

Polygyny, census and effective population size in the threatened frog, *Rana latastei*

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Abstract

Effective population size (N_e) is a key determinant of genetic diversity of populations. In amphibians, the ratio effective population size/census size (N_e/N) is often very low, raising concerns for the long-term persistence of genetic diversity in isolated populations. It has been proposed that the phenomenon of 'genetic compensation' increases the ratio N_e/N in small populations, but the underlying mechanisms are poorly understood. Polygyny can decrease N_e/N because of the negative relationship between polygyny and N_e . We used genetic information (microsatellites) to evaluate the relationship between census size, polygyny and N_e in populations of the threatened Italian agile frog *Rana latastei*. We reconstructed parentage in tadpoles from nine populations with eight to 32 breeding females, using a likelihood-based method; we analysed simulated datasets with known properties to confirm the reliability of this approach in reconstructing polygyny. Furthermore, we estimated N_e using approximate Bayesian computation. The level of polygyny differed strongly among populations (average number of mates per breeding male: 2–6.4). Polygyny was greater in populations with larger census sizes. Moreover, variance in male mating success was larger in large populations. Effective population size increased with population size, but was negatively related to polygyny; as polygyny increased in large populations, this was associated with reduced N_e/N . In polygynous species, increasing levels of polygyny in large populations may explain the low N_e/N values, with important implications for the conservation of genetic diversity and for long-term population persistence.

Introduction

Effective population size (N_e), a basic parameter of population genetics, is important because it determines largely the amount of genetic variation that can be maintained in a population. Effective population size is equivalent to the census size, N , of an ideal population in which mating is at random and every individual has the same chance to reproduce. In real populations, however, deviations from this ideal situation may result in a reduction in N_e and thus, an increased loss of genetic variation (Frankham, Ballou & Briscoe, 2002). Assuming a negative relationship between genetic erosion and the probability of population persistence, decreasing values for N_e or the ratio N_e/N should increase the risk of loss of population-averaged fitness and possible population extinction through inbreeding (Frankham *et al.*, 2002; Spielman, Brook & Frankham, 2004; O'Grady *et al.*, 2006; Ficetola, Garner & De Bernardi, 2007; Palstra & Ruzzante, 2008). As such, small N_e or N_e/N

values are considered to be indications of the potential for loss of genetic variability within a population; the assessment of N_e in wild populations, in conjunction with population size estimates, is an extremely valuable tool for the conservation management of populations.

Empirical studies of wild populations have shown that N_e is often much smaller than the population census size, and there is large variation of N_e/N in nature (Frankham, 1995; Palstra & Ruzzante, 2008). In amphibians, N_e/N can be particularly small; values of N_e/N are often described well below 0.1 (Scribner, Arntzen & Burke, 1997; Rowe & Beebee, 2004; Jehle *et al.*, 2005; Schmeller & Merilä, 2007; Palstra & Ruzzante, 2008). Metapopulation dynamics and concurrent gene flow can maintain N_e (Matocq, 2004; Schmeller & Merilä, 2007) and amphibian populations often exhibit metapopulation dynamics or are maintained through post-metamorphic dispersal (see Smith & Green, 2005). Thus, anthropogenic habitat alteration and landscape fragmentation that reduces connectivity among populations and associated gene flow may directly and negatively affect N_e (Hitchings & Beebee, 1997; Johansson *et al.*, 2005; Cushman, 2006; Ficetola *et al.*, 2007).

[Correction added after online publication, 30 November 2009: addition of '*Rana latastei*' to article title].

At the population level, several factors can influence the N_e/N ratio: periodic fluctuations in census size, an unequal sex ratio and high variance in reproductive success (Frankham, 1995). Several researchers have observed that N_e/N may often be larger in small populations (Palstra & Ruzzante, 2008). This phenomenon is referred to as genetic compensation (Ardren & Kapuscinski, 2003), and has been described in small populations of several species from various taxonomic lineages, including insects, fish and amphibians (Rowe & Beebe, 2004; Jehle *et al.*, 2005; Watts *et al.*, 2007, reviewed in Palstra & Ruzzante, 2008). An inverse correlation between N_e/N ratio and N can be caused by increasing the standardized variance in family size (Hedrick, 2005) or by increasing the variance in breeding success (Ardren & Kapuscinski, 2003) as N increases, but the mechanisms underlying genetic compensation may be complex and are not completely understood (Palstra & Ruzzante, 2008). Nevertheless, genetic compensation can have important consequences for the maintenance of genetic diversity in small populations and a greater understanding of the mechanisms of genetic compensation provides important information for the conservation of population genetic diversity.

Ecological and demographic features can strongly affect mating systems (Lott, 1991), which, in turn, influences the number of breeding adults (N_b), their reproductive success and N_e (Anthony & Blumstein, 2000). Both polygyny and polyandry are frequently reported for amphibians (Jones, Adams & Arnold, 2002; Lodé & Lesbarrères, 2004; Vieites *et al.*, 2004; Liebgold *et al.*, 2006; Broquet, Jaquière & Perrin, 2009), and in many of these studies, skewed male reproduction has been detected. Increased polyandry and/or polygyny associated with reproductive skews will reduce N_b and N_e , through increased variance in reproductive success of either sex (Frankham, 1995; Anthony & Blumstein, 2000). However, assessing individual reproductive success in wild populations of amphibians has proved challenging, because of the sheer numbers of adults present at breeding assemblages. Fortunately, genetic markers can allow reliable reconstruction of parentage (Garant & Kruuk, 2005), and therefore provide unique information on the actual levels of polygyny and N_b in wild populations; but the role polygyny plays in determining the relationship between N , N_b and N_e has received little attention in wild populations of endangered species (but see Broquet *et al.*, 2009; also see Anthony & Blumstein, 2000; Kaeuffer *et al.*, 2007 for theoretical and simulation analyses).

In this study, we used microsatellite DNA markers to reconstruct parentage of offspring sampled from populations of the threatened Italian agile frog *Rana latastei* [Vulnerable, B2ab (iii), <http://www.iucnredlist.org/>]. *Rana latastei* exists in small, isolated populations threatened through increasing fragmentation and exploitation of terrestrial habitat (Ficetola & De Bernardi, 2004, 2005). Previous work has shown that population genetic variability can be severely depleted in this species (Garner, Angelone & Pearman, 2004), and that population isolation as well as historical processes contribute to the pattern observed (Ficetola *et al.*, 2007). To date, the role population size and mating system have played in determining

population genetic structure has not been assessed; research suggests mating system should influence genetic structure (Hettyey & Pearman, 2003). In this study, we develop and utilize marker information to infer parentage, which along with estimates of population size derived from clutch counts, is used to estimate the mating success of males, and test two hypotheses that can explain low N_b/N and N_e/N in larger populations: (1) polygyny is greatest in larger populations; (2) the variance of male reproductive success is positively related to population size. We followed this by estimating N_e for our study populations and evaluated if variation of N_e with respect to population size mapped with male reproductive patterns.

Methods

Rana latastei is a small brown frog endemic of floodplains of northern Italy and adjacent countries (Fig. 1). *Rana latastei* is threatened by multiple factors, including loss of habitat, increased isolation of populations and loss of genetic diversity (Ficetola & De Bernardi, 2004; Pearman & Garner, 2005; Barbieri & Mazzotti, 2006; Ficetola *et al.*, 2007). It is considered globally vulnerable by the IUCN (Andreone, Garner & Schmidt, 2004). *Rana latastei* exhibits explosive breeding behaviour, mating only lasts several hours and each female produces a single egg mass (*c.* 1200 eggs clutch⁻¹, Barbieri & Mazzotti, 2006; Seglie *et al.*, 2008). Clutches are globular, hold their shape and are deposited onto submerged tree roots and sticks within 1 m of the water surface (Barbieri & Mazzotti, 2006). Thus, they are easily spotted and recognized and correspond to the



Figure 1 Distribution of study populations, within the range of *Rana latastei*. Distribution range redrawn from Andreone *et al.* (2004).

number of breeding females. Clutch counts, a standard census method for brown frogs, are therefore a reliable means to census the number of breeding females (see Salvidio, 2009): analyses on the closely related *Rana dalmatina* showed that clutch counts correspond exactly to the total number of females in a pond (Lodé, Holveck & Lesbarrères, 2005). Multiple paternity in a *R. latastei* clutch has not been described; therefore, each clutch was assumed to be a full-sib family. Some studies have described multiple paternity in other European brown frog species (*R. dalmatina* and *Rana temporaria*, respectively, Lodé & Lesbarrères, 2004; Vieites *et al.*, 2004), but the proportion of offspring attributable to polyandrous mating was small for both species. In *R. dalmatina*, 18% of clutches were sired by two males, and the secondary male sired only 24% of eggs, that is, 4% of all eggs are sired by a secondary male (Lodé & Lesbarrères, 2004). In *R. temporaria*, clutch piracy affected 84% of clutches, but pirate males fertilized only 26% of pirated clutches, and in these clutches 24% of embryos were fertilized by pirates, that is, 5% of all eggs were sired by a secondary male (Vieites *et al.*, 2004). Even if multiple paternity was present for *R. latastei*, we estimate no more than two sampled embryos per population would be from extra-pair insemination, adding only 5% error to our estimates. Thus, ignoring the possibility of multiple paternity would not have significantly affected our study.

During the breeding season (March 2001, 2003 and 2004), we collected a small portion of eggs from all egg masses from eight populations (ponds) with different numbers of clutches (Table 1). Sampled populations covered a large proportion of the species' range (Fig. 1). Further details on populations are reported elsewhere (Garner *et al.*, 2004; Ficetola *et al.*, 2007); here, we included only populations for which all clutches were sampled. Population CU was sampled both in 2003 and 2004, and we included both samples separately in the dataset. We estimated the number of adults per population (N) assuming a sex ratio of 1.5 (i.e. $N = N \text{ clutches} \times 2.5$; Barbieri & Bernini, 2004). This is the average of estimates of field sex ratios during the post-breeding

season from three studies (range: 1.2–1.7). All studies have always found a male-biased sex ratio in *R. latastei*. To address potential variation among populations, we also estimated N using the maximum and minimum values of sex ratio reported in the literature. As a small proportion of adult females may not breed in a given year, these values may underestimate N . Eggs were reared to hatch and a single larva per clutch was euthanized for DNA extraction. Embryos were genotyped at six microsatellite loci (Garner & Tomio, 2001). We used GENETIX (Belkir, 2004) to calculate measures of genetic variability of populations (expected heterozygosity and average number of alleles per locus). Genotyping procedures and further details on populations are described in other studies (Garner *et al.*, 2004; Ficetola *et al.*, 2007).

Estimation of polygyny

We used COLONY, a likelihood-based method, to reconstruct the half-sibling groups of sampled tadpoles using their multilocus genotypes (Wang, 2004). We assumed the rate of genotyping error to be 2% (Ficetola *et al.*, 2007). As we sampled only one embryo per clutch, we assumed that all embryos had different mothers but that different embryos may have been sired by the same male. We performed three runs per population using different seeds for the random number generator and in all cases, obtained the same family structures. In some populations, up to four microsatellite markers were monomorphic (Garner *et al.*, 2004; Ficetola *et al.*, 2007). To account for these differences in genetic information among populations and determine if they affected our results, we used a jack-knife procedure. To do this, we removed each microsatellite marker from the dataset in turn, reanalysed the data for the remaining five microsatellites, repeating this procedure for all markers.

We examined the reconstructed sibships to assess the number of clutches sired by each male. Embryos that did not have half-sibs were treated as the offspring of males that sired only one clutch; similarly, we assessed the number of

Table 1 Summary statistics of the sampled populations

Population	N clutches	NPL	Ar	H_E	Polygyny	Var. success ^a	N_e (95% CL)	N_e/N
VM	16	6	4.17	0.501	2.3	1.6	15.6 (12.1–24.6)	0.39 (0.36–0.44)
CO	20	5	2.33	0.221	4.0	5.7	17.4 (12.1–28.3)	0.35 (0.32–0.40)
A1	24	5	2.67	0.232	4.0	13.2	23.3 (15.0–41.0)	0.39 (0.36–0.44)
CU_2003	8	3	1.67	0.180	2.0	2.0	13.5 (7.9–28.4) ^b	0.47 (0.44–0.54)
CU_2004	20	3	1.67	0.186	3.3	5.8		
AL	32	3	2.17	0.237	6.4	6.8	17.8 (11.0–38.8)	0.22 (0.21–0.25)
T1	30	2	2.00	0.181	6.0	7.5	23.5 (11.7–54.1)	0.31 (0.29–0.36)
T2	28	2	0.17	0.166	5.6	12.3	17.2 (8.3–36.0)	0.25 (0.23–0.28)
T3	25	2	1.50	0.169	6.3	12.3	8.6 (4.7–19.5)	0.14 (0.13–0.16)

N_e/N is estimated assuming a sex ratio = 1.5 (see 'Methods'); in parentheses, values calculated assuming the maximum and minimum sex ratio values observed for the species (minimum = 1.2; maximum = 1.7) (Barbieri & Bernini, 2004).

^aVariance of breeding success of reproductive males, calculated as the variance in number of matings per male.

^bFor population CU, N_e estimated by pooling data of 2003 and 2004; average N calculated using harmonic mean.

NPL, number of polymorphic loci; Ar, allelic richness averaged across all six loci; H_E , expected heterozygosity; CL, 95% credible limits.

clutches sired by each polygynous male by identifying all the half-sibling embryos. As we sampled all clutches in each population, this approach allowed us to estimate how many clutches are sired by each breeding male. We calculated the population-level polygyny as the average number of clutches sired by reproductive males. Similarly, we calculated the population-level variance in reproductive success as the variance of the number of clutches sired by breeding males, in each population. We used Spearman's rank correlation r_s to evaluate bivariate relationships among variables (N , population-level polygyny, variance in reproductive success). We used Spearman's correlation as it is more robust than Pearson's correlation and more appropriate when relationships are not expected to be strictly linear (Sokal & Rohlf, 1995).

COLONY shows good performance in reconstructing parentage when using a large number of variable microsatellites (≥ 6 , Wang, 2004). However, several endangered species, including *R. latastei*, suffer from limited genetic variability (Gebremedhin *et al.*, 2009). To address the potential shortcomings of our limited number of markers in parentage reconstruction, we used simulations to test whether the limited number of variable markers might bias the estimation of polygyny. We generated the simulated data mimicking the real data in the amount of marker information (number of markers and polymorphisms) and in the level of polygyny. We considered three polygyny levels (two, four, six females per breeding male), and three levels of marker information (two markers with five and three alleles; four markers with four, three, three, three alleles; six markers with nine, five, three, three, three, two alleles), assuming an even allele frequency. We generated 1000 replicate datasets per each combination of parameters. We then analysed the simulated data using COLONY, to see whether the simulated parameter values (number of females mated per male) are recovered or not.

Estimation of N_e

We used ONeSAMP to estimate short-term N_e , as inferred from single time samples. ONeSAMP is a recently developed method, estimating N_e and 95% credible limits (CL) using approximate Bayesian computation (Tallmon *et al.*, 2008). This method generates 50 000 simulated populations with N_e between an upper and a lower limit specified by the prior. For each simulated population, ONeSAMP draws samples with number of individuals and loci identical to those of the test datasets. N_e of simulated populations with summary statistics close to the test population were accepted and used to infer N_e of the target populations (Tallmon *et al.*, 2008). As a prior, we assumed $2 < N_e < 100$; runs with different priors, assuming maximum N_e proportional to the number of clutches observed, gave very similar results (difference in estimated $N_e \leq 1$). For these analyses data collected during the 2 years for population CU were pooled; we used the harmonic mean of N to estimate the average N over the 2 years (Rowe & Beebee, 2004) because generation interval in *R. latastei* is ≥ 2 years (Guarino *et al.*,

2003; Barbieri & Bernini, 2004). Subsequently, we used multiple linear regressions to evaluate the relative role of N and polygyny in explaining effective population size of populations.

Results

Polygyny and variance in reproductive success

COLONY converged to a single, maximum likelihood solution for all populations. We observed a strong variation among males for the number of mates; reproductive males fertilized one to 11 clutches. Furthermore, the degree of polygyny differed strongly among populations and ranged from 2 to 6.4 (Table 1) and increased significantly with increasing population size ($r_s = 0.94$, $N = 9$, $P < 0.001$) (Fig. 2a). Jackknifing showed these results were robust, as each marker

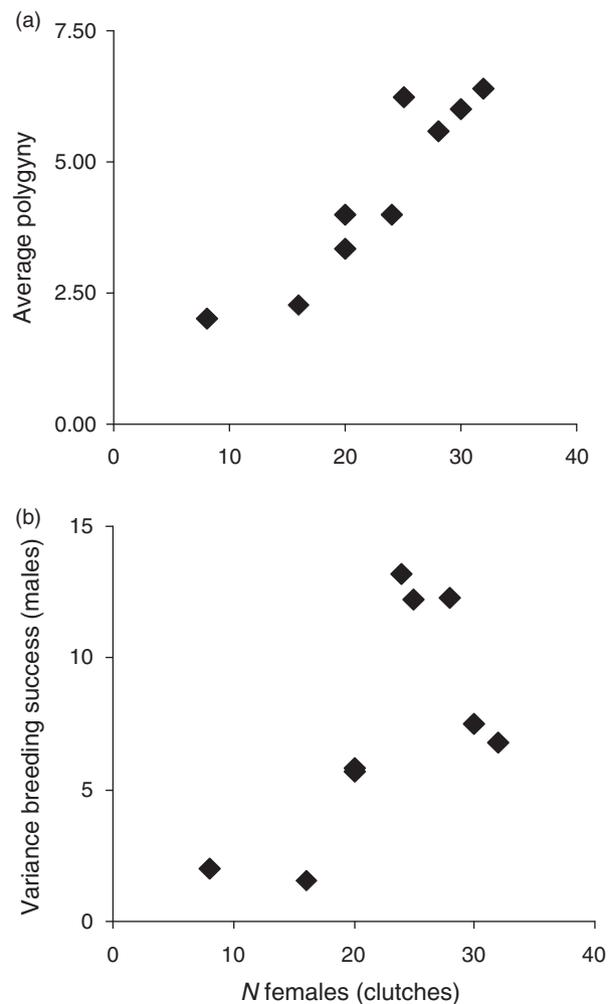


Figure 2 Bivariate relationship between the number of breeding females and (a) population-level polygyny and (b) variance in reproductive success of males, across *Rana latastei* populations.

removal returned equivalent correlations between N and degree of polygyny ($0.83 \leq r_S \leq 1$, all $P < 0.006$). Variance in male reproductive success (calculated as the variance among males in number of matings) increased with population size ($r_S = 0.66$, $N = 9$, $P = 0.05$, Fig. 2b). This positive relationship between population size and variance in male reproductive success was to a large extent confirmed by jack-knifing datasets ($0.49 \leq r_S \leq 0.80$, $P \leq 0.05$ for three of six correlations), suggesting that these results are robust.

Results of simulations

Although a small number of variable loci (2–4) sometimes led to imperfectly reconstructed family structures, they still provided sufficient information to reliably estimate the level of polygyny (Fig. 3). When only two loci were available, the estimated polygyny tended to be higher than the true value (Fig. 3a); this occurred because some unrelated tadpoles may have similar genotypes at the two loci by chance and are thus incorrectly classified as half siblings. Nevertheless, this possible bias did not affect the results of our analysis, as the relationship between population size, polygyny and variance in reproductive success remained unchanged if populations with only two variable microsatellites (T1, T2, T3) are excluded from the analysis (correlation N females – polygyny: $r_S = 0.96$, $P = 0.003$; correlation N females – variance: $r_S = 0.75$, $P = 0.08$).

Estimates of N_e

ONEsAMP estimated finite N_e for all populations; N_e obtained using ONEsAMP ranged between 8.6 and 23.5. For all populations, the estimated N_e was smaller than N , but the credible limits were large (Table 1). N_e was positively related to population size, but not significantly so ($r_S = 0.52$, $N = 8$, $P = 0.18$). For all populations, N_e/N was < 1 ; despite the weak positive relationship between N_e and N , N_e/N tended to be smaller in large populations ($r_S = -0.79$, $N = 8$, $P = 0.02$) (Table 1, Fig. 4). Multiple linear regression relating N_e to N and polygyny explained a large amount of variation in N_e ($R^2 = 0.63$). Effective population size was positively related to the adult population size ($F_{1,5} = 8.5$, $P = 0.03$); when population size was controlled, N_e was

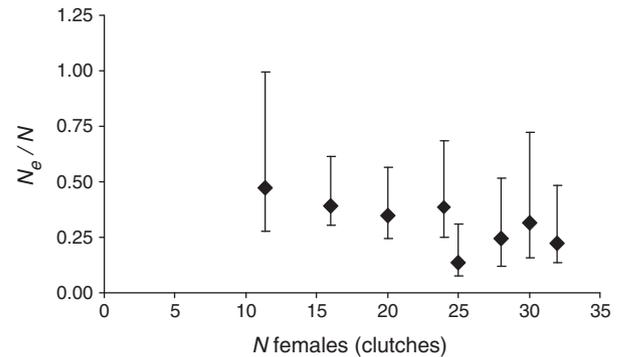


Figure 4 Relationship between the number of breeding females and the ratio N_e/N , across *Rana latastei* populations. Error bars represent 95% Bayesian credible limits.

smaller in populations with higher levels of polygyny ($F_{1,5} = 6.5$, $P = 0.05$).

Discussion

Population size is generally treated as an indicator of the probability of population persistence; the larger the population, less prone it should be to extinction through stochastic and other processes. This perspective is not as entrenched in amphibian conservation, where population extinction is commonly accepted as a component of amphibian metapopulation dynamics and the measure of species 'health' may better be increasing site occupancy in a landscape rather than large size populations at any one location (Marsh, 2008). Nevertheless, the goal of amphibian conservation, for the most part, is population viability across a landscape, and the probability of persistence of a population within a patch is still explicitly linked to the number of individuals that occur in the population (Hels & Nachman, 2002). The rate of population loss is expected to accelerate when population sizes are generally smaller (Lande, Engen & Sæther, 2003), and small populations are also commonly assumed to be more likely to exhibit decreased population genetic variation (Frankham *et al.*, 2002). However, our results show that increasing population size is associated with increased variance of male breeding success, increased monopolization of females by highly polygynous males and a

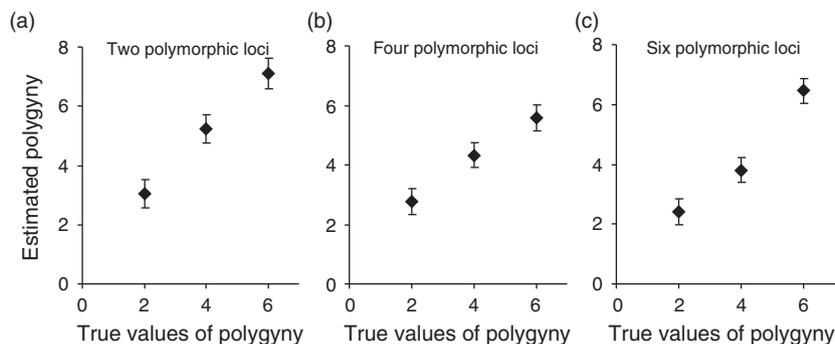


Figure 3 Reconstruction of polygyny levels using simulated data (a) two, (b) four and (c) six markers compared with known polygyny levels (two, four and six mates per male). Error bars are the coefficient of variation of the mean.

concurrent decrease in N_e/N . Palstra & Ruzzante (2008) urge caution in interpreting results when plotting N_e/N against its denominator; however, the fact we have measured similar patterns for both population-level polygyny and variance in male reproductive success means that the N_e/N and N relationship are unlikely to be because of a spurious correlation.

Our analysis suggests a joint effect of census size and polygyny on N_e ; we observed larger N_e in populations with large N and weak polygyny. However, the present-day N_e clearly depends on levels of N and polygyny experienced by populations in the past; relationships observed using present-day parameters may reflect both recent and historical processes. A relationship between short-term N_e and current demographic parameters (polygyny, census size) might therefore seem surprising, as amphibian populations can certainly fluctuate broadly in time (Pechmann & Wilbur, 1994). Analyses of time series have revealed significant autocorrelation and convergence towards demographic stability in *R. latastei* populations (Salvidio, 2009). The small size of many populations is mostly caused by a lack of suitable habitat, as shown by rapid population rebounds when management increases habitat availability (Ficetola & De Bernardi, 2005; G. F. Ficetola, unpubl. data). Demographic stability and dependence on habitat features might explain the strong relationship between N_e and the present-day parameters. Furthermore, although the N_e estimated by ONeSAMP from six summary statistics is vaguely an average effective size over some undefined number of generations before the sampling point, it is most strongly affected by the recent N_e .

The increased polygyny that we detected in large populations (Fig. 2) affected N_e (Anthony & Blumstein, 2000), suggesting that, in larger populations, a few, successful males have the ability to monopolize either access to females or sites where females choose to mate and oviposit. The breeding phenology of *R. latastei* and many other temperate amphibian species involves males arriving first to the breeding site followed by a more staggered female arrival pattern (Lodé *et al.*, 2005). Males are afforded some opportunity to interact before all females have come to the pond (Seglie *et al.*, 2008), and this may result in certain males achieving dominance over others, or exposing weaker males to male–male interactions that may cause them to be less effective at acquiring females and maintaining amplexus when other males compete for females. Two of us (G. F. F. and T. W. J. G.) have observed multiple males attempting amplexus with single females. Also, *R. latastei* clutches are not widely distributed across a breeding site. Instead, egg clutches tend to be closely clustered on emergent tree roots and branches at pond verges; it is not uncommon to see several clutches attached to a single branch trailing in the water (Pozzi, 1980). Whatever the mechanism, it appears that there is more opportunity for male–male competition in larger populations and further behavioural studies are needed to confirm this hypothesis.

Loss of genetic variation in populations of any size may or may not be of concern, as genetic stochasticity is thought only to become an issue when populations are both small and lacking the benefits of gene flow (Palstra & Ruzzante,

2008). Hence, are our results important for the conservation of *R. latastei*? While some populations of this frog are large (> 100 adult frogs), predominantly in the eastern part of the species range, all the populations used for this study are comprised of fewer than 100 frogs, as are many other populations located in the western half of the range (Barbieri & Bernini, 2004; Ficetola *et al.*, 2007). Some of these small populations are locally adapted and are considered independent conservation units (Ficetola & De Bernardi, 2005). Gene flow is to a large extent restricted as a result of habitat fragmentation across much of the species distribution and has resulted in reduced population genetic variability in more isolated populations (Ficetola *et al.*, 2007). Two studies have positively correlated population genetic variability to components of fitness that have the ability to regulate population size (Pearman & Garner, 2005; Ficetola *et al.*, 2007) and one showed that the deleterious effects of decreased genetic variability manifest at a clutch, as well as population, level (Pearman & Garner, 2005). *Rana latastei* populations, for the most part meet the criteria for increased importance of genetic stochasticity as a threat to population persistence. As variation in the mating system among populations can affect N_e , shifting levels of polyandry may influence the stability of genetic diversity, and should be considered in viability analyses and plans for the conservation of populations of *R. latastei*.

Census sizes remain the predominant data available for most conservation efforts and are the core value from which widely accepted conservation assessments are made (IUCN, 2001). In the past two decades, population genetics has become more entrenched as a conservation tool; however, conservation as a whole still suffers from a lack of integration between demography and population genetics. It was only in the late 1990s when the first comprehensive works attempting to integrate behaviour with conservation were published (reviewed by Caro, 2007), and most progress in linking behaviour with demography and population genetics has been achieved using a limited number of mammals and birds as study organisms. Demography and population genetics are well studied in many amphibians, yet attempts to integrate these with behaviour, into a cohesive picture for the purposes of animal conservation, are few and far between. This may be due to the difficulties associated with assessing behaviour in often cryptic amphibian species. Direct field observation of mating systems remain the standard, but in the event such observations cannot be performed reliably, genetic information can help to develop useful information that infers behaviour and also provides new and valuable conservation information. Understanding how social systems may vary in amphibian populations of different sizes and the way this can affect genetic diversity, we feel is a key topic for amphibian conservation.

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