs between the egg and metamorph stages. Thus, we might consider other factors such as year-to-year fluctuations in population size. Of course, our results are from one year in a single population and may not be typical. Water levels in the pond were very high in 2006, which may have contributed to unusually high survival of entire egg masses. Such an environmental effect was also noted by Schmitter and Merila (2007), who suggested that high egg-to-metamorph mortality during a short growing season may have been responsible for low N_e/N ratios in two populations of *R. temporaria*. Together, these observations suggest the interesting hypothesis that the N_b/N ratio varies from year to year (or from population to population) depending on habitat quality. Indeed, N_b/N and N_e/N might even be predictable from environmental measurements.

This study is the first attempt to determine where in the ranid life cycle N_b (and by extension, N_e) is reduced. More studies will be needed before a consensus is reached about the importance of different factors. Here we provide some of the first data on the subject, and suggest the hypothesis that N_b/N might vary substantially in time and space owing to habitat conditions that influence the survival of eggs and larvae. The approach of estimating N_b using genetic data from a single cohort at more than one life stage should prove valuable in future studies on the determinants of effective population size in amphibians and other taxa.

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