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Combining spawn egg counts, individual photo-ID and genetic fingerprinting to estimate the population size and sex ratio of an Endangered amphibian.

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Running title: Population size and sex ratio

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Abstract

For pond breeding amphibians, spawn egg counts are frequently used for population abundance estimation. Numbers of spawn clumps are taken as a proxy of breeding females whilst the likely sex ratio is often to estimate male numbers. Sex ratios, however, are rarely at parity rendering extrapolation of total population estimates from egg counts dubious. This is a problem when dealing with declining species where accurate population estimation is important for informing conservation. We monitored the breeding activity of the Natterjack toad (*Epidalea calamita*, Laurenti 1768), combining egg string counts and individual photo-identification with Capture-Mark-Recapture population size and operational sex ratio estimation. Male Natterjack toads were identified by the pattern of natural markings with repeated ID of the same individual confirmed for 10% of the samples using genetic fingerprinting. We identified 647 unique individuals within a closed study population at Caherdaniel, Co Kerry. Population estimates derived from egg string counts estimated a breeding population of 350 females (95%CI 331-369) and Capture-Mark-Recapture estimated a breeding population of 1,698 males (95%CI 1,000 - 2,397). The female:male sex ratio was conservatively estimated at 1:6 (95%CI 1:3 - 1:7) where $62 \pm 6\%$ of females were assumed to spawn. These substantially departed from any *priori* assumption of 1:1 which could have underestimated the breeding population by up to 83%. Where amphibian absolute population size estimation is necessary, methods should include empirical survey data on operational sex ratios and not rely on assumptions or those derived from the literature which may be highly population and/or context-dependent.

44 Introduction

45 Amphibians are the most threatened vertebrate group with 41% of species threatened with
 46 extinction. Population declines have been detected even in common and widespread
 47 species (Young *et al.* 2001; Stuart *et al.* 2004; Nyström *et al.* 2007; Adams *et al.* 2013;
 48 Grant *et al.* 2016; Petrovan & Schmidt 2016). Potential causes for the observed declines
 49 include habitat destruction and fragmentation (Cushman 2006), contamination (Mann *et*
 50 *al.* 2009; Brühl *et al.* 2013), spread of pathogens (Berger *et al.* 1998; Lips 1999; Daszak
 51 *et al.* 2003; Martel *et al.* 2013), climate change (Carey & Alexander 2003), invasive
 52 species (Johnson *et al.* 2011), illegal harvest and trade (Schlaepfer *et al.* 2005; Chan *et*
 53 *al.* 2014) or interaction among several factors (Pounds *et al.* 2006). Methods supporting
 54 monitoring and surveillance are, therefore, particularly important when dealing with
 55 threatened and declining species to inform conservation (Schmidt 2004). Most studies on
 56 amphibian demographics, distribution and dynamics use indirect count data, for example,
 57 counts of egg strings or spawn clumps (for females) or call vocalisations (for males). Such
 58 measures are proxies for abundance, are vulnerable to survey bias and errors and are likely
 59 imperfect reflections of absolute numbers (Schmidt 2005; Mazerolle *et al.* 2007; Wagner
 60 *et al.* 2011). Methodological bias and error, both in field surveys but also subsequent
 61 statistical extrapolation, based on assumptions that have not been empirically tested, are
 62 likely to yield unreliable abundance estimates generating spurious population trends
 63 misinforming conservation management programmes (Kéry & Schmid 2004; Schmidt
 64 2005). For example, chorus size of male European tree frogs (*Hyla arborea*, Linnaeus
 65 1758) taken as an indirect measure has little relationship to abundance estimates derived
 66 from recapture studies and fails to detect significant interannual population change (Pellet
 67 *et al.* 2007). Indirect count data have been shown by numerous studies to underestimate
 68 true population size (e.g. Mazerolle *et al.* 2007; Dodd & Dorazio 2004) Thus, survey and
 69 analytical methods should incorporate variation in detection probability, for example,
 70 adopting Capture-Mark-Recapture or Distance-Sampling techniques, to provide more
 71 robust estimates of population parameters (Schmidt 2003).

72 Indirect measures such as egg counts can be a reliable survey technique for
 73 estimating the number of reproductive females (Windmiller 1996; Crouch & Paton 2000;
 74 Brede & Beebee 2006). For some species, like the Natterjack toad (*Epidalea calamita*,

Laurenti 1768), egg string counts can be a good indicator of the number of breeding females as the species usually lays just one egg string per year (Danton & Beebee 1996). However, this method still relies on assumptions regarding the proportion of females that breed annually which depends on weather conditions and availability of breeding sites (Smith & Skelcher 2019). Transferring egg counts into population size estimates can be problematic and highly inaccurate due to extreme variation in sex ratios in amphibians. For instance, the female:male sex ratio of the common toad (*Bufo bufo*) in Great Britain and Sweden are known to vary between 1:2 and 1:8 (Gittins 1983; Reading 1991; Reading 2001; Scribnner *et al.* 2001). Hence, extrapolating male population estimates from egg counts without knowledge of operational sex ratios is not to be recommended.

Capture-Mark-Recapture (CMR) provides a robust population size estimate with associated margins of error but relies on the reliable recognition of individuals where the species has individually recognisable markings e.g. natural ventral markings (Mettouris *et al.* 2016), passive DNA collecting allowing the recognition of individuals by genetic fingerprinting e.g. using microsatellite markers (Boersen *et al.* 2003; Ringler *et al.* 2015) or invasive techniques such as toe clipping (Grafe *et al.* 2011) or the use of tags such as Passive Integrated Transponder (PIT) tags (Grant & Raymond 2011; Weber *et al.* 2019). Due to animal welfare implications and potential impacts on behaviour including movements, modern studies tend to favour passive techniques. CMR analyses provide robust estimates of demographic parameters while simultaneously accounting for an imperfect detection. If the assumptions of CMR models are not met, then the magnitude and direction of the bias can be quantified and population estimates statistically adjusted accordingly (Manly *et al.* 1999; Schmidt *et al.* 2002; Schmidt 2004).

The Natterjack toad is the rarest and most range restricted amphibian in Ireland, currently Regionally Red-listed as Endangered (King *et al.* 2011). The species is listed under Annex IV of the EU Habitat and Species Directive (92/43/EEC) with EU member states required under Article 17 to report regularly to the European Commission on species' population size and trend. Methods for estimating the species total population size for the assessment of trends (Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019) have relied on egg string counts extrapolated using assumptions for the number of females that spawn annually (following Audry & Emmerson 2005, derived from Denton & Beebee 1993), and the operational sex ratio typically assumed to be 1:1 (following

Dentn & Beebee 1996; Buckley *et al.* 2014). Egg counts are known to be a reasonably effective method in monitoring reproductive females (Crouch & Paton 2000) but amphibian sex ratios are well known to be highly male-biased at breeding sites (e.g. Elmberg 1990; Friedl & Klump 1997; Loman & Madsen 2010) suggesting this may be the greatest source of potential error when estimating the absolute breeding population size.

This study aimed to empirically estimate the operational sex ratio of a (closed) breeding Natterjack toad population in Ireland (with no immigration or emigration due to the study population's geographical isolation). We adopted egg string counts to estimate the female breeding population size while the male breeding population was estimated (with associated confidence intervals) by CMR using passive photo-identification to recognise individuals from their ventral markings verified by molecular genetic fingerprinting using microsatellite markers on DNA recovered from skin swabs. We combine these techniques to estimate the total breeding population size over two years and the sex ratio allowing us to quantify the degree of departure from an assumed 1:1 sex ratio and the error such an assumption might produce. Amphibian sex ratios are difficult to estimate with any degree of accuracy; thus, our approach will not only inform the conservation of the Endangered Natterjack toad in Ireland but also methods for other amphibian demographic studies.

Materials and methods

Study area

We conducted the study at Caherdaniel, Co. Kerry (51.7592°N, -10.1230°E); the most southerly Natterjack toad population in Ireland (Figure 1). The site was selected as it was isolated from all other Natterjack toad populations ensuring it was closed with no immigration or emigration. There are only three breeding ponds (C1= 0.20ha, C2= 0.33ha and C3= 0.03ha) located within 200 m of each other; thus, surveys were able to cover the entire breeding site thoroughly. The habitat was sand dune with a short maritime grass sward.

Field surveys

We conducted fieldwork in 2017 and 2018 during the Natterjack toad's breeding season (April -July) following Reyne et al. (2019). We visited breeding ponds every seven to ten days through the duration of the breeding season. This visit interval was chosen to ensure that egg strings could not be deposited and hatch between visits thus being missed. We recorded the total number of egg strings in each pond by walking the perimeter searching shallow water and aquatic vegetation. Surveys of deeper water away from the perimeter were made using a zigzag transect method. For each pond we mapped egg string locations and stages of development based on Gosner stages (Gosner 1960) in order to avoid double counting during consecutive visits. The earliest stage (Gosner stages 2-6) consisted of two lines of recently laid eggs; the second stage (Gosner stages 7-14) had a single line of eggs; and the third stage near (Gosner stage 16) consisted of well-developed embryos with defined tails. It was assumed that each breeding female deposited one egg string, thus, egg string counts were taken as equivalent to the minimum number of breeding females.

We plotted the cumulative number of egg strings / minimum number of females at each pond visit over the full survey season for each year and the asymptote calculated using a Generalized Additive Model (GAM) which allowed 95% Confidence Intervals to be associated with counts. The lower confidence limit (LCL) accounted for potential double counting where the real number was lower than that counted and the upper confidence limit (UCL) accounted for egg strings potentially being missed (perhaps at depth or within dense vegetation) where the real number was lower.

We conducted a CMR study to estimate the male population size. We sampled breeding aggregations of males at the three breeding ponds by conducting thorough searches for one hour after dusk and capturing by hand all male toads seen (identified by the presence of purple colouration on the neck) or heard calling. We cleaned all toads with water prior to their ventral surface being photographed and swabbed to collect DNA. After sampling, all toads were returned to the breeding site safely and no animals were harmed.

Photo-ID

We placed each male toad in a transparent box and secured them with a light sponge in order to standardize image acquisition. We took photographs using Nikon D3400 and

stored them in a digital catalogue. Individuals were identified based on natural ventral markings without the need of invasive techniques such as toe-clipping. Each toad was assigned with a unique numeric identifier. Photographs of captured individuals were compared by single eye matching and a capture history for each individual was constructed.

Genetic fingerprinting

We collected DNA using synthetic sterile cotton swabs (CamLab). Skin swabs have been shown to be an efficient and reliable method for collecting non-invasive samples from amphibian species (Prunier *et al.* 2012). We gently brushed the ventral side of each toad to collect skin cells, avoiding poison glands to prevent contaminating the samples with potential PCR inhibitors. Swabs were stored in 100% ethanol at ambient temperature. Approximately 10% of the samples were used for genetic fingerprinting in order to verify and provide quality assurance with respect to photo-ID. We used a high salt DNA extraction protocol following Aljanabi and Martinez (1997) as a reliable and cheap alternative to commercial kits (Müller *et al.* 2013). We added a few additional steps to the extraction protocol in order to remove PCR inhibitors (Protocol S1 & S2). We amplified each sample using seven fluorescently labelled microsatellite markers developed by Rowe *et al.* (1997, 2000) and Faucher *et al.* (2016) (Table S1). Microsatellite markers were chosen based on high levels of polymorphism, low genotyping errors and low probability of the set of molecular markers failing to differentiate between two randomly selected individuals (<0.001). Forward primers were labelled with 6-FAMTM, NEDTM and VICTM fluorescent dye (Applied Biosystems, Integrated DNA Technologies). We run 10µl reactions containing 1µl of genomic DNA, 5 µl Type-it Multiplex PCR Master Mix (Qiagen) and primer mix of labelled forward and reverse with 0.1 – 0.3 µM final concentrations. PCR cycling program had an initial denaturation of 95°C for 15min; 35 cycles of 94 °C for 30sec, annealing temperature 58°C for 90sec, and 72°C for 60sec, and final extension at 60°C for 30min. Samples were randomized during genotyping analysis to avoid bias. Amplification was confirmed for PCR products on 100ml 1% agarose gel stained with ethidium bromide under UV light. PCR product was diluted ten times with ddH₂O. Diluted PCR product (1 µl) together with 8.95µl Hi-DiTM Formamide (Thermo Scientific) and 0.05 µl GeneScanTM 600LIZ ladder (Applied Biosystems) were pooled together for fragment analysis. The microsatellite

genotyping was performed on ABI 3730xl Genetic Analyzer (Applied Biosystems). Alleles were scored with GeneMarker® V1.8 and Peak Scanner™ Software v1.0 (Life Technologies, Inc.). We rounded all genotype calls to an even or odd number based on the respective locus. Differences between assigned and actual allele size were between 0.1 and 1 bp.

We genotyped all samples between three and seven times to identify real genotypes as skin swab samples had a fairly high genotyping error rate. Alleles had to be identical across a minimum two repetitions for heterozygote individuals and three repetitions for homozygotes. We calculated the probability of genotyping errors using PEDANT v10 software (Johnson & Haydon 2007). We used 10,000 search steps to calculate the maximum likelihood error rate of allelic dropout (ADO) and false allele (FA). Contamination with genetic material of a different individual was assigned in cases where additional alleles were present (three alleles per locus). We calculated the percentage of genotyping success over all loci and all repetitions. We calculated the combined non-exclusion identity probability for the chosen set of markers using Cervus v3.07. We used GENECAP, a Microsoft Excel macro (Wilberg *et al.* 2004), to produce a capture history where matching genotypes are considered to belong to the same individual, hence are classified as recaptures. Finally, we compared the number of identified individuals from genetic fingerprinting and photo-ID.

Population size and sex ratio estimation

We used egg string counts to estimate the breeding female population size of the Natterjack toad based on methods used by previous studies in Ireland (Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019). Notwithstanding rare instances of double or multiple clutching (Beebee & Denton 1996), females typically deposit one egg string only (Buckley & Beebee 2004); thus, an accurate egg string count represents the minimum number of females breeding in one year. Denton & Beebee (1993) derived from Denton (1991) studied a Natterjack population in Hampshire (UK) over 5 years and concluded “in any one year only 44-64% of females spawned”. Stephan *et al.* (2001) working in Brandberge, Germany from 1992-1999 reported an average estimate of 63% of females spawning annually. Drivers of reproductive activity in females are likely highly context dependent varying between ponds, populations and with weather conditions and

invertebrate prey abundance. No empirical data were available for Ireland, however, from the published literature (Table 1) we estimated the median $\pm$ 95% CIs of the percentage of females that breed annually to be $62 \pm 6\%$ (95% CIs 56 - 68%). Thus, to convert egg strings counts into a female population estimate, the lower confidence limit (LCL) was derived as $F_{LCL} = S/0.52$, the mean estimate by $F_{mean} = S/0.58$ and the upper confidence limit (UCL) by $F_{UCL} = S/0.65$, where F = the estimated female population and S = the total number of egg strings counted. This provided some estimate of the potential error in estimating the female population size only. Initial population size models (following Bécart *et al.* 2007; Sweeney *et al.* 2013; Reyne *et al.* 2019) assumed a sex ratio of 1:1 (following Buckley *et al.* 2014), therefore, we doubled the estimated female population size to estimate the total breeding population.

We performed CMR analysis using the software program MARK (White & Burnham 1999). The software uses Maximum Likelihood procedures to estimate population parameters (Cooch & Gary 2019). Natterjack toads have a prolonged breeding season (April – July annually). The number of individuals at the breeding sites varies throughout the breeding season with no period when all individuals of the population are present. Even though our study population was closed at the global level (no immigration or emigration), Wagner *et al.* (2011) recommends using an open population modelling approach for amphibians with a prolonged breeding season where individuals can come and go from the breeding site as a better match to their phenology. For our analysis, we used an open population modelling approach based on a Cormack-Jolly-Seber model (Jolly 1965; Seber 1965). We performed analyses using the computer package POPAN (sub-module of MARK) developed by Schwarz and Arnasan (1996). Parameterization included N representing the size of a super-population, that is the total number of individuals in the area, ϕ was the survival rate, p was the probability of capture and b was the probability of an animal from the super-population entering the sub-population (i.e. toads accruing at the breeding site). In the model, (t) and $(.)$ represent time-dependant and constant parameters respectively. The first step in the analysis was to verify if our data met the two main model assumptions: all marked individuals have the same probability of capture and survival between i and $i+1$ sampling events. We used the RELEASE function to run a Goodness-of-Fit (GOT) test for a fully time dependant model $p(t) \phi(t) b(t)$. In case the model assumptions were not met, we calculated a *post-hoc* Variance

Inflation Factor (VIF) to adjust for the lack of fit, resulting in a Quasi-Akaike Information Criterion corrected for small sample size (QAIC_C) value. We constructed models based on different combinations of time-dependant and consistent parameters (ϕ , p and b). The single best appropriating model (model with the lowest QAIC_C value) was used to estimate the breeding male population size (with associated 95% Confidence Intervals).

The estimated breeding female and male populations were summed to get the total breeding population. The operational sex ratio was expressed as the ratio of females:males. The total population derived from combined egg string counts and genetically verified photo-ID CMR was contrasted with a traditional population model derived from egg string counts and assuming a 1:1 sex ratio expressed as the percentage difference between the models (propagating 95% CIs to obtain likely ranges).

Results

Egg string counts / minimum no females

In total, we conducted 30 pond visits throughout the Natterjack toad breeding season during 2017 ($n=17$) and 2018 ($n=13$). We recorded a total of 231 egg strings / minimum number of females during 2017 and 224 egg strings / minimum number of females during 2018. There was a significant sigmoidal accumulation of egg strings (Figure 2) during both 2017 ($F=5674$, $p<0.001$, $r^2=0.906$) and 2018 ($F=9439$, $p<0.001$, $r^2=0.960$) resulting in $\pm 4.1\%$ error during 2017 (95%CI 212 – 250) and $\pm 1.2\%$ error during 2018 (95%CI 218 - 229).

Photo-ID

Over the course of nine survey nights (four during 2017 and five during 2018), we collected, photographed and swabbed a total of 884 male toads. We identified 647 unique individuals based on photographs of ventral marking comparisons (Figure 3) enabling recapture histories to be constructed for each individual and the total population (Table 2). The recapture rate was 26%. The individual accumulation curve showed no sign of asymptote increasing linearly throughout the study i.e. a roughly similar number of new male individuals were identified at each successive capture event (Figure 4).

Genetic fingerprinting

In total, we genotyped 65 samples (equating to a 10% sample of individual males). Final DNA concentration ranged between 1.1 and 32.4 ng/μl (mean ± SE: 10.3 ± 8.2). The genotyping success rate overall was low with an average of 33% (Table S2). Genotyping success for one of the markers (BC22) was 8%, hence we excluded the marker from further analysis. The average probability that the final set of microsatellite markers failed to differentiate between two randomly chosen individuals was 0.014, suggesting that the selected assay had an adequate power for individual discrimination. Average rate of genotyping error per allele due to ADO and FA was 0.023 and 0.001 respectively (Table S2). We discarded samples that either contained DNA from more than one individual (5%) or DNA that failed to amplify for two or more loci (37%) as the probability of successful identification of individuals based on four or fewer markers was low. Hence, we retained 39 samples to create a capture history. Results suggested a total of 45 alleles with an average of 6.4 alleles per locus. GENECAPI identified 16 individual recaptures and 25 unique genotypes that matched the individual recapture identities assigned from photo-ID by comparison of images by eye. Thus, male toads recognised as different individuals by their ventral markings were also identified as different individuals by their microsatellite genotypes and *vice versa* providing a measure of quality assurance for photo-ID recapture histories.

Male population size

For the mark-recapture analysis we tested eight different models. Three models failed to achieve numerical convergence: 1) $p(t) \phi(t) b(\cdot)$, 2) $p(t) \phi(\cdot) b(\cdot)$ and 3) $p(\cdot) \phi(t) b(\cdot)$. The Goodness-of-fit test for the fully time dependant model suggested the presence of overdispersion violating one of the main assumptions of the model i.e. equal survival of all individuals from i to $i+1$ sampling events (TEST2 + TEST3, Table S3). We calculated and applied a *post-hoc* VIF of $\hat{c} = 1.62$. Based on QAIC_C, the model we selected was $p(t) \phi(\cdot) b(t)$ where detection probability and survival were constant while capture probability varied with time (Table 3). The single best approximating model estimated the breeding male population at 1,698 (95%CI = 1,000 - 2,397) individuals. Separate models were not fitted for each year as the number of capture events per year was low resulting in the failure of the Goodness-of-test for 2017 whilst the model for 2018 yielded 95%CI of low utility given their width.

Total population size

Combining egg string counts to derive a female population estimate and photo-ID verified by genetic fingerprinting to derive a male population estimate (Table 4), we estimated the operational sex ratio (propagating and compounding the 95% CIs associated with both) at 1:7 (95% CI 1:5 - 1:10) where egg string numbers were taken as the minimum number of females. Adjustment for the percentage of females likely to spawn ($62 \pm 6\%$) estimated the sex ratio at 1:5 (95% CI 1:3 - 1:7). Both substantially departed from an *a priori* assumption of 1:1 which would have underestimated the total breeding population by 76% (95% CI 68 - 82%) where egg string numbers were taken as the minimum number of females or by 78% (95% CI 69 - 83%) where 62% of the female population was assumed to spawn annually.

Discussion

Population size estimates for the Natterjack toad population at Caherdaniel, Co. Kerry, Ireland derived from egg spawn counts and CMR data differed substantially. Our results demonstrated clearly that assuming a 1:1 sex ratio could underestimate the population by up to 83%. Such method provides a weak basis for understanding population dynamics and could easily fail to detect temporal changes in the population trend and would, therefore, be highly unreliable when developing conservation and management strategies.

Egg spawn counts are widely used in amphibian studies (e.g. Buckley & Beebee, 2004; Loman & Andersson 2007; Hartel 2008) and can be an effective mean for monitoring reproductive females (Crouch & Paton 2000). However, the variability in probability of detecting spawns is largely unknown. It is likely influenced by a variety of biotic and abiotic factors (e.g. cloud cover, aquatic vegetation and wind affecting visibility through the water surface). Grant *et al.* (2005) examined the spawn detection probability among different observers and breeding pool characteristics (size, depth and vegetation). Detection varied spatially and temporally where no consistent sets of variables were able to explain the observed variation. Hence, assessment of variables that may influence the detection probabilities associated with specific sites or observers are essential in reducing bias in population size estimates. Another source of uncertainty when using spawn counts is the number of clutches deposited annually. Females of some

species, like the Natterjack toad, deposit a single egg string, thus the ratio between the number of breeding females and number of egg strings is 1:1. However, a second smaller egg string can be produced leading to overestimation of the number of breeding females, though this is rare (Denton & Beebee 1996). Many temperate and tropical species deposit between 2 and 12 clutches annually (Wells 2007) making it even more difficult to quantify population size and long-term trends.

Spawn counts represent variation of the proportion of females choosing to breed each year and strongly depends on availability of suitable breeding ponds and weather conditions, especially for species like the Natterjack toad that breeds in ephemeral ponds (Banks & Beebee 1988). A 31-year study of a Natterjack toad population in north-west England found a strong positive relationship between spring rainfall and annual spawn counts. Hence, large variation in the number of egg strings was observed between years as a result of differences in rainfall with some females skipping breeding seasons (Smith & Skelcher 2019). A study of the common toad (*Bufo bufo*, Linnaeus 1758) suggested that on average 41% of the females skipped breeding in any particular year, while the corresponding estimate for males was less than 5% (Loman & Madsen 2010). Hence, the total spawn counts represent annual fecundity (breeding effort) and not the total number of females in a population *per se*.

Errors in estimating breeding female number are likely to be less than errors in sex ratio assumptions when calculating population size. A male-biased sex ratio is often observed at amphibian breeding sites (e.g. Reading 2001; Loman 2010). This can be explained by lower female survival rates (Elmberg 1990; Friedl & Klump 1997) and females maturing at a later age (Reading 1991; Miaud *et al.* 1999). However, this is not always the case as large variation has been observed among populations. For instance, the female:male ratio for the Natterjack toad has been observed to vary between 1:0.3 and 1:11 (Table 5). Such is the likely variation between ponds, populations and years that sex ratio is likely the greatest source of biases and error in estimating total population size. The estimate from the current study ranged from 1:3 to 1:11 (depending on assumptions) encompassed most of the variation from previous studies whilst our average of 1:5 was substantially more male-biased than the mean or median from previous studies (Table 5). Thus, empirical estimates of sex ratio are essential for obtaining reliable demographic data for rare amphibian species.

CMR data and associated statistical models are robust methods for estimating confidence around population estimates. CMR requires identification of individuals; but not all species have natural unique markings. Toe clipping has been widely used as a marking technique for amphibians (Waichaman 1992; Halliday 1996), however, concerns have been raised regarding the ethical treatment of animals and its impact on survival (McCarthy & Parris 2004). Marking techniques widely used with other taxa like banding and PIT tags are often not suitable for amphibians (Halliday 1996; Funk *et al.* 2005). Over the past decades non-invasive genetic sampling has emerged as an alternative option for Mark-Recapture sampling (e.g. Petit & Valiere 2006; Solberg *et al.* 2006; Mondol *et al.* 2009). Despite its great potential, many challenges still exist regarding low amplification success rate, high genotypic errors and shadow effects (lack of power to distinguish individuals) leading to low reliability in obtaining genotypes. Collected DNA is often degraded, low quantity and contaminated with PCR inhibitors (Idaghdour *et al.* 2003; Lampa *et al.* 2013). Skin swabbing is a preferred method as it is easier and less invasive than buccal swabs, however, skin samples can have a high degree of contamination, especially if samples are collected during the breeding season when amphibians aggregate (Müller *et al.* 2013). In the current study, almost half of the genotyped samples (40%) were discarded due to cross-contamination and poor amplification success rate despite a high number of replications per sample ($n=7$). Thus, collecting high numbers of genetic samples is necessary while genotyping costs should be considered when planning study designs. Other issues that can arise when working with Mark-Recapture data is low recapture rate especially for animals that are hard to detect and capture, temporary emigration, uneven survival and detectability. In this study, the discovery curve increased linearly throughout the study with new individuals being regularly identified. Perhaps, it can be explained, by the duration of toad searches (one hour after dusk) and high number of males at the breeding site. If the searches had continued throughout of hours of darkness (prohibited by logistics) we would have captured a greater proportion of the male population.

Previous estimates of the Natterjack toad population size in Ireland relied solely on egg string counts with subsequent extrapolations (Becart *et al.* 2006; Sweeney *et al.* 2013). As demonstrated in the current study, such estimates are likely to be unreliable with numbers underestimated when failing to account for detection probability, annual

variation in proportion of breeding females and operational sex ratio. Given the scale of the amphibian extinction crisis and the need for robust population monitoring and surveillance techniques, there is need to promote study designs that account for differences between meta-populations including spatial and temporal variation in describing population declines, extinction probabilities and as a guide when informing conservation planning (Stephan *et al.* 2001; Minin & Griffiths 2011). Our results suggest spawn counts cannot be extrapolated with any reliability to produce precise population estimates but they do provide insights into variation in fecundity likely reflecting the effective (rather than absolute) population size. From an evolution perspective only individuals contributing to the gene pool of the next generation are important (Wright 1931), hence conservation management goals should be focused on maintaining genetic diversity within the reproductive population (Hedrick 2001). In this case, the effective population size of the Natterjack toad population at Caherdaniel, Co. Kerry Ireland is likely limited by the number of breeding females given they are the rarer sex. Hence, simply taking egg string counts as a measure of changes in the reproductive effort and population health is probably the easiest solution for annual monitoring rather than rolling out sex ratio estimation to produce absolute population estimates.

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Table 1. The estimated percentage of female Natterjack toads that breeds annually reported by two empirical studies.

Source	Location	Year	% females reproductive
Denton & Beebee (1993)	Hampshire, England	1988	55
		1989	64
		1990	44
		1991	63
		1992	61
Stephen <i>et al.</i> (2001)	Brandberge, Germany	1992-1999	63
Mean (95%CI)			58 (52 - 65)
Median (95%CI)			62 (56 - 68)

Table 2. Study-level recapture history of the Natterjack toads were N number of captures per dated sampling event.

Survey night	N	Recapture history							
		20-May-2017	10-Jun-2017	30-Jun-2017	14-Apr-2018	21-Apr-2018	05-May-2018	19-May-2018	02-Jun-2018
06-May-2017	26	4	4	1	4	4	3	3	2
20-May-2017	50		9	0	5	10	5	7	7
10-Jun-2017	81			1	7	14	7	8	5
30-Jun-2017	5				0	0	1	2	0
14-Apr-2018	89					20	17	12	10
21-Apr-2018	189						50	23	17
05-May-2018	186							32	20
19-May-2018	137								18
02-Jun-2018	121								
TOTAL	884	4	13	2	16	48	83	87	79

Table 3. Candidate model selection and abundance estimates for the male Natterjack toad breeding population size where p = capture and recapture probability, ϕ = survival probability, b = probability of first appearance (population entry), (t) = parameter changes over time, $()$ = constant over time, ω = the Akaike weight of each model, K = the number of parameters, N = is the estimated male population size, LCI and UCI provide the lower and upper limit of the 95% CI. The single best approximating model is marked with an asterisk.

[illegible]

Table 4. Population estimates for female and male breeding populations at three ponds at Caherdaniel, Co. Kerry derived from egg string counts (confidence intervals from GAM plus those associated with the assumed percentage of females that breed) and photo-ID verified by genetic fingerprinting (confidence intervals from CMR). Note that Confidence Intervals associated with each total population estimate are compound.

Year	Abundance estimate			Sex ratio		
	N	LCI	UCI	F:M	LCI	UCI
a) Egg string count / Min no females						
2017	231	212	250			
2018	224	218	229			
Mean	228	215	240			
b) Estimated no females (assuming $62 \pm 6\%$ spawning)						
2017	373	379	368			
2018	361	389	337			
Mean	368	384	353			
c) Total pop estimate¹ (Min no females + 1:1 Sex ratio)						
2017	462	424	500	1:1	1:1	1:1
2018	448	436	458	1:1	1:1	1:1
Mean	456	430	480	1:1	1:1	1:1
d) Total pop Estimate² ($62 \pm 6\%$ females spawning + 1:1 sex ratio)						
2017	746	758	736	1:1	1:1	1:1
2018	722	778	674	1:1	1:1	1:1
Mean	736	768	706	1:1	1:1	1:1
e) Estimated no males (Mark-Recapture)						
2017	1,698	1,000	2,397			
2018	1,698	1,000	2,397			
Mean	1,698	1,000	2,397			
f) Total pop estimate³ (min no females + estimated males)						
2017	1,929	1,212	2,647	1:7.4	1:4.7	1:9.6
2018	1,922	1,218	2,626	1:7.6	1:4.6	1:10.5
Mean	1,926	1,215	2,637	1:7.4	1:4.7	1:10
g) Total pop estimate⁴ ($62 \pm 6\%$ females spawning + estimated males)						
2017	2,071	1,379	2,765	1:4.6	1:2.6	1:6.5
2018	2,059	1,389	2,734	1:4.7	1:2.6	1:7.1
Mean	2,066	1,384	2,750	1:4.6	1:2.6	1:6.8

Table 5. Published Natterjack toad sex ratios as reported in the literature.

Source	Location	Year	Sex ratio			Comments
			Female	:	Male	
Denton and Beebee (1993)	Hampshire, England	1988	1	:	0.30	All sightings
			1	:	0.56	Known individuals only
		1989	1	:	0.33	All sightings
			1	:	0.47	Known individuals only
Stephan et al. (2001)	Halle, Germany	1992-1999	1	:	1.30	Newly hatched
Gunther and Meyer (1996)	Various	-	1	:	0.84	Lowest reported
			1	:	11.20	Highest reported
Mean (95%CI)			1	:	2.14 (000 – 5.11)	
Median (95%CI)			1	:	0.56 (000 – 3.53)	

Figure legends

Fig. 1 Map of the study area and satellite image of Natterjack toad breeding ponds sampled in the study.

Fig. 2 Cumulative egg string counts / minimum number of females at Caherdaniel, Co. Kerry from weekly surveys from April-July during **a)** 2017 and **b)** 2018. Dashed lines represent a Generalized Additive Model (GAM) and shading its 95% Confidence Intervals

Fig. 3 Individual variation in male Natterjack toad ventral markings used for photo-ID **A-B)** shows the same individual caught in 2017 and 2018 with identical markings highlighted by a red circle and **C-D)** show two different individuals caught during 2018.

Fig. 4 Individual accumulation curve (black) for discovery of new male Natterjack toads from photo-ID fitted with a linear regression (grey dash).

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Supplementary Materials

Protocol S1. High salt DNA extraction protocol for skin swabs, modified from Miller *et al.* (1988).

1. Centrifuge the swab samples for 30 minutes at full speed (14,000 rpm).
2. Remove the ethanol. Dry the swabs and sampling tubes for 24 hours at room temperature.
3. Add 600 μ l of TNES buffer* and 20 μ l of Proteinase-K (>600 mAU/ml) to the swabs. Mix the samples by inverting the tube.
4. Incubate the samples for 12 hours at 54°C.
5. Remove the swabs.
6. Add 167 μ l of 6M NaCl and centrifuge the samples at full speed (14,000 rpm) for 5 minutes.
7. Remove supernatant to a new 1.5 ml micro centrifuge tube.
8. Add 800 μ l ice cold 100% ethanol (C_2H_6O) and centrifuge at full speed (14,000 rpm) for 15 minutes.
9. Remove the supernatant and keep the DNA pellet.
10. Add 500 μ l of 100% ethanol (C_2H_6O) at room temperature and centrifuge at 6,000 rpm for 1 minute.
11. Repeat steps 9 and 10.
12. Leave the samples to dry for 2 hours at room temperature and re-suspend the DNA in 50 μ l dH_2O .

*Solution recipes for 100 ml TNES buffer: 5 ml TRIS (1M, 7.5 pH), 8 ml NaCl (5M), 20 ml EDTA (0.5M) and 10 ml SDS (0.5%). Top up with dH_2O , vortex and autoclave the solution.

Protocol S2. Ethanol clean-up.

1. To 15 µl extracted DNA add 1.5 µl 3M Sodium acetate and 60 µl 100% ice-cold ethanol.
2. Vortex the samples and incubate at -20°C for 15min.
3. Centrifuge at full speed (14,000rpm) for 30min.
4. Remove the supernatant and add 200 µl ice-cold 75% ethanol.
5. Centrifuge at full speed (14,000rpm) for 10min.
6. Remove the supernatant, leave samples to dry for 2 hours at room temperature and re-suspend the DNA in 50 µl dH₂O.

Table S1. Characterization of the seven microsatellite markers used in the study. A is the number of alleles and C_F is the final primer concentration in the PCR reaction.

Locus	Repeated structure	Primers (5'- 3')	Allelic size range (bp)	A	C_F	Dye	GenBank	Reference
BC02	(GATA) ₁₄	F: TTGCTTGAGAAAAGTCCAACA R: ACTTGCCAACTCTCCCAGAA	191 - 218	7	0.3µM 0.3µM	VIC	KX237585	Faucher <i>et al.</i> 2016
BC08	(TAGA) ₁₁	F: CTCTTGCAAGATCTCTGGG R: TACTGACTGCTGCCCTCTCC	241 - 279	9	0.1µM 0.1µM	FAM	KX237574	Faucher <i>et al.</i> 2016
BC22	(ATCT) ₉	F: TGCAGATTGCCAGCAGTTTA R: CACTTCCTCAAGGTGGTGCT	314 - 339	10	0.1µM 0.1µM	FAM	KX237578	Faucher <i>et al.</i> 2016
Bcalµ1	(AT) ₄ (GT) ₂₂	F: TGGGAATCCTTAGTGGTGAGCC R: TGAACCCATCTTGTAATGGCC	122 - 138	11	0.1µM 0.1µM	VIC	X99281	Rowe <i>et al.</i> 1997
Bcalµ3	(TC) ₂₁	F: TGGGTGTCATGTTAGATTCCC R: TGGACACTATTTGGGACTTGC	109 - 129	13	0.3µM 0.3µM	FAM	X99283	Rowe <i>et al.</i> 1997
Bcalµ8	(CT) ₆ GT(CT) ₄ GT(CT) ₂₄ ATAC(AT) ₇	F: TGCTAGGGAATAACTGGAGAGC R: GTGAACAGAAATGGTTTAGGGC	153 - 179	12	0.3µM 0.3µM	NED	X99288	Rowe <i>et al.</i> 1997
Bcalµ11	(AG) ₁₄	F: TCATAGGTCAGTGGAAGAGCA R: CGTCAACTTCAATTCGCTCA	165 - 193	12	0.1µM 0.1µM	FAM	AF267240	Rowe <i>et al.</i> 2000

Table S2. Genotyping error rate at 7 loci estimated with PEDANT version beta 1.3 with 10,000 search steps for enumerating each error rate. ϵ_1 is the rate of allelic dropout (ADO), ϵ_2 is the rate of false alleles (FA), GER is genetic error rate and GS is genotyping success.

Locus	Sample size	ϵ_1	ϵ_2	GER	GS
BC02	320	0.039	0.000	0.039	40.31
BC08	320	0.025	0.000	0.025	26.56
BC22	320	0.000	0.000	0.000	8.44
Bcal μ 1	320	0.008	0.000	0.008	41.88
Bcal μ 3	320	0.049	0.005	0.054	39.69
Bcal μ 8	320	0.015	0.004	0.019	38.13
Bcal μ 11	320	0.027	0.000	0.027	37.81

Table S3. Program RELEASE goodness-of-fit test results for the fully time dependant Cormack-Jolly-Seber model tested on Mark-Recapture data of male Natterjack toads using open population POPAN parameterization in program MARK. $\hat{c}$ is a *post-hoc* variance inflation factor (VIF). TEST2 tested for equal detection, TEST3 for equal survival between sampling events, TEST3.SR tested if the survival depended on whether an individual was caught previously, and TEST3.SM tested if an individual recapture depended on whether the individual was caught previously. The asterisk indicates significant deviation from the model's main assumption.

Test	Chi-square	<i>d.f.</i>	<i>p</i>	$\hat{c}$
TEST2	16.0276	10	0.099	1.603
TEST3	19.6078	12	0.075	1.634
TEST3.SR	17.1103	7	0.017	2.444
TEST3.SM	2.4974	5	0.777	0.499
TEST2 + TEST3	35.6354	22	0.033*	1.620