**RESEARCH ARTICLE** 



# Effective to census population size ratios in two Near Threatened Mediterranean amphibians: *Pleurodeles waltl* and *Pelobates cultripes*

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**Abstract** Efforts to mitigate amphibian declines are hindered by a lack of information about basic aspects of their biology and demography. The effective to census population size ratio  $(N_e/N_c)$  is one of the most important parameters for the management of wildlife populations because it combines information on population abundance and genetic diversity and helps predict population viability in the long term. Few studies have calculated this ratio in amphibians, which sometimes show low ratios, associated with a higher extinction risk. Here we integrate field-based (capture-mark-recapture studies, egg string counts) and molecular approaches (estimation of the effective number of breeders

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 $(N_{h})$  and the effective population size  $(N_{e})$  based on genotypes from larval cohorts and candidate parents) to produce the first estimates of the  $N_e/N_c$  and  $N_b/N_c$  ratios in two amphibians, the Iberian ribbed newt Pleurodeles waltl and the western spadefoot Pelobates cultripes. Additionally, we investigate sex-biased dispersal in both species based on direct (field observations) and indirect (genetic) evidence. Both species showed similar ratios, slightly lower in Pleurodeles (0.21–0.24) than in Pelobates (0.25–0.30). Observed displacement rates were low in both species (P. *waltl*=0.51%; *P. cultripes*=1.23%). We found no evidence for sex-biased dispersal in P. cultripes, but both direct and indirect evidences suggest a tendency for female-biased dispersal in P. waltl. We discuss differences in the genetic estimates of  $N_{\rho}$  and  $N_{h}$  provided by three inference methods and the implications of our findings for the management of these species, characteristic of Mediterranean wetlands in the Iberian Peninsula and listed as Near Threatened.

**Keywords** Abundance · Capture-mark-recapture · Conservation · Genetic monitoring · Linkagedisequilibrium method · Sibship frequency method · Sexbiased dispersal

# Introduction

The effective population size  $(N_e)$  is an important parameter linking evolutionary and conservation biology. It is defined as the size of an 'ideal' population that experiences the same rate of change of allele frequencies or heterozygosity as the target population (Wright 1931) and thus its estimation can help predict the long-term viability of populations (Luikart et al. 2010). Populations with a small size, in the absence of migration, experience a loss of genetic diversity determined by genetic drift, and are subject to increased inbreeding, making them more vulnerable to extinction due to stochastic processes (Frankham et al. 2002).  $N_e$  can be affected by several demographic factors: sex ratio, variation in offspring number, inbreeding, age and stage structure, changes in population size, and spatial and genetic structure (Charlesworth 2009). As a consequence, direct estimates of  $N_e$  are hard to obtain for many species in the wild, and most studies have increasingly relied on the use of molecular approaches for indirect calculation of  $N_e$  (Baalsrud et al. 2014; Kamath et al. 2015; Wang et al. 2011; Wilson et al. 2014). Currently, several methods are able to estimate contemporary  $N_e$  based on samples from a single generation (e.g. Do et al. 2014; Jones and Wang 2010; Tallmon et al. 2008), as opposed to the traditionally used two-sample method, that requires the use of time-distant samples (Fisher 1930; Nei and Tajima 1981; Turner et al. 2001).

From a conservation perspective, estimates of contemporary  $N_e$  provide very useful information on population status. In addition, calculation of the ratio between the effective and census population size ( $N_c$ ) can provide more accurate forecasts of population viability (Frankham 1995; Nunney and Elam 1994). In fact, this ratio is considered one of the most important parameters for the management of wildlife populations (Luikart et al. 2010). The ratio  $N_e/N_c$  tends to be low in general, but shows wide variation across taxa (Frankham 1995). Despite the importance of this parameter, most studies estimating  $N_e$  do not calculate  $N_c$ , mostly on account of logistic limitations related to obtaining sufficient and accurate demographic information from target species (Palstra and Fraser 2012).

Amphibians are considered the most endangered group of vertebrates, with 41% of species included in some of the IUCN categories representing threat of extinction (Hoffmann et al. 2010). Causes of declines at the global level are multiple and, in some cases, poorly understood. At the base of this knowledge gap there is an incomplete understanding of basic aspects of the biology of most species, including demographic parameters like population sizes or migration rates. Previous studies analyzing the relationship  $N_e/N_c$ have found generally low ratios, although in some cases higher ratios have been found in small populations, which has been associated with mechanisms of genetic compensation (Álvarez et al. 2015; Beebee 2009; Brede and Beebee 2006; Easteal 1985; Gill 1978; Jehle et al. 2001; Scribner et al. 1997).

Studies in amphibians are still scarce, and results are difficult to compare due to differences in methodological approaches. For instance, amphibian populations often comprise overlapping generations, which complicates estimation of  $N_e$ . Some studies have estimated  $N_e$  by grouping samples from different cohorts, but this methodology has been criticized (Waples et al. 2014), since large biases can be introduced depending on two biological traits (maximum longevity and adult life span) that are usually not considered. Alternatively, other studies have estimated the effective number of breeders  $(N_b)$  based on sibship reconstruction, and used direct records of breeding activity (such as egg mass counts) to assess the reliability of results (Sánchez-Montes et al. in review). However, in many species eggs are laid individually rather than in masses and  $N_b$ can only be estimated via genetic methods. In spite of the difficulties, valuable insights on population demography to inform conservation programs can be obtained from the integration of field-based information (capture-mark-recapture data, counts of egg masses) and molecular approaches.

Amphibians in Mediterranean areas face a high extinction risk in the medium to long term (Araújo et al. 2011). Many species, including several geographically restricted endemics, depend on ephemeral water bodies for reproduction, which availability is expected to decrease due to increased aridification and changes in land use. Attempts to model potential responses of Mediterranean pond-breeding amphibians to future scenarios (e.g. Fortuna et al. 2006) are however hindered by the previously mentioned lack of information on basic demographic parameters in most species. In this study, we provide new field- and molecular- based data on  $N_e$ ,  $N_b$  and  $N_c$  for two syntopic, pondbreeding amphibians that are characteristic of Mediterranean wetlands: the Iberian ribbed newt, Pleurodeles waltl Michahelles, 1830 and the western spadefoot, Pelobates cultripes (Cuvier, 1829). In addition, we test for sex-biased dispersal in each species based on a combination of field observations and molecular tools. The two study species are broadly co-distributed across the Iberian Peninsula (García-París et al. 2004), but their populations are declining due to habitat fragmentation, aquatic habitat loss, road mortality and the introduction of invasive species (Montori et al. 2002; Tejedo and Reques 2002), and consequently they are listed as Near Threatened (NT) by the IUCN (Beja et al. 2009, 2016). Our aim is to provide information to help design genetic monitoring programs accounting for patterns of local abundance and connectivity and prevent the loss of genetic diversity and evolutionary potential in the long term.

#### Methods

# Study area, sampling, and adult population size estimates

The study area is a semi-permanent aquatic system comprising a large pond (Laguna de Valdemanco) and the surrounding flooded meadows, with a maximum extension of 12,800 m<sup>2</sup>, and a maximum depth of 1 m (Sánchez-Montes and Martínez-Solano 2011), and is located near Valdemanco (Madrid, central Spain). We estimated the annual adult population size ( $N_a$ ; Frankham 1995), an approximation to  $N_c$ , for P. cultripes and P. waltl based on capturemark-recapture (CMR) data. Capture sessions were performed during nocturnal surveys in the breeding seasons of 2010 to 2013. In P. cultripes, the breeding season usually extends from February to April, whereas in P. waltl it is a bit longer, from December to May. Adults of both species were captured by hand, sexed based on external morphological features and marked with subcutaneous PIT tags (8 mm AVID M.U.S.I.C. chips, EzID, Greeley, Colorado, USA) that provided them with an individual identifying code, readable with an AVID Minitracker II device. All specimens were released back into their place of capture after marking and we stored tissue samples from one toe clip of each specimen in 100% ethanol for subsequent genetic analyses.

CMR data were analyzed following Pollock's "Robust Design" procedure (Pollock 1982) in program MARK 7 (White and Burnham 1999). This multimodel approach is well suited to estimate  $N_a$  in amphibians (see Sánchez-Montes et al. in review). It relies on some primary sampling periods (in this case, the different years) and some secondary periods within them (in this case, the capture sessions within each year's breeding season). Individual capture histories comprised thirteen capture sessions in the case of *P. cultripes*, and sixteen for *P. waltl*. Catchability parameters are modeled within each year provided that population closure can be assumed across all secondary sampling periods within each primary period. This information is then used to estimate the annual survival rate and  $N_a$ .

We tested the likelihood of different models in which annual survival was modeled either as constant or as time and/or sex dependent. We also modeled the probability of individuals entering or exiting the "surveyable fraction of the population" (i.e. adult breeders) from year to year. This parameter accounts for individual migration in or out of the study area but also for the possibility that some adults might skip some breeding season(s). The probability of entering (or exiting) the adult breeding fraction was modeled either as random, dependent of the last state of the individual, or zero. All models were ranked based on their Akaike Information Criterion (AICc) (Akaike 1974; Burnham and Anderson 2002). Annual estimates of  $N_a$  for each sex of each species were calculated as the weighted average of estimates from the candidate models.

#### Molecular methods and estimation of $N_b$ and $N_e$

A subset of adult, marked individuals of the two species (55 males and 52 females for *P. waltl*; 47 males and 48 females in *P. cultripes*) as well as a sample of the offspring of each species (95 larvae of *P. waltl* collected in 2010 and 183 tadpoles of *P. cultripes* collected in 2013) were genotyped to estimate  $N_b$  and  $N_e$ . *Pleurodeles waltl* females lay eggs individually, attaching them to the vegetation, whereas *P. cultripes* females produce egg strings that can be counted and taken as a proxy for the number of breeding females, thus allowing calibration of genetic estimates of  $N_b$ . Particular care was devoted to ensuring that offspring samples provided an unbiased representation of most full sib and half sib families present in the study area by comprehensive sampling throughout the pond (*P. waltl*) and by including samples from all recorded egg strings of *P. cultripes* in the season (75 in 2013).

Genomic DNA was extracted from tissue samples (tail tips of larvae and toe clips of adults) with NucleoSpin Tissue-Kits (Macherey-Nagel). Sixteen and 21 specific polymorphic microsatellite loci were used for genotyping individuals of *P. cultripes* and *P. waltl*, respectively (Gutiérrez-Rodríguez et al. 2014; Gutiérrez-Rodríguez and Martínez-Solano 2013; van de Vliet et al. 2009, see Supplemental Tables 1, 2). PCR reactions were performed using Type-it Microsatellite PCR kits (Qiagen). All reactions were carried out in a total volume of 15 µl, containing 7.5 µl of Master Mix, 1.2 µl of primer mix (0.2 µM of each primer) and 25 ng of template DNA. Multiplex reactions have previously been described in Gutiérrez-Rodríguez and Martínez-Solano (2013) and Gutiérrez-Rodríguez et al. (2014). PCR products were genotyped on an ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems), and fragments were scored and binned using GENEMAPPER v4.0 (Applied Biosystems).

Micro-Checker v2.2.3 (van Oosterhout et al. 2004) was used to test for evidence of stuttering, large allele dropout and presence of null alleles in each species, using a 99% confidence interval and 1000 randomizations. Deviations from Hardy–Weinberg equilibrium (HWE) and evidence of linkage disequilibrium (LD) were tested between all pairs of loci using Genepop on the Web (Raymond and Rousset 1995). These analyses were carried out using only adult genotypes for each species. Significance values were adjusted for multiple testing by applying a sequential Bonferroni correction (Rice 1989).

We estimated the number of alleles, observed and expected heterozygosities for each species with GenAlEx v6.5b5 (Peakall and Smouse 2012). We also calculated the Probability of Identity with and without accounting for related individuals in the sample (PI and PISibs, respectively). The reciprocal of the mean squared deviations of relatedness estimates (RMSD) was calculated with KinInfor v1 (Wang 2006) using one moment estimator (Wang 2002). This method allows estimation of information content in the microsatellite markers used in each species. We estimated  $N_b$  and  $N_e$  in *P. waltl* and *P. cultripes* with the single-sample methods implemented in programs Colony v2.0.6.2 (Jones and Wang 2010), OneSamp v1.2 (Tallmon et al. 2008), and NeEstimator v2 (Do et al. 2014). These analyses were based on the full dataset in *P. waltl*, whereas for *P. cultripes* only fourteen loci were used in OneSamp and NeEstimator, because the potential presence of null alleles in two loci (see "Results") may bias results obtained with these two programs. In contrast, we used the full marker set of *P. cultripes* in Colony analyses, since this program can accommodate genotyping errors such as those arising from the presence of null alleles.

Colony applies the sibship frequency (SF) method (Wang 2009), where  $N_b$  is estimated using inferred family relationships between offspring samples as full-sibs, half-sibs or non-sibs, that is, individuals sharing two, one or zero parents, respectively (Jones and Wang 2010). SF analyses were carried out including the offspring sample, and genotyped adults of both sexes as candidate parents. The average prior probability for genotyped adults actually siring some offspring was inferred from census estimates estimated by software Mark (see "Results"), as the ratio between the number of genotyped adults and the corresponding estimate of  $N_a$  for each sex. Colony analyses were performed assuming a polygamous mating system in both species. For each species, one run was performed implementing the full-likelihood method of Wang (2004) with "very high" likelihood precision, "very long" run length and using the "weak" sibship size prior = 1.

 $N_b$  can also be calculated in iteroparous species using Onesamp and NeEstimator when all samples belong to a single cohort (Beebee 2009; Waples 2005), as is the case of our offspring samples of *P. waltl* and *P. cultripes*.  $N_b$  estimates were calculated in NeEstimator based on the biascorrected version of the linkage disequilibrium method (LD) of Waples and Do (2008). Following the recommendations of Waples and Do (2010), rare alleles with a frequency of less than 0.02 were excluded. We also used the random mating prior and the parametric method to estimate confidence intervals.

To explore the effect of sample size on our final  $N_b$  estimates based on the SF and LD methods, we produced sets of subsampled data representing increasing proportions of our final offspring sample sizes and analyzed them in Colony and NeEstimator, with five and ten replicates per subsample, respectively. The subsamples were produced using R v3.0.3 (R Core Team 2014), and  $N_b$  estimates were calculated as in the full dataset, except for SF analyses, which were run with "medium" likelihood precision and "medium" run length. Mean  $N_b$  across replicates and percent root-mean-squared bias (RMSB; Waples et al. 2014) were estimated for each subsampled set.

We computed OneSamp estimates of  $N_b$ , which are based on the calculation of eight summary statistics using an approximate Bayesian computation (ABC) approach (Tallmon et al. 2008). We used default priors and defined different lower and upper bounds for  $N_e$ , informed by our  $N_a$  estimates: 50–200 and 50–500 in *P. cultripes*, and 20–500 and 20–1000 in *P. waltl*. Three replicates were run for each prior combination, and mean values across runs were calculated and compared with the results of the SF and LD methods.

In addition to  $N_b$ , estimates of  $N_e$  in iteroparous species can be obtained by combining adult samples from different generations (Tallmon et al. 2008; Waples and Do 2010). Thus, we calculated  $N_e$  using Onesamp and NeEstimator including only adult genotypes, with the same settings and priors described above. Recently, however, Waples et al. (2014) have criticized this approach, proposing several formulas to estimate  $N_b$  and  $N_e$  from single-cohort LD estimates using information about the adult life span (AL) and age at maturity ( $\alpha$ ) of the target species:

$$N_{b}(\text{Adj2}) = \frac{\text{raw } N_{b}}{1.103 - 0.245 \times \log (\text{AL}/\alpha)}$$
$$N_{e}(\text{Adj2}) = \frac{N_{b}(\text{Adj2})}{0.485 + 0.758 \times \log (\text{AL}/\alpha)}$$

where raw  $N_b$  is the unadjusted estimate obtained using the LD method from a single-cohort sample.

In the case of *P. cultripes*, data on age at maturity and adult life span have been estimated in 2 and 12 years, respectively, in a population near our study area in Madrid (Talavera 1990). No published information is available for *P. waltl*, but skeletochronological studies in progress have produced preliminary values of 2 and 11 years in Valdemanco (Gutiérrez-Rodríguez et al., unpublished data). Thus, we used these values to apply Waples et al.'s (2014) formulas and calculate  $N_e$  in the two species.

#### Sex-biased dispersal

We used two different methods to test for differences in dispersal between sexes. First, an assignment index correction (AIc) developed by Favre et al. (1997) and extended by Mossman and Waser (1999) was calculated using Genalex v6.5b5. This analysis only accounts for complete genotypes, so individuals with missing data were excluded. Secondly, analyses comparing mean values of relatedness between sexes were carried out with Coancestry v 1.0.1.5 (Wang 2011) using five moment (Lynch 1988; Lynch and Ritland 1999; Queller and Goodnight 1989; Ritland 1996; Wang 2002) and two likelihood estimators (Milligan 2003; Wang 2007). These analyses accounted for the possibility of inbreeding and assumed a 1% genotyping error rate. We

used 1000 bootstraps to construct confidence intervals for the estimates. In both methods, statistically significant differences between sexes indicate higher dispersal of the sex exhibiting lower AIc or average relatedness values.

Finally, we used field observations of recorded displacements in the two species based on CMR monitoring data to compare with indirect (genetic) estimates of sex-biased dispersal. We performed nocturnal transects in an area of about 0.5 km<sup>2</sup> encompassing the main breeding site for the two species (Laguna de Valdemanco), surrounding terrestrial habitat, and additional minor breeding sites in the vicinities (see detailed description of the study area in Sánchez-Montes and Martínez-Solano 2011). These transects were inspected on an opportunistic basis over an 8-year period (2009–2016), out of the main breeding season but when weather conditions (temperature, humidity) provided good conditions for amphibian activity.

# Results

#### $N_a$ estimates

Between 2010 and 2013 we captured 356 adults of *P. waltl* (245 males and 111 females) and 385 adults of *P. cultripes* (242 males and 143 females). Recapture rates overall were 30.6% for male *P. waltl*; 23.4% for female *P. waltl*; 44.6% for male *P. cultripes*, and 32.2% for female *P. cultripes*.

 $N_a$  estimates for 2011 and 2013 obtained with program Mark are shown in Fig. 1. Unfortunately, we could not obtain precise estimates in 2010 (the first year of marking) and 2012, an unusually dry year (see also Sánchez-Montes et al. in review). The precision of estimates of  $N_a$  for both species increased over the years. In the 2 years with estimable parameters, estimates of the number of *P. waltl* males ranged between 278 and 349, and the number of females, between 243 and 245. In *P. cultripes*, estimates ranged between 166 and 186 males, and between 138 and 166 females in 2011 and 2013, respectively.

#### Estimates of $N_b$ and $N_e$

Possible null alleles were detected for *P. cultripes* in loci Pc3.3, Pc4.3, Pc4.7 and Pc4.11. Of these, only loci Pc4.7 and Pc4.11 were discarded for subsequent analyses (except those in Colony, as indicated), because they have also been shown to present possible null alleles in other populations (Gutiérrez-Rodríguez et al. in review). We found no potential null alleles in loci used for genotyping in *P. waltl*, but there were significant deviations from Hardy–Weinberg equilibrium (HWE) in loci Pleu2.35 and Pleu3.14. We also found evidence of linkage disequilibrium (LD) between loci Ppl7—Pleu3.5, and



**Fig. 1** Estimates of the annual number of adults ( $N_a$ , separately for each sex) in *P. waltl (left)* and *P. cultripes (right)* in 2011 and 2013. Squares and triangles represent males and females, respectively. Mean values are shown in *black dots* and 95% confidence intervals (CI) in grey dashed lines

Ppl5—Pleu2.35. These disequilibria did not affect other populations (Gutiérrez-Rodríguez et al. in review) and thus are considered to represent population-specific processes rather than physical linkage.

The mean number of alleles, observed and expected heterozygosity were 4.238, 0.419 and 0.440 in *P. cultripes* and 5.127, 0.551 and 0.543 in *P. waltl*. Individual identification was possible in both species, since all adults, larvae and tadpoles presented exclusive genotypes. In *P. waltl*, individual identification with 95% confidence only required two loci (four when accounting for possible relatives in the sample), whereas two (five) loci were necessary in *P. cultripes* (see Supplemental Fig. 1). Using the complete marker sets, the probability of two specimens having the same genotype was  $3.0 \times 10^{-14}$  (and  $1.7 \times 10^{-6}$  when accounting for relatives in the sample) in *P. waltl* and  $2.6 \times 10^{-7}$  (and  $10^{-3}$ ) for *P. cultripes*. The estimated RMSD values were 46.1 in the *P. waltl* microsatellite set and 26.1 in *P. cultripes*.

Estimates of  $N_b$  and  $N_e$  are shown in Table 1. In *P. waltl*,  $N_b$  estimates ranged from 96 to 123, with overall congruence across methods and most estimates slightly underestimating  $N_b$  as compared to the adjusted ( $N_b$ (Adj2)) formula. In *P. cultripes*,  $N_b$  estimates ranged from 84 to 230, with estimates based on the ABC method showing strong upward deviations with respect to the adjusted  $N_b$ (Adj2) estimate.

Replicate analyses performed to explore the effect of offspring sample size on final  $N_b$  estimates showed stabilization of LD-based estimates with 40 and 50% of total sample sizes in *P. cultripes* and *P. waltl*, respectively (Supplemental Fig. 2). In the case of the SF method,  $N_b$  estimates **Table 1** Estimates of  $N_b$  andapproximation to  $N_e$  for *P. waltl*(top) and *P. cultripes* (bottom)in Laguna de Valdemanco

Method	Priors	N <sub>b</sub>		$N_b/N_a$		N <sub>e</sub>		N <sub>e</sub> /N <sub>a</sub>	
		Estimate	95% CI	2011	2013	Estimate	95% CI	2011	2013
P. waltl									
ABC (Onesamp)	20-500	100	84–157	0.17	0.19	42	36–55	0.07	0.08
	20-1000	109	72–219	0.18	0.21	42	38–59	0.07	0.08
LD (NeEstimator)	0.02	115	89–160	0.19	0.22	153	119–208	0.26	0.29
	Adj	123		0.21	0.24	122		0.21	0.23
SF (Colony)	P–P Weak	96	72-132	0.16	0.18				
P. cultripes									
ABC (Onesamp)	50-200	195	145-241	0.55	0.64	78	59-107	0.22	0.26
	50-500	230	167–400	0.65	0.76	84	64–133	0.24	0.28
LD (NeEstimator)	0.02	84	67–108	0.24	0.28	136	84-281	0.39	0.45
	Adj	91		0.26	0.30	87		0.25	0.29
SF (Colony)	P–P Weak	93	71–126	0.26	0.31				

For a description of the different priors used see "Methods". The ratios  $N_b/N_a$  and  $N_e/N_a$  are calculated for 2 different years (2011 and 2013). P–P indicates the assumption of polygamy for both males and females, and Weak—weak sibship size prior in SF analyses. Adj.—adjusted estimate

increased with increasing sample size, and showed lower variance between replicates than in the LD method.

Estimates of  $N_e$  from samples combining individuals from different cohorts differed across methods. In *P. waltl*, the ABC method produced an estimate of 42, whereas the LD method estimated 153. The latter was closer, but upwards biased by 28% with respect to the adjusted  $N_e$ (Adj2) (Table 1). In *P. cultripes*, estimates based on the ABC method were also lower than those from the LD method, which was upwards biased by over 50%. In this species, however, ABC-based estimates were closer to the adjusted  $N_e$  estimate, with a slight downward bias.

Adjusted values of  $N_b$  and  $N_e$  were similar within each species (123 and 122 in *P. waltl* and 91 and 87 in *P. cultripes*). The resulting  $N_b/N_a$  ratios were similar across species, although slightly lower in *P. waltl* (0.21–0.24 vs. 0.25–0.30).

#### Sex-biased dispersal

AIc estimates were different between sexes in *P. waltl* (see Fig. 2), but results were not statistically significant. Mean bias assignment was lower in females than in males, indicating a tendency to female-biased dispersal. In the case of *P. cultripes*, no differences between sexes were observed. On the other hand, four out of the seven relatedness estimators calculated by Coancestry showed statistically significant differences between sexes in *P. waltl*. In all cases, the results showed higher relatedness in males than in females, also supporting female-biased dispersal. In *P. cultripes*, there were no significant differences between sexes in average relatedness.

Over an 8-year period (2009–2016), out of a total of 1172 and 1293 recapture events, we recorded six (frequency = 0.51%) and sixteen (frequency = 1.23%) movements longer than 250 m in *P. waltl* and *P. cultripes*, respectively. Four of these displacements (three females and one male) exceeded 700 m in *P. waltl*. In the case of *P. cultripes*, eight displacements to nearby breeding sites were recorded, with five of them (two females and three males) involving distances longer than 700 meters.

# Discussion

We present the first estimates of the effective to census population size ratio in two pond-breeding amphibians characteristic of Mediterranean wetlands. Prior to our study there was little information on their population biology or demography, so our results are a valuable contribution towards evidence-based conservation actions directed to minimize local extinction risk in these species. In this regard, a potential limitation of our results is to what extent they are generalizable to other populations and regions, since variation in  $N_b$ ,  $N_e$  and their ratio with  $N_a$  is expected, especially in widespread species. While it is hard to anticipate the magnitude of differences in these parameters across populations, here we focused on large (hundreds of adults) and apparently healthy (with no record of the presence of exotic species or infectious diseases) populations. Furthermore, sampling took place in years with optimal conditions for breeding success (based on our field observations, including a long hydroperiod maximizing survival to metamorphosis and low egg mortality). This is an important factor, since the ephemeral ponds in which





Fig. 2 Mean and 95% CI values of seven estimators of Relatedness (*Top*), and mean and standard error of Assignment Index correction (AIc) values (*Bottom*) in *P. waltl* (*left*) and *P. cultripes* (*right*).

Squares and triangles represent males and females, respectively. Significant differences are marked with *asterisks* 

both species usually breed are subject to strong variation in hydroperiod across years, with dry years typically resulting in reduced breeding and recruitment (and therefore in lower  $N_b$  or  $N_e$  estimates). Therefore, our results should be representative of populations of both species in good conservation status and comparable to those from other locations. Smaller, isolated populations should also be studied to search for evidence of genetic compensation effects and quantify their magnitude with respect to large, healthy populations. Additionally, the obtained  $N_b/N_a$  ratios could be used to approximate abundance  $(N_a)$  based on  $N_b$  estimates, which are easier to obtain, given that  $N_a$  estimates usually require years of mark-recapture efforts.

Considering the effect of sample size is critical to obtain accurate estimates of  $N_e$  and  $N_b$  based on LD methods (Kamath et al. 2015; Waples and Do 2010), especially if sample sizes are lower than the actual values of  $N_e$  and  $N_b$ . In our case,  $N_b$  estimates from subsampled datasets based on the SF method increased linearly without reaching stabilization. However, differences between replicates were smaller than across  $N_b$  estimates based on the LD method, which stabilized at around 40–50% of the final sample size for both species (Supplemental Fig. 2). This represents minimum sample sizes of around 70 (*P. cultripes*) and 50 (*P. waltl*) single-cohort offspring samples, higher values than those previously recommended (Waples 2006; Waples and Do 2010). This is probably caused by lower levels of polymorphism in our marker sets (RMSD: *P. waltl*: 46.1 and *P. cultripes*: 26.1) in comparison to those usually employed in simulations.

In iteroparous species with overlapping generations,  $N_{h}$  estimates have been more generally applied than  $N_{e}$ , because most methods attempting to estimate the latter rely on the assumption of discrete, non overlapping generations. This assumption does not apply for the estimation of  $N_b$ , which partially summarizes the effects of the sizes of age classes and the variance in reproductive success within and among age and sex classes on inbreeding and drift in a population with overlapping generations (Wang 2016). Here we used three different methods (LD, ABC and SA methods) to estimate  $N_h$  using single-cohort samples. The results were consistent across methods, except for estimates obtained with Onesamp for P. cultripes, which doubled LD and SA estimates (Table 1). The ABC method is sensitive to priors (Pérez-Figueroa et al. 2015), but adjusting the prior to reflect realistic estimates has been shown to improve estimates (Holleley et al. 2014). Here we specified boundaries for the priors based on our  $N_a$  estimates, but perhaps they were still too wide to provide accurate estimates (although wider prior boundaries in *P. waltl* did not affect precision of  $N_b$  estimates).

Estimates based on the LD method with single cohort samples relate to  $N_b$  (Waples 2005). However, Waples et al. (2014) have suggested that  $N_b$  estimates using this method in iteroparous species may present biases due to unaccounted age structure (Waples et al. 2013). Thus, we adjusted our LD-based estimates using the equations in Waples et al. (2014). By accounting for information on the adult life span and age at maturity of both species, we obtained slightly higher values compared to unadjusted LD estimates (7.0% in P. waltl and 8.3% in P. cultripes). The adjusted LD estimate of  $N_b$  was very similar to the SF estimate in *P. cultripes*, but slightly larger in the case of *P.* waltl. Wang (2009) suggested that the SF method could underestimate  $N_h$  when markers are less informative and sample size is small, especially in polygamous systems (Wang 2016), whereas  $N_h$  estimates are more accurate when sample size is large relative to  $N_h$  (Wang 2009). Our markers are more informative in *P. waltl* (RMSD=46.1) than in *P. cultripes* (RMSD=26.1), but sample size in the former was about half of that in P. cultripes, which could partially explain this underestimation.

Studies estimating  $N_c$  in some explosive-breeding amphibians have used counts of egg strings as a proxy for the adult census size (number of egg strings  $\times 2 = N_c$ ), which is based on the assumptions that (1) most females in the population breed and they do so only once during the season, and (2) there is a balanced sex ratio (Beebee 2009). It is worth asking whether a similar inference could be made for P. cultripes, because egg strings are easy to find and count exhaustively in this species. In 2013 we counted 75 egg strings, but estimated an adult population size of around 140 females, suggesting a relatively low female breeding success, which contrasts with the high success recorded in other species in the same area (close to unity in B. calamita, in line with the findings of Beebee 2009, see Sánchez-Montes et al. in review). The corresponding  $N_b$  estimate for *Pelobates* in 2013 was around 90 according to the LD and SF methods. Consistency across both methods supported the reliability of the estimates, in contrast to that obtained with the ABC method. This result suggests some degree of polygamy in this species, as also shown by family relationships reconstructed with software Colony. This is surprising in view of the short breeding season of the species, which provides few opportunities for multiple matings, and the mating behavior, in which amplectant males fertilize the eggs as they are released by the female. A question worth further exploration is the possibility of multiple fertilization of different egg strings by the sperm of several males meeting in breeding aggregations in small pools; these aggregations are often observed in our study area.

Several methods have been proposed to estimate  $N_e$ with single-sample estimators in iteroparous species: the LD method (Waples et al. 2014; Waples and Do 2010), an ABC-based approach (Tallmon et al. 2008), the estimator by parentage assignments (EPA) (Wang et al. 2010), and the method implemented in software AgeNe (Waples et al. 2011). The last two methods could not be applied here, since information on the individual age, age-specific survival rates or relative fecundity was unavailable for both species. Thus, we focused on the LD and ABC methods, which produced different results. Mixed cohort adult samples produced strongly upwards-biased results compared to the adjusted estimates of  $N_{e}$ : 28% in P. waltl and 56% in P. cultripes. These results contrast with those obtained by Waples et al. (2014), where uncorrected estimates of  $N_{a}$ based on mixed cohorts were lower than adjusted ones in all species analyzed. However, Ruzzante et al. (2016) found a wider ratio of adjusted to unadjusted estimates based on mixed adult samples, ranging from 0.69 to 4.77.

On the other hand, the ABC method implemented in Onesamp has been also applied to estimate  $N_{\nu}$  with multiple-cohort samples in iteroparous species (Phillipsen et al. 2011; Tallmon et al. 2008). In our study, estimates of  $N_a$ based on mixed cohort adult samples in Onesamp were similar to adjusted LD estimates in P. cultripes, but very different (less than half than adjusted LD estimates) in P. *waltl.* These discrepancies are hard to interpret, but may reflect the different timescales associated with ABC and LD-based estimates (Wang 2016; Wang et al. 2016). For instance, one of the statistics used to estimate  $N_{e}$  with the ABC approach is the M-ratio, which is able to detect demographic changes in the previous 100 generations (Garza and Williamson 2001), so it is hardly comparable to estimates based on the LD method, which estimates  $N_e$  in the parental generation for sampled individuals (Waples 2005).

Keeping in mind the reported differences across methods, the  $N_b/N_a$  and  $N_e/N_a$  ratios obtained in the two species were similar (range 0.21–0.30 considering LD estimates), and near the average estimate (0.22–0.23) calculated by Palstra and Fraser (2012) across disparate taxa. In amphibians, strong differences have been observed between species with different life-history traits. For instance, in anurans, Rowe and Beebee 2004 reported ratios of 0.02–0.17 in *Bufo calamita*, whereas Schmeller and Merilä 2007 estimated ratios between 0.23 and 1.67 in *Rana temporaria*. Similar variation has been reported in urodeles, with low ratios in *Triturus marmoratus* (0.09; Jehle et al. 2001) contrasting with high ratios in *Salamandra salamandra* (0.8; Álvarez et al. 2015), although in the latter case genetic compensation effects were invoked.

How do our estimated ratios relate to population viability? First, our results suggest that adult abundance may be a poor surrogate of population status, with populations with hundreds of breeding adults having an effective population size close to 100 individuals in the two species (around 90 in Pelobates and 120 in Pleurodeles). Frankham et al. (2014) proposed a minimum value of  $N_e$  of 100 to avoid or minimize problems of inbreeding depression in wild populations. If large and healthy populations such as those in our study area are characterized by  $N_a$  estimates below or barely above that critical value, the situation is probably much worse for smaller populations, although in these cases there may be genetic compensation mechanisms at play. In any case, additional estimates of the  $N_a/N_e$  ratio in other populations are required for a better understanding of the actual range of variation in this key parameter.

Finally, our results also provide some insights on dispersal in the two species, which may be helpful when designing management strategies to revert the effects of habitat fragmentation. The observed rates of long displacement events were quite low in both species (*P. waltl* = 0.51%; *P.* cultripes = 1.23%), in line with the low general dispersal capacity of amphibians and their tendency to philopatry, suggesting high vulnerability to natural or artificial fragmentation of their habitats. The higher frequency of displacements in P. cultripes is consistent with their more terrestrial behaviour and with the higher dispersal capacity of anurans compared to urodeles (Smith and Green 2005). On the other hand, our genetic-based results regarding sexbiased dispersal suggest a tendency for female-biased dispersal in P. waltl (Fig. 2). The same tendency was also suggested by our direct observations of dispersive individuals, in which three out of four recorded long-distance movements were performed by females. This result contrasts with general reports of male biased dispersal in urodeles (Helfer et al. 2012; Liebgold et al. 2006), but similar observations of female-biased dispersal have been recently reported in the newt Triturus marmoratus (Trochet et al. 2017). These authors hypothesize that this bias may result from the territorial behaviour of males during their aquatic phase and /or from a tendency of females to move further away from breeding sites in search of high quality terrestrial foraging grounds with reduced intraspecific competition. Both explanations may also apply to Pleurodeles, but need to be tested with field and experimental evidence. At any rate, low dispersal rates combined with low  $N_e$  values increase the risk of inbreeding (Johnson et al. 2007). Considering in addition that habitat fragmentation is one of the major threats identified in the two species, we argue that conservation measures should focus on improving population connectivity to minimize the risk of loss of genetic diversity and evolutionary potential.

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