ORIGINAL ARTICLE

<PE-AT>An integrative demographic study of the Iberian painted frog (*Discoglossus galganoi*): inter-annual variation in the effective to census population size ratio, with insights on mating system and breeding success
Running title: Integrative demography of the Iberian painted frog

Óscar Olarte, Gregorio Sánchez-Montes, Íñigo Martínez-Solano*

Department of Biodiversity and Evolutionary Biology, Museo Nacional de Ciencias Naturales, CSIC

c/ José Gutiérrez Abascal, 2, 28006 Madrid, Spain

* Corresponding author; Iñigo Martínez-Solano,

e-mail: inigomsolano@mncn.csic.es

Abstract

In the face of worldwide amphibian declines, integrative studies combining individual-based

information and genetic data represent a powerful approach to produce robust, reliable and

comparable assessments of demographic dynamics. The Iberian painted frog (*Discoglossus galganoi*)

is endemic to Spain and Portugal and shows decreasing population trends across its range, but few

studies have attempted to estimate census sizes or assess genetic diversity in wild populations, and

little is known about their reproductive biology. We applied an integrative approach based on the

combination of capture-mark-recapture data and multilocus genotypes to monitor a breeding

population of *D. galganoi* in central Spain during two consecutive breeding seasons, focusing on the

estimation of demographic parameters and their temporal variation. Specifically, we estimated the

number of adults (N_a), the effective population size (N_e) and the effective number of breeders (N_b),

as well as survival and migration rates. We documented a >50% decrease in the estimated number

of adults of both sexes between the breeding seasons of 2018 and 2019, probably associated with

reduced rainfall in the latter. Estimates of N_b and the N_b/N_a ratio were low in both seasons, with a 20-30% decrease in N_b and a 47% increase in the N_b/N_a ratio in 2019. Based on the reconstruction of pedigrees from larval and adult genotypes, we provide the first genetic evidence of polygamy in males and females of *D. galganoi* and the first estimates of breeding success in the species.

Key words: Amphibians, demography, effective population size, genetic diversity, breeding success, polygamy.

<PE-FRONTEND>

1. Introduction

The global amphibian crisis has brought to light a major knowledge gap on amphibian demography and population dynamics, which hinders attempts to infer ultimate causes of decline (Stuart *et al*. 2004; Hoffmann *et al*. 2010). To address this shortfall, there is an urgent need for solid knowledge about the natural history and biology of amphibians, including detailed information about their demographic trends, habitat requirements and reproductive biology (Gascon *et al*. 2007; Toledo *et al*. 2014). The continuous development of analytic formulations for individual-based data and the widespread availability of molecular resources for ecological research have set a powerful framework for integrative approaches providing robust, reliable and comparable estimates of key demographic parameters (Clutton-Brock & Sheldon 2010; Allendorf 2017). Integrative studies represent a cornerstone in the assessment of population trends and in the detection of potential declines before populations plunge towards extinction (Brooks *et al*. 2004; Verdade *et al*. 2012).

Accurate and robust estimates of census size (N_c) or the number of adults of the population (N_a) are essential to characterize amphibian demographic dynamics (Frankham 1995; Witmer 2005), as well as survival and migration rates, which help contextualize observed fluctuations in population size over time. Capture-mark-recapture studies provide the most accurate estimates, although N-

mixture models have proven a reliable alternative when intensive individual marking effort is not feasible (Ficetola *et al.* 2018). Additionally, accounting for temporal variation in patterns of genetic diversity provides key information on long-term population viability (Nunney & Campbell 1993; Frankham 1995; Luikart *et al.* 2010; Banks *et al.* 2013). Important parameters in this context include the effective population size (N_e), which is proportional to the capacity of a population to maintain its genetic diversity (Wright 1931; Merrel 1981), and the effective number of breeders (N_b), which represents the genetic contribution of adults generating an offspring cohort in a single breeding season (Waples *et al.* 2014). The relationship between the potential of a population to maintain genetic diversity and its size can be approximated by the effective to census population size ratio (N_e/N_c, Palstra & Fraser 2012). This ratio provides information on the long-term viability of populations (Frankham 1995; Luikart *et al.* 2010) and can be used to estimate the minimum viable population size (Nunney & Campbell 1993). The ratio between the effective number of breeders (N_b) and the number of potentially breeding adults of a population (N_a) can be regarded as an approximation to the N_e/N_c ratio in a single breeding season (Sánchez-Montes *et al.* 2017). The estimation of this set of demographic parameters in integrative population monitoring programs represents a powerful asset to improve our understanding of amphibian population dynamics.

Modelling population turnover rates and the maintenance of genetic diversity also requires knowledge about the biology of target species (Balloux & Lehmann 2003; Holman & Kokko 2013; Gao *et al.* 2019), including mating system and variation in breeding success (Byrne & Roberts 2012). However, this information is lacking for most amphibian species in the wild (O'Brien *et al.* 2018). Pedigree information from direct records or parentage reconstruction from genetic methods can provide robust inferences about the number of successfully breeding males and females and the rates of polygamy of each sex, therefore allowing exploration of the mating system and breeding success of target species (Vieites *et al.* 2004; Rovelli *et al.* 2015; Levine *et al.* 2019). The sibship frequency method infers the parentage of an offspring sample jointly with an estimate of the effective population size (Wang 2009b; Mangold *et al.* 2015). However, the accuracy of

reconstructed parentage should be calibrated with direct observations (for example from capture-mark-recapture studies), which are seldom available for wild amphibian populations (Sánchez-Montes *et al*. 2017). As a consequence, the possibilities of integrative approaches remain underexploited and basic aspects of the reproductive biology of many amphibian species worldwide remain unknown (O'Brien *et al.* 2018).

The Iberian Peninsula is home to a rich amphibian fauna, including many endemic species that attest to the long and complex tectonic and climatic history of the region (Abellán & Svenning 2014). Local extinctions and population declines have been reported in several species, mostly as a result of habitat loss (Márquez & Lizana 2002). However, for many of these species there is scarce information about basic aspects of their natural history, demography or genetic diversity, which calls for integrative demographic studies to understand their population dynamics. Here we adopted an integrative approach, based on the combination of capture-mark-recapture data and multilocus genotypes, to monitor a breeding population of the Iberian painted frog, *Discoglossus galganoi* Capula, Nascetti, Lanza, Bullini, & Crespo (1985). Two major lineages occupying western and eastern Iberia have been identified in previous studies, first considered at the species level (*D. galganoi* and *D. jeanneae*) and currently as subspecies: *D. g. galganoi* and *D. g. jeannea*e (Carretero *et al*. 2014; Dufresnes *et al*. 2020). Populations of *D. galganoi* are in decline, especially in the east, where populations are highly fragmented and local extinctions have been reported in the recent past (Martínez-Solano & García-París 2002). *Discoglossus galganoi* is a generalist species, present in a broad variety of habitats, including urban parks (Sánchez *et al*. 2015), but little is known about its breeding biology, population sizes or genetic diversity (Galán 1999; Martínez-Solano 2014).

We monitored a breeding population of *D. galganoi* in central Spain during two consecutive breeding seasons (2018 and 2019) to estimate several demographic parameters, namely the number of adults (N_a), the effective population size (N_e), the effective number of breeders (N_b) and survival and migration rates. We also investigated other aspects of the reproductive biology of the species,

including the breeding success and polygamy rates in both sexes, based on the reconstruction of pedigrees from genotypes of adults and larval cohorts for each breeding season. We discuss the benefits of our integrative approach for the study of amphibian populations.

2. Materials and Methods

2.1. Study area

The study area is located in *Monte de El Pardo, Madrid*, central Spain (40°30'N, 3°44'W). The habitat is characterized by an anthropized landscape of promenade parks and olive tree crops. A population of *D. galganoi* breeds in a small (3x2m) spring and its effluent, semi-permanent stream that runs superficially for 100-150 meters before filtering underground. This breeding habitat is shared with another amphibian species, the Iberian common toad (*Bufo spinosus*), and two alien predators: the red swamp crayfish (*Procambarus clarkii*) and the goldfish (*Carassius auratus*).

2.2. Capture-mark-recapture (CMR) and tissue sampling

We conducted nocturnal transects in search of adults of *D. galganoi* every week from September 2017 to June 2019, except during the dry summer months (June-August), when the species is not active. Thus, our sampling sessions included the breeding seasons (March to May, approximately) of 2018 and 2019. We chose nights with favorable conditions for amphibian activity: mild temperatures, high humidity and little or no wind, and actively and exhaustively searched for adults along stream margins and potential shelters, including leaf litter and rocks. All captured individuals were sexed based on sexually dimorphic characters (García-París *et al*. 2004; Martínez-Solano 2014). We also measured their snout to vent length and body mass and obtained a tissue sample corresponding to the terminal 2-3 phalanges of the fourth toe of the right hind leg. All individuals were marked for identification with a passive integrated transponder (PIT) tag, then released back into the place of capture.

In addition to the nocturnal sampling sessions for CMR data of adults, we sampled larval tissues at the end of the 2018 and 2019 breeding seasons (in June 2018 and 2019, respectively), in order to obtain a representation of the offspring cohort of each year. We clipped a small fragment of the tail of each sampled tadpole (n = 81 individuals in the 2018 cohort and n = 44 in the 2019 cohort). All tissue samples were preserved in absolute ethanol until their subsequent processing for genetic analyses.

2.3. Estimates of N_a and survival and migration rates

Capture histories of adult individuals were used to estimate N_a for males and females, jointly with survival and migration rates. We first constructed a complete capture history matrix, including the sex of each individual and coding whether it was captured (1) or not captured (0) in each sampling session. This matrix was then analyzed with two different formulations implemented in software MARK (White & Burnham 1999): the POPAN formulation, suited to estimate the size of open populations (Schwarz & Arnason 1996), and the robust design, developed to model fluctuations in population size mediated by survival and migration rates (Pollock 1982).

Two POPAN analyses were performed, each aimed to estimate the number of adult individuals that were present at the study area during the hydrometeorological year encompassing either one of the two breeding seasons of study. Accordingly, the first POPAN analysis included sampling sessions from September 2017 to June 2018 (hereafter the 2017-2018 period, thus encompassing the 2018 breeding season), and the second POPAN analysis included sampling sessions from September 2018 to June 2019 (hereafter the 2018-2019 period, thus encompassing the 2019 breeding season). In both analyses, we discarded all sampling sessions with less than five captures to avoid overparameterization of models. Thus, final datasets included a total of 20 sampling sessions for the 2017-2018 period (from November 24, 2017 to May 29, 2018), and 21 sampling sessions for the 2018-2019 period (from September 26, 2018 to June 13, 2019).

Models in POPAN analyses were constructed by combining the parameters p (probability of capture), φ (apparent survival) and *pent* (probability of entrance of new individuals in the study area) as either constant (.) or dependent on sex (g), time (t) or both factors and their interaction (g*t). Because males of pond-breeding amphibians often spend more time near breeding sites than females and their detectability is thus higher, the p parameter was modeled only as dependent on sex (g), or sex and time along with their interaction (g*t). Also, since attendance to breeding sites is higher during the reproductive season in the spring (March-May), we modeled *pent* as time-dependent in all cases (models (t) and (g*t)). The resulting 12 models were constructed, run and finally ranked according to the Akaike Information Criterion corrected for small sample size or AICc (Akaike 1974), which takes into account the likelihood of each model penalized by the number of parameters. Global estimates of N_a for each period and partial estimates of N_a derived for each session were calculated as the weighted average of model estimates based on their AICc.

Unlike the POPAN formulation, the robust design approach allows estimation of the probability of temporary migration of individuals outside the study area (γ'') and their probability of return (1-γ'). The robust design is a combination of the Cormack-Jolly-Seber (CJS) model (Cormack 1964; Jolly 1965; Seber 1965) and closed capture-recapture models, which assume that the population does not grow or decrease during a particular period of time. This combination is implemented through the hierarchical organization of primary and secondary sampling occasions, each primary occasion encompassing several secondary occasions close in time, with the assumption that the population size remains constant during that period (Table S1). Accordingly, the intervals between primary occasions should be sufficient for the population to grow (by births and immigration) or decrease (by deaths and emigration), while the interval between secondary occasions within each primary occasion should be short enough to assume that no changes in size occur in the population (Kendall & Nichols 1995).

A single robust design analysis was conducted for the entire study period, covering from November 2017 to June 2019. For this purpose, we selected 31 sampling sessions organized in 6 primary occasions (approximating climatic seasons: autumn, winter and spring for each period) with a minimum of one month separation between each consecutive pair (Table S1). Twelve models were built combining 1) the probabilities of emigration and immigration as either dependent on the last state of the individual ("markovian" model: $\gamma'' \neq \gamma'$), random ("random movement" model: $\gamma'' = \gamma'$) or null ("no movement" model: $\gamma'' = \gamma' = 0$); and 2) the (real) probability of survival (S) as either constant (.) or dependent on sex (g), time (t), or sex, time and their interaction (g*t). Final estimates of the parameters and derived estimates of N_a for each primary occasion were obtained by weighted average of the models based on their AICc.

To check whether capture histories conform to the assumptions of the corresponding formulation, we ran goodness of fit (GOF) tests on the input data matrices of both POPAN and robust design analyses using the program U-Care (Choquet *et al*. 2005). The 3.SR test was used to verify the possible existence of transience effects caused by dispersing individuals, which would violate the assumption that all individuals in the population have the same probability of being recaptured (Pradel *et al*. 1997). The 2.CT test was run to test the assumption that marking has no effect on the probability of recapture (*i.e.*, there is no trap-dependence), neither by increasing it (trap-happiness) nor by decreasing it (trap-shyness) (Pradel & Sanz-Aguilar 2012).

2.4. DNA extraction and genotyping

Genomic DNA from all larval (n = 125) and adult (n = 212) tissue samples was isolated with commercial Qiagen DNeasy Blood & Tissue Kits following the instructions of the manufacturer. For genotyping, we selected 15 microsatellites among the set described in Gutiérrez-Rodríguez *et al*. (2014). These loci were grouped into three multiplex reactions (Table S2) with Multiplex Manager 1.0 (Holleley & Geerts 2009), establishing a minimum distance of 20 base pairs between loci.

Microsatellite amplification was implemented by polymerase chain reaction (PCR) in a total volume

of 15.2 µL, with 7.5 µL of Type-it Master Mix (Qiagen), 1.2 µL of primer mix, 5.3 µL of RNase-free

H_2O, and 1.2 µL of template DNA.

PCR cycling conditions consisted of an initial denaturation step at 95ºC for 5 minutes,

followed by 30 denaturation cycles for 30 seconds at 95 ºC, annealing for 90 seconds at 60ºC and

extension for 30 seconds at 72ºC, with a final extension phase of 10 minutes at 60ºC. The quantity

and quality of PCR products was verified through electrophoresis in 2% agarose gels, including

negative controls in each PCR to detect possible contamination. PCR products were genotyped in an

ABI PRISM 3730 sequencer with the GeneScan 500 LIZ size standard (Applied Biosystems). Finally,

the multilocus genotype of each individual was assigned based on the size of the amplified

fragments using GENEMAPPER v4.0 (Applied Biosystems).

2.5. Genetic diversity, N_e, N_b, breeding success and polygamy rates

We used GenAlEx 6.503 (Peakall & Smouse 2006, 2012) to calculate the number of alleles or allelic

richness (*AR*), observed heterozygosity (H_O) and expected heterozygosity (H_E) for each locus, and the

corresponding multilocus estimates and the number of private alleles (*PA*) for samples of the 2017-

2018 and 2018-2019 periods separately.

We then used software Colony (Jones & Wang 2010) to estimate N_e and N_b and to

reconstruct parentage of larval samples (Wang 2009b), which was in turn the source information

used to infer the breeding success and polygamy rates of individuals of both sexes. For N_e, we

obtained a single estimate based on the analysis as offspring sample of the genotypes of all adults

captured. N_b analyses were carried out separately for each of the periods of study (2017-2018

including the 2018 breeding season and 2018-2019 including the 2019 breeding season), including

the larvae sampled in each period as offspring sample, and all the genotyped males and females as

candidate parental and maternal samples, respectively.

Analyses were performed with different *a priori* probabilities that the progenitors of sampled larvae are among the genotyped samples of males and females. These *a priori* probabilities were calculated for males and females separately by dividing either 1) the number of individuals of the corresponding sex captured in each period by the POPAN estimate of N_a of the corresponding sex in the same period (see Results), or 2) the total number of genotyped individuals of the corresponding sex by the POPAN estimate of N_a of the same sex in the corresponding period. According to this procedure (see Results), *a priori* probabilities of presence of actual parents among the candidate parents were calculated as follows: 1) fathers: 88/116 = 0.76 and mothers: 77/135 = 0.57 for 2017-2018, and fathers: 40/49 = 0.82 and mothers: 45/65 = 0.69 for 2018-2019; and 2) fathers: 101/116 = 0.87 and mothers: 111/135 = 0.82 for 2017-2018, and fathers: 101/49 >1 (a probability of 0.99 was used) and mothers: 111/65 >1 (a probability of 0.99 was used) for 2018-2019.

In all N_e and N_b analyses we allowed for the possibility of polygamy in both sexes. The final analysis was performed with the maximum run length and using the Full-Likelihood (FL) analysis method with maximum precision. Two runs of each analysis were conducted to assess the consistency of results. In addition, different analyses were carried out using or not a weak maternal and paternal sibship size prior = 1 (*i.e.*, *a priori* information about the average number of representatives of each family in the offspring sample, see Sánchez-Montes *et al*. 2017). Monomorphic loci (see Table S2) were excluded from analyses and, for polymorphic loci, *a priori* genotyping error rates were set to 0.01.

3. Results

3.1. Estimates of N_a and survival and migration rates

The GOF tests of POPAN datasets showed evidence of transience and trap-dependence effects in males in 2017-2018 (standardized log odds ratio (LOR) statistic for transience = 2.146, *p* = 0.032; LOR for trap-dependence = -2.452, *p* = 0.014), and trap-dependence in males in 2018-2019 (LOR = -2.052,

p = 0.040). The model {p(g*t) φ(g) $pent$(t)} was the best ranked with an AICc weight > 0.9 both in 2017-2018 and 2018-2019 (Table 1). Global estimates of N_a showed a sex-ratio in favour of females in both periods and a substantial decrease (>50%) in the number of individuals of both sexes in 2018-2019 compared to the previous period (Table 2). Derived N_a estimates for each sampling session showed a strong association with the dynamics of individual entries into the breeding area, represented by parameter $pent$ (Fig. 1). Values of this parameter were inferred to be similar across sexes in both years, with models with higher AICc weights sharing a time-dependence for $pent$ (Table 1). An initial decline in N_a was observed when the probability of entrance of individuals to the breeding area was virtually reduced to zero between December 2017 and February 2018. At the end of March 2018, when $pent$ increased to almost 0.4, the number of males increased from 18 (95% CI: 11-25) to 70 (53-88), and the number of females from 16 (9-24) to 73 (47-99). In the period 2018-2019, two main entrance events were inferred on December 4 and February 7, although they did not result in strong increases in N_a as in the previous period.

None of the GOF tests showed evidence of significant deviations from model assumptions in any of the seasons of the robust design dataset. The model with the best AICc was the "markovian" with time-dependent survival, with an AICc weight of 0.7 (Table 3). The model {Random Movement S(t)} also ranked high in the results table, with an AICc weight of 0.28. The first three models, which amounted to > 99% of the AICc weight, shared time-dependence on survival (Table 3). Estimates of N_a showed different fluctuations on the number of males and females across the two study periods. In the first period there was an important increase in the number of individuals of both sexes from winter to spring. Estimates then decreased in the following autumn and did not recover in the spring (Table 2), which was characterized by low precipitation. Monthly survival estimates were high over the two years of study, except between winter 2017-2018 and spring 2018 (Fig. 2). Migration rates differed between periods and seasons, and also between sexes. No informative estimates of immigration rates were obtained (1-γ'), as their 95% confidence intervals were very wide in most cases (for this reason they are not represented in Fig. 2).

3.2. Genetic diversity, N_e, N_b, breeding success and polygamy rates

The four indices of genetic diversity showed very low and nearly identical values in both periods of study (Table 4). The estimate of effective population size (N_e) ranged between 40 and 50 individuals, depending on the use (mean: 50, 95%CI: 35-74) or not (mean: 40, 95%CI: 27-62) of the sibship size prior. Estimates of N_b were robust to the priors, with values in the 2019 breeding season being 20-30% lower than those in 2018 breeding season (Table 5). The corresponding N_b/N_a ratios were higher in 2018-2019 compared to the previous period (0.22 and 0.15, respectively, based on baseline pedigrees, see below).

We defined one baseline pedigree for each of the two breeding seasons of study by selecting the pedigrees showing higher concordance with most of the remaining pedigrees among all N_b analyses (Appendices 3 and 4). The two selected pedigrees were reconstructed without the use of sibship size prior, setting *a priori* probabilities of the presence of progenitors among the genotyped adult sample of 0.76 for males and 0.57 for females in 2018 and 0.82 for males and 0.69 for females in 2019. Breeding success, calculated as the proportion of individuals of each sex that was inferred to be the parent of at least one tadpole in the pedigrees, was low and similar across sexes: 19/116 = 0.16 for males and 17/135 = 0.13 for females in 2018, and 12/49 = 0.24 in males and 16/65 = 0.25 in females in 2019. Among individuals who bred successfully in 2018, 89.47% of males and 76.47% of females produced offspring with more than one partner. In 2019, 66.67% and 62.50% of successfully breeding males and females, respectively, mated with more than one partner (Fig. 3).

4. Discussion

Amphibians are characterized by strong apparent fluctuations in abundance, usually in response to external factors like temperature or precipitation (Skelly *et al*. 2003; Greenberg *et al*. 2018). Also, recruitment of juveniles is highly variable over the years (Pechmann *et al*. 1991; Pechmann & Wilbur 1994; Trenham *et al*. 2003), which has a strong impact on population dynamics (Berven 1990). Our results, while limited in timeframe, are the first data on population size variation (at least the breeding portion of the population) in *D. galganoi*. GOF tests of the POPAN (but not robust design) dataset revealed slight but significant trap-happiness and transience effects in males, indicating that they are more likely recaptured after being marked, and that there are individuals migrating in and out of the breeding area. This suggests that the population dynamics of the species is best modeled by robust design analyses, which account for the possibility of temporary migration of individuals through estimation of *γ* parameters. At any rate, estimates from POPAN and robust design analyses coincide in showing a reduction in the number of individuals of both sexes during the second year of monitoring. This decrease in the number of individuals present at the breeding area in the second year may reflect an actual, ongoing decline or may be part of a natural fluctuation. Extending our integrative monitoring approach further in time will allow a more comprehensive characterization of population size variation in *D. galganoi*, but our preliminary results suggest a major role of the variation in climatic conditions in the abundance of breeding adults.

The main difference between the population size estimates in both years is driven by a major entry event of breeding males and females to the breeding area in the spring of 2018 that did not occur in the following breeding season. This is associated with differences in the precipitation regimes between the springs of 2018 (very rainy) and 2019 (virtually no precipitations) (Fig. 1), which suggests that spring rainfall may have had an impact on the breeding success of the population. This potential link should be further tested with data from a longer time series. Although *D. galganoi*

does not present a true hibernation (Martínez-Solano 2014), adults often remain inactive when temperature and humidity conditions are unfavorable (García-París 1985; Barbadillo 1987; Salvador & García-París 2001). Our results suggest that some individuals in the population reduce or cease their activity in the winter and summer, when conditions are more adverse, and subsequently re-activate and enter the breeding area when climate ameliorates in autumn and spring. However, adults of *D. galganoi* can also be active with low air temperatures, between 1ºC and 5ºC (Pleguezuelos & Moreno 1990), and our results indeed show that at least part of the population remains active all year round. In addition to the re-activation of part of the population, the arrival of individuals from terrestrial refugia or other populations nearby for reproduction may also result in higher estimates of *pent* in some periods. However, we have not detected displacements of marked individuals from/to the closest known breeding site, which is located at about 2 km and has also been subject to periodical monitoring. Little is known about the dispersal capacity of *D. galganoi*, but the related species *D. pictus* has been shown to be able to expand rapidly outside of its native range (Llorente *et al*. 2015). Future studies should focus on quantifying connectivity at short spatial scales in order to improve our understanding of population dynamics in *D. galganoi*.

Our results illustrate the usefulness of integrative approaches to the study of population dynamics in the context of intrinsic factors like the reproductive biology of the species and its population genetic diversity. Previous studies estimating N_e or N_b in anurans have mainly focused on ranids and bufonids, showing wide variation among populations and species (Hoffman *et al*. 2004; Beebee 2009; Phillipsen *et al*. 2011). In general, bufonid species are characterized by low N_e values, generally below 100 (Scribner *et al*. 1997; Rowe & Beebee 2004; Brede & Beebee 2006; Wang 2009a). A study estimating N_e in a temporal series using the sibship frequency method revealed a 2.3-fold difference across a 6-years interval in a population of *Bufo bufo* (Coles *et al.* 2019). Whenever census size has been approximated, resulting N_e/N_c ratios are usually very low, in the range of 0.02-0.2 (Rowe & Beebee 2004; Brede & Beebee 2006; Coles *et al.* 2019), but higher N_e/N_c ratios have also been documented in small populations, suggesting genetic compensation

mechanisms (Beebee 2009). In ranid frogs, on the other hand, N_e reports are in the range of hundreds to a few thousands, although much lower estimates are also common (Hoffman *et al*. 2004; Brede & Beebee 2006; Schmeller & Merilä 2007; Ficetola *et al.* 2010; Phillipsen *et al*. 2011; Hinkson & Richter 2016). The N_e/N_c ratios in ranids are usually higher and more variable than in bufonids, with reported estimates in the range of 0.1-1, and even >1 (Hoffman *et al*. 2004; Schmeller & Merilä 2007; Ficetola *et al.* 2010). Our N_e (40-50) and N_b/N_a (0.15-0.22) estimates for *D. galganoi* are within the range reported for bufonids and in the lower range of estimates in ranids, suggesting a high risk of erosion of genetic diversity. Indeed, genetic diversity in our study population is lower than in other studied populations of *D. galganoi* (Gutiérrez-Rodríguez *et al*. 2014).

In small and genetically depauperated populations, genetic compensation mechanisms may contribute to increase N_b and N_e via an increment in the average breeding success of individuals (Beebee 2009; Sánchez-Montes *et al.* 2017), and this in turn may be related with higher polygamy rates (Byrne & Roberts 2012; Gao *et al.* 2019). In our study population we observed high polygamy rates in both males and females, according to inferred pedigrees. This is the first genetic evidence of polygamy and breeding success in *D. galganoi*, although reconstructed pedigrees must be taken with caution due to the low levels of genetic polymorphism detected. The high numbers of inferred mating partners per breeding individual are consistent with the breeding behaviour reported for *D. galganoi*, characterized by the concentration of large numbers of adults in small ponds, with short amplexus times and high numbers of eggs that are laid by females and can be fertilized by multiple males (García-París 1985; González de la Vega 1988; Galán 2003; Díaz-Paniagua *et al*. 2005; Martínez-Solano 2014). However, despite the high inferred polygamy rates, average breeding success in males and females (13-25%) was still relatively low in comparison with other anurans (e.g. 30-94% reported in Broquet *et al.* 2009; Mangold *et al.* 2015; O'Brien *et al.* 2018). Low average breeding success is probably the main factor contributing to the low estimated values of N_e.

The combination of field data, with intensive monitoring and marking of hundreds of adult individuals, and genetic data (genotypes of large samples of adults and larvae) provided the first estimates of population size in the species (N_a), as well as the effective population size and the variation in the effective number of breeders (N_b) between breeding seasons. This in turn allowed estimation of the N_b/N_a ratio, which provides relevant information about the evolutionary potential of the population (Frankham 1995). Moreover, inferred pedigrees allowed thorough exploration of the mating system of *D. galganoi*, suggesting possible intrinsic mechanisms for the observed demographic pattern. Further integrative studies applying similar procedures to characterize the natural variation of demographic parameters in different species and in long-term programs will complement our understanding of amphibian population dynamics, with wide potential implications for evolutionary and conservation research.

ACKNOWLEDGMENTS

References

Abellán P, Svenning J-C (2014). Refugia within refugia – patterns in endemism and genetic divergence are linked to Late Quaternary climate stability in the Iberian Peninsula. *Biological Journal of the Linnean Society* **113**, 13–28.

Akaike H (1974). A new look at the statistical model identification. *IEEE Transactions on Automatic Control* **19(6)**, 716–723.

Allendorf FW (2017). Genetics and the conservation of natural populations: allozymes to genomes. *Molecular Ecology* **26(2)**, 420–430.

Balloux F, Lehmann L (2003). Random mating with a finite number of matings. *Genetics* **165(4)**, 2313–2315.

Banks SC, Cary GJ, Smith AL, *et al.* (2013). How does ecological disturbance influence genetic diversity? *Trends in Ecology and Evolution* **28(11)**, 670–679.

Barbadillo LJ (1987). *La guía de INCAFO de los anfibios y reptiles de la Península Ibérica, Islas Baleares y Canarias.* INCAFO, Madrid.

Beebee TJC (2009). A comparison of single-sample effective size estimators using empirical toad (*Bufo calamita*) population data: genetic compensation and population size-genetic diversity correlations. *Molecular Ecology* **18(23)**, 4790–4797.

Berven KA (1990). Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). *Ecology* **71(4)**, 1599–1608.

Brede EG, Beebee TJC (2006). Large variations in the ratio of effective breeding and census population sizes between two species of pond-breeding anurans. *Biological Journal of the*

Linnean Society **89(2)**, 365–372.

Brooks T, Da Fonseca GAB, Rodrigues, ASL (2004). Species, data, and conservation planning. *Conservation Biology* **18(6)**, 1682–1688.

Broquet T, Jaquiéry J, Perrin N (2009). Opportunity for sexual selection and effective population size in the lek-breeding European treefrog (*Hyla arborea*). *Evolution* **63(3)**, 674–683.

Byrne PG, Roberts JD (2012). Evolutionary causes and consequences of sequential polyandry in anuran amphibians. *Biological Reviews* **87(1)**, 209–228.

Capula M, Nascetti G, Lanza B, Bullini L, Crespo EG (1985). Morphological and genetic differentiation between the iberian and the other west mediterranean *Discoglossus* species (Amphibia Salientia Discoglossidae). *Monitore Zoologico Italiano - Italian Journal of Zoology* **19(1–2)**, 69–90.

Carretero MA, Martínez-Solano Í, Ayllón E, Llorente G (2014). *Lista patrón de los anfibios y reptiles de España (Actualizada a diciembre de 2014)*. Asociación Herpetológica Española, Madrid.

Choquet R, Reboulet AM, Lebreton JD, Gimenez O, Pradel R (2005). *U-CARE 2.2 User's Manual*. CEFE, Montpellier, France.

Clutton-Brock T, Sheldon BC (2010). Individuals and populations: the role of long-term, individual-based studies of animals in ecology and evolutionary biology. *Trends in Ecology and Evolution* **25(10)**, 562–573.

Coles RS, Reading CJ, Jehle R (2019). Linking effective population size dynamics to phenotypic traits in the common toad (*Bufo bufo*). *Conservation Genetics* **20**, 987–995.

Cormack RM (1964). Estimates of Survival from the Sighting of Marked Animals. *Biometrika* **51**, 429–438.

Díaz-Paniagua C, Gómez-Rodríguez C, Portheault A, de Vries W (2005). *Los anfibios de Doñana (Naturaleza y Parques Naturales)*. Organismo Autónomo de Parques Nacionales, Madrid.

Dufresnes C, Pribille M, Alard B *et al.* (2020). Integrating hybrid zone analyses in species delimitation: lessons from two anuran radiations of the Western Mediterranean. *Heredity* **124(3)**, 423–438.

Ficetola GF, Padoa-Schioppa E, Wang J, Garner TWJ (2010). Polygyny, census and effective population size in the threatened frog, *Rana latastei*. *Animal Conservation* **13(s1)**, 82–89.

Ficetola GF, Barzaghi B, Melotto A *et al.* (2018). *N*-mixture models reliably estimate the abundance of small vertebrates. *Scientific Reports* **8,** 10357.

Frankham R. (1995). Effective population size/adult population size ratios in wildlife: a review. *Genetics Research* **66(2)**, 95–107.

Galán P (1999). *Conservación de la herpetofauna gallega*. Universidade da Coruña, A Coruña.

Galán P (2003). *Anfibios y Reptiles del Parque Nacional de las Islas Atlánticas de Galica. Faunistica, Biologia y Conservación*. Ministerio de Medio Ambiente, Madrid.

Gao J, Santi F, Zhou L, Wang X, Riesch R, Plath M (2019). Geographical and temporal variation of multiple paternity in invasive mosquitofish (*Gambusia holbrooki*, *Gambusia affinis*). *Molecular Ecology* **28(24)**, 5315–5329.

García-París M (1985). *Los anfibios de España*. Ministerio de Agricultura, Pesca y Alimentación, Madrid.

García-París M, Montori A, Herrero P (2004). Amphibia, Lissamphibia. In: Ramos MA, Alba J, Bellés i Ros X, *et al.*, eds. *Fauna ibérica.* Museo Nacional de Ciencias Naturales–CSIC, Madrid.

Gascon C, Collins JP, Moore RD, Church DR, Mckay JE, Mendelson JR III, eds (2007). *Amphibian Conservation Action Plan*. IUCN/SSC Amphibian Specialist Group, Gland, Switzerland and

Cambridge, UK.

González de la Vega JP (1988). *Anfibios y Reptiles de la Provincia de Huelva*. Ertisa, Huelva.

Greenberg C, Zarnoch S, Austin J (2018). Long term amphibian monitoring at wetlands lacks power to detect population trends. *Biological Conservation* **228**, 120–131.

Gutiérrez-Rodríguez J, Salvi D, Geffen E, Gafny S, Martínez-Solano Í (2014). Isolation and characterisation of novel polymorphic microsatellite loci in Iberian painted frogs (*Discoglossus galganoi* and *D. jeanneae*), with data on cross-species amplification in *Discoglossus* and *Latonia*. *Herpetological Journal* **24(4)**, 261–265.

Hinkson KM, Richter SC (2016). Temporal trends in genetic data and effective population size support efficacy of management practices in critically endangered dusky gopher frogs (*Lithobates sevosus*). *Ecology and Evolution* **6(9)**, 2667–2678.

Hoffman EA, Schueler FW, Blouin MS (2004). Effective population sizes and temporal stability of genetic structure in *Rana pipiens*, the northern leopard frog. *Evolution* **58(11)**, 2536–2545.

Hoffmann M, Hilton-Taylor C, Angulo A, *et al.* (2010). The impact of conservation on the status of the World's vertebrates. *Science* **330(6010)**, 1503–1509.

Holleley CE, Geerts PG (2009). Multiplex Manager 1.0: a cross-platform computer program that plans and optimizes multiplex PCR. *BioTechniques* **46(7)**, 511–517.

Holman L, Kokko H (2013). The consequences of polyandry for population viability, extinction risk and conservation. *Philosophical Transactions of the Royal Society B: Biological Sciences* **368(1613)**, 20120053.

Jolly GM (1965). Explicit estimates from capture-recapture data with both death and immigration stochastic model. *Biometrika* **52**, 225–247.

Jones OR, Wang J (2010). COLONY: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources* **10(3)**, 551–555.

Kendall WL, Nichols JD (1995). On the use of secondary capture-recapture samples to estimate temporary emigration and breeding proportions. *Journal of Applied Statistics*, **22(5-6)**, 751–762.

Levine BA, Douglas MR, Adams AAY *et al.* (2019). Genomic pedigree reconstruction identifies predictors of mating and reproductive success in an invasive vertebrate. *Ecology and Evolution* **9(20)**, 11863–11877.

Llorente G, Montori A, Pujol-Buxó E (2015). El sapillo pintojo mediterráneo (*Discoglossus pictus*) en la península ibérica. *Boletín de la Asociación Herpetologógica Española* **26**, 15-19.

Luikart G, Ryman N, Tallmon DA, Schwartz MK, Allendorf FW (2010). Estimation of census and effective population sizes: the increasing usefulness of DNA-based approaches. *Conservation Genetics* **11(2)**, 355–373.

Mangold A, Trenkwalder K, Ringler M, Hödl W, Ringler E (2015). Low reproductive skew despite high male-biased operational sex ratio in a glass frog with paternal care. *BMC Evolutionary Biology* **15(1)**, 181.

Márquez R, Lizana M (2002). Conservación de los Anfibios y Reptiles de España. In: Pleguezuelos JM, Márquez R, Lizana M, eds. *Atlas y Libro Rojo de los Anfibios y Reptiles de España*. Dirección General de Conservación de la Naturaleza-Asociación Herpetologica Española, Madrid, pp. 417–453.

Martínez-Solano, Í (2014). Sapillo pintojo ibérico - *Discoglossus galganoi*. In: Salvador A, Martínez-Solano Í, eds. *Enciclopedia Virtual de los Vertebrados Españoles*. Museo Nacional de Ciencias Naturales, Madrid.

Martínez-Solano Í, García-París M (2002). *Discoglossus jeanneae*. In: Pleguezuelos JM, Márquez R, Lizana M, eds. *Atlas y Libro Rojo de los Anfibios y Reptiles de España*. Dirección General de Conservación de la Naturaleza – Asociación Herpetológica Española, Madrid, pp. 88–90.

Merrel DJ (1981). *Ecological Genetics*. Longman, London.

Nunney L, Campbell KA (1993). Assessing minimum viable population size: demography meets population genetics. *Trends in Ecology and Evolution* **8(7)**, 234–239.

O'Brien DM, Keogh JS, Silla AJ, Byrne PG (2018). The unexpected genetic mating system of the red-backed toadlet (*Pseudophryne coriacea*): a species with prolonged terrestrial breeding and cryptic reproductive behaviour. *Molecular Ecology* **27(14)**, 3001–3015.

Palstra FP, Fraser DJ (2012). Effective/census population size ratio estimation: a compendium and appraisal. *Ecology and Evolution* **2(9)**, 2357–2365.

Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288–295.

Peakall R, Smouse PE (2012). GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research – an update. *Bionformatics* **28**, 2537–2539.

Pechmann JH, Scott DE, Semlitsch RD, Caldwell JP, Vitt LJ, Gibbons J W (1991). Declining amphibian populations: the problem of separating human impacts from natural fluctuations. *Science* **253**, 892–895.

Pechmann JH, Wilbur HM (1994). Putting declining amphibian populations in perspective: natural fluctuations and human impacts. *Herpetologica* **50**, 65–84.

Phillipsen IC, Funk WC, Hoffman EA, Monsen KJ, Blouin MS (2011). Comparative analyses of effective population size within and among species: ranid frogs as a case study. *Evolution* **65(10)**, 2927–2945.

Pleguezuelos JM, Moreno M (1990). *Atlas herpetológico de la provincia de Jaén*. Agencia de Medio Ambiente, Junta de Andalucía, Sevilla.

Pollock KH (1982). A capture-recapture design robust to unequal probability of capture. *Journal of Wildlife Management* **46**, 752–757.

Pradel R, Hines JE, Lebreton JD, Nichols JD (1997). Capture-recapture survival models taking account of transients. *Biometrics* **53(1)**, 60–72.

Pradel R, Sanz-Aguilar A (2012). Modeling trap-awareness and related phenomena in capture-recapture studies. *PLoS ONE* **7(3)**, e32666.

Rovelli V, Randi E, Davoli F, Macale D, Bologna MA, Vignoli L (2015). She gets many and she chooses the best: polygynandry in *Salamandrina perspicillata* (Amphibia: Salamandridae). *Biological Journal of the Linnean Society* **116(3)**, 671–683.

Rowe G, Beebee TJC (2004). Reconciling genetic and demographic estimators of effective population size in the anuran amphibian *Bufo calamita*. *Conservation Genetics* **5(3)**, 287–298.

Salvador A, García-París M (2001). *Anfibios Españoles. Identificación, Historia Natural y Distribución*. Esfagnos, Talavera de la Reina.

Sánchez-Montes G, Wang J, Ariño AH, Vizmanos JL, Martínez-Solano Í (2017). Reliable effective number of breeders/adult census size ratios in seasonal-breeding species: opportunity for integrative demographic inferences based on capture-mark-recapture data and multilocus genotypes. *Ecology and Evolution* **7(23)**, 10301–10314.

Sánchez A, Talavera A, Hinckley A (2015). Descripción y conservación de una población urbana de *Discoglossus galganoi*. *Boletín Asociación Herpetológica Española* **26(1)**, 72–75.

Schmeller DS, Merilä J (2007). Demographic and genetic estimates of effective population and breeding size in the amphibian *Rana temporaria*. *Conservation Biology* **21(1)**, 142–151.

Accepted Article

Schwarz CJ, Arnason AN (1996). A general methodology for the analysis of capture-recapture experiments in open populations. *Biometrics* **52(3)**, 860–873.

Scribner KT, Arntzen JW, Burke T (1997). Effective number of breeding adults in *Bufo bufo* estimated from age-specific variation at minisatellite loci. *Molecular Ecology* **6**, 701–712.

Seber GAF (1965). A note on the multiple recapture census. *Biometrika* **52**, 249–259.

Skelly DK, Yurewicz KL, Werner EE, Relyea RA (2003). Estimating decline and distributional change in amphibians. *Conservation Biology* **17(3)**, 741–755.

Stuart SN, Chanson JS, Cox N, *et al.* (2004). Status and trends of amphibian declines and extinctions worldwide. *Science* **306(5702)**, 1783–1786.

Toledo LF, Becker CG, Haddad CFB, Zamudio KR (2014). Rarity as an indicator of endangerment in neotropical frogs. *Biological Conservation* **179**, 54–62.

Trenham PC, Koenig WD, Mossman MJ, Stark, SL, Jagger LA (2003). Regional dynamics of wetland-breeding frogs and toads: turnover and synchrony. *Ecological Applications* **13**, 1522–1532.

Verdade VK, Valdujo PH, Carnaval AC, *et al*. (2012). A leap further: the Brazilian amphibian conservation action plan. *Alytes* **29(1–4)**, 27–42.

Vieites DR, Nieto-Román S, Barluenga M, Palanca A, Vences M, Meyer A (2004). Post-mating clutch piracy in an amphibian. *Nature* **431(7006)**, 305–308.

Wang IJ (2009a). Fine-scale population structure in a desert amphibian: landscape genetics of the black toad (*Bufo exsul*). *Molecular Ecology* **18(18)**, 3847–3856.

Wang J (2009b). A new method for estimating effective population sizes from a single sample of multilocus genotypes. *Molecular Ecology* **18(10)**, 2148–2164.

Waples RS, Antao T, Luikart G (2014). Effects of overlapping generations on linkage disequilibrium

estimates of effective population size. *Genetics* **197(2)**, 769–780.

White GC, Burnham KP (1999). Program MARK: survival estimation from populations of marked animals. *Bird Study* **46(s1)**, 120–139.

Witmer GW (2005). Wildlife population monitoring: some practical considerations. *Wildlife Research* **32(3)**, 259.

Wright S (1931). Evolution in Mendelian populations. *Genetics* **16**, 97–159.

SUPPLEMENTARY MATERIALS

Table *S*1 Structure of primary and secondary occasions *used in robust design analyses*

Table S2 Set of microsatellites used to genotype D. galganoi individuals (original reference: Gutiérrez-Rodríguez et al. (2014)). Locus name, multiplex reaction, volume of 100 μM stock of each F and R primer required for 250 μL of primer mix, allelic richness (AR), expected heterozygosity (H_E) and observed heterozygosity (H_O) for each locus.

Table S3 Pedigrees for the 2018 D. galganoi larvae according to the final baseline analysis, and concordance in replicated analyses 1) with the same parameters (changing the random seed (Seed)), 2) with different a priori probability of the presence of parents among the sample of genotyped adults (Prob) and 3) using the weak sibship size prior = 1.

Table S4 Pedigrees for the 2019 D. galganoi larvae according to the final baseline analysis, and concordance in replicated analyses 1) with the same parameters (changing the random seed (Seed)), 2) with different a priori probability of the presence of parents among the sample of genotyped adults (Prob) and 3) using weak sibship size prior = 1.

Table 1 Best POPAN models (AICc weight>0) for the 2017-2018 and 2018-2019 periods. Param:

number of parameters of the model

Period	Model	AICc	AICc weight	Param.	Deviance
	{p(g*t) φ(g) *pent*(t)}	1234.20	0.9908	62	-144.62
2017-	{p(g) φ(t) *pent*(t)}	1243.69	0.0086	41	-77.60
2018	{p(g) φ(g) *pent*(t)}	1249.65	0.0004	24	-30.37
	{p(g*t) φ(t) *pent*(t)}	1251.50	0.0002	77	-173.68
2018-	{p(g*t) φ(g) *pent*(t)}	941.31	0.9142	65	164.58
2019	{p(g) φ(g) *pent*(t)}	946.05	0.0858	25	291.56

Table 2 Average N_a estimates based on POPAN and robust design analyses with their 95% confidence intervals (in parentheses)

Period	N_a POPAN		Season	N_a Robust design	
	Males	Females		Males	Females
			Autumn 1	**27** (21-33)	**97** (1-194)
2017-2018	**116** (99-134)	**135** (102-169)	Winter 1	**24** (20-28)	**29** (21-36)
			Spring 1	**69** (60-78)	**59** (43-76)
			Autumn 2	**26** (13-39)	**19** (11-27)
2018-2019	**49** (39-59)	**65** (48-82)	Winter 2	**20** (4-36)	**47** (0-107)
			Spring 2	**25** (24-26)	**22** (20-24)

Table 3 Best robust design models (AICc weight>0) ranked by their AICc. Param: number of parameters of the model

Model	AICc	AICc weight	Param.	Deviance
{Markovian S(t)}	2321.19	0.7002	81	1955.11
{Random Movement S(t)}	2323.02	0.2817	75	1973.54
{No Movement S(t)}	2329.02	0.0140	68	1998.36
{Markovian S(.)}	2332.76	0.0022	79	1972.26
{Markovian S(g*t)}	2334.80	0.0008	86	1954.53
{Markovian S(g)}	2335.39	0.0006	80	1972.11
{Random Movement S(g*t)}	2335.63	0.0005	80	1972.34
{No Movement S(g*t)}	2339.41	0.0001	73	1995.37

Table 4 Estimates of the number of alleles (AR), number of private alleles (PA), observed (H$_O$) and expected heterozygosity (H$_E$) with their 95% CIs for the breeding populations of D. galganoi in 2017-18 and 2018-19.

Period	AR	PA	H$_O$	H$_E$
2017-2018	**3.4** (2.24-4.56)	**0.07** (0-0.2)	**0.35** (0.22-0.49)	**0.37** (0.23-0.50)
2018-2019	**3.4** (2.27-4.53)	**0.07** (0-0.2)	**0.36** (0.22-0.51)	**0.37** (0.23-0.51)

Table 5 N_b estimates (with their 95% CI) for the breeding populations of D. galganoi in 2018 and 2019. Different analyses used different a priori probabilities of parents being included in the genotyped sample (Prob) and were conducted using or not a weak sibship size prior = 1 (Sib. Prior).

Breeding season	Prob	Sib. Prior	N_b
2018	males: 0.76 females: 0.57	No	**37** (24-59)
			33 (22-55)
		Weak	**37** (24-59)
			38 (25-31)
	males: 0.87 females: 0.82	No	**35** (21-58)
			33 (21-53)
		Weak	**34** (22-56)
			38 (25-60)
2019	males: 0.82 females: 0.69	No	**25** (15-46)
			24 (15-44)
		Weak	**30** (19-52)
			26 (15-46)
	males: 0.99 females: 0.99	No	**25** (15-46)
			24 (14-44)
		Weak	**26** (15-47)
			26 (16-46)

Figure 1 Temporal dynamics in the probability of entrance of individuals into the population (pent) and partial estimates of N_a for each session according to POPAN results of the best ranked model over the two periods of study. Columns represent accumulated precipitation (in mm) in each hydrometeorological year.
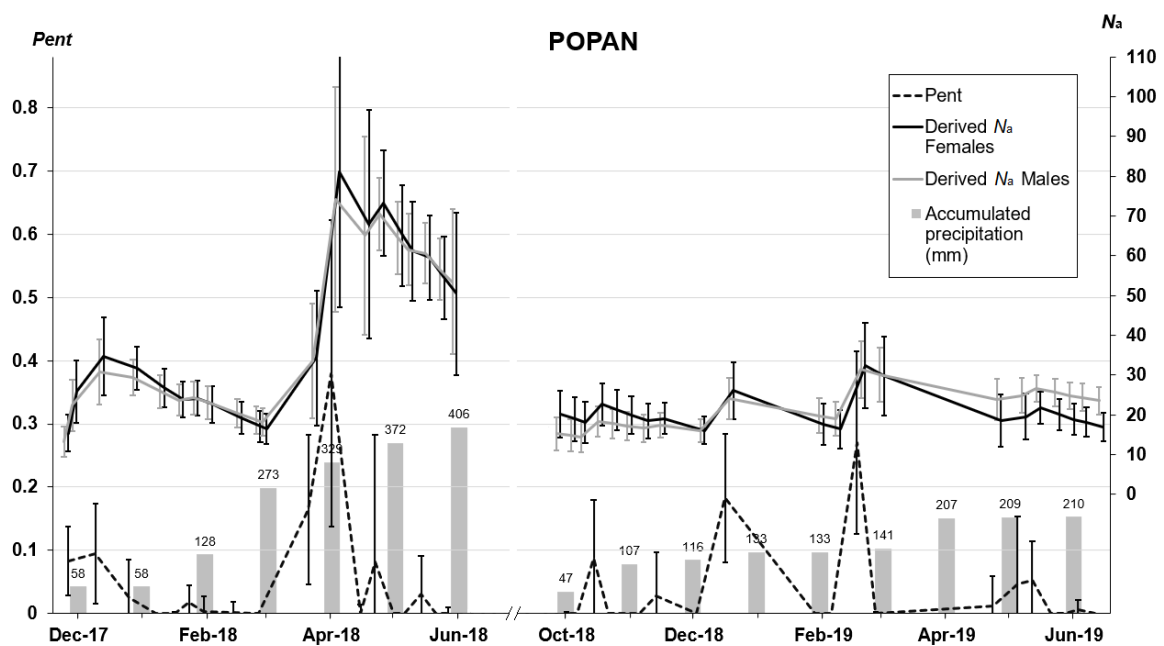
Figure 2 Dynamics of N$_a$, survival (S) and migration rates (γ'') estimated from robust design analyses.
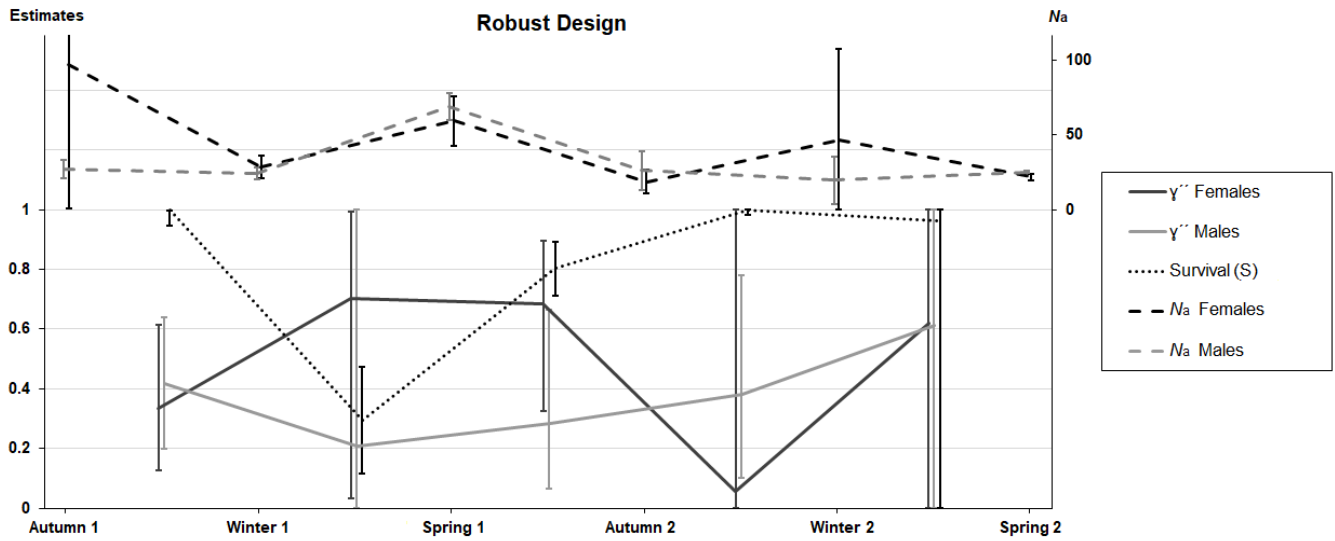
Figure 3 Number of partners per successfully breeding individual inferred from the reconstructed pedigrees for the 2018 (left) and 2019 (right) breeding seasons.