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A method for estimating fecundity in the spadefoot toad, *Pelobates fuscus*, through full and partial egg-counting

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The fecundity of female anurans is one of the key parameters facilitating predictions on population status and dynamics. A common methodological approach to its estimation is based on a full count of eggs per clutch already laid at a spawning site (Kaplan 1987, Berven 1988, Rafińska 1991, Lizana et al. 1994, Jaafar et al. 1999, Lips 2001, Lardner & Loman 2003, Richter et al. 2003, Prado & Haddad 2005, Räsänen et al. 2008, Mirabile et al. 2009, Cadeddu & Castellano 2012, Bruce 2014). Such a technique is considered least invasive in terms of conservation and perhaps applicable to rare or endangered anuran species or species with low or unstable populations in a particular region. At the same time, the quality of data obtained from a freshly laid egg clutch is highly affected by a number of external and internal factors. For example, it is virtually impossible in a natural ecosystem to eliminate the influence of predators consuming amphibian clutches, e.g., fish, insect larvae, etc. (Caldwel et al. 1980, Skelly & Werner 1990, Miaud 1993, Lardner 2000, Relyea 2003, Portheault et al. 2007). Furthermore, it is difficult to estimate the exact time of oviposition and fertilization and, as a result, standardization of the obtained results is hampered by the rapid onset of development of fertilized eggs and certain consumption of nutrients for embryogenesis. These factors may obviously influence the variance of original data.

The second technique, although invasive but widely used for fecundity evaluation studies, is based on a full count the number of eggs contained in ovaries and ready to be laid in females migrating to their spawning sites (Kuramoto 1978, Cummins 1986, Reading 1986, Montori 1989, Cogălniceanu & Miaud 2004, Camargo et al. 2005, Hero et al. 2005). It can be applied to more common amphibian species only, where the removal of a small sample will have no major impact on the population, which is especially important in long-term studies. Both of these methodological approaches require major research effort and are quite time-consuming.

Recently, some researchers have applied a so-called volumetric method (e.g., Lyapkov et al. 2001, 2002, Korzikov & Ruchin 2013) based on counting the number of eggs in a standardized portion of an anuran clutch and extrapolating these counts on the total number of eggs in the entire clutch. However, this method has been subjected to reasonable criticism (Ishchenko 2003) since the obtained clutch parameters have no significant correlation with the body's dry weight and thus cannot be used to assess the reproductive effort. Moreover, some authors use partial counting of eggs per clutch to evaluate the fertility of amphibian females (Liedtke et al. 2014) even though the accuracy of this method has not previously been tested. It is obvious that the introduction of any new sampling method whose application entails judgements on the number of eggs per anuran clutch in total (the general population) must be accompanied by a detailed analysis of the deviation of the calculated parameters from reference values, i.e., a convergence assessment.

The aim of this work was to quantify the accuracy level of estimates of female fecundity by counting eggs in samples of common spadefoot toads (*Pelobates fuscus*).

Female *Pelobates fuscus* used for the fecundity estimation were captured during the breeding migration in April of 2014 by linear drift fences with bucket traps (following, e.g., Gibbons & Semlitsch 1981, Corn 1994; Dodd & Scott 1994, Kupfer & Kneitz 2000, Yermokhin & Tabachishin 2011a) set up around three breeding sites in the Medveditsa River valley (between the settlements of Uritskoye and Ataevka, Lysogorsky District, Saratov region, Don

Basin, southwestern Russia), at Lakes Sadok (51°21'31" N, 44°48'11" E), Kruglen'koe (51°21'55" N, 44°49'58" E), and Cherepashie (51°21'50" N, 44°49'03" E). Females for study (N = 32) were selected from a larger sample using a random number generator (ANSI algorithm in the software package Attestat 12.5 (Gaydyshev 2001). The examined sample obtained in this manner included mature females of various size and body mass categories. This particular treatment of the sampling was chosen due to the previously established dependence of the fecundity of *P. fuscus* females on their size and body mass (Yermokhin & Tabachishin 2011b). Specimens were killed by drowning in 20% alcohol solution, fixed in 10% formalin, and stored in 70% ethanol (Pisani 1973, McDiarmid 1994). The specimens of *P. fuscus* studied are stored in the collection of the Zoological Museum, Saratov State University, Saratov, Russia.

To estimate fecundity, ovaries were extracted and the numbers of eggs contained were counted. The count was taken as the reference value (N_r) and then compared with the calculated value obtained on the basis of the mean dry weight of the eggs in samples of a sub-sample of ova extracted from the ovaries (N_{calc}). Samples to obtain the calculated values of the number of eggs per clutch were taken by successively dividing the set of eggs into fractions, ascending in number: 25, 50, 75, 100, 150, 200, 250, 300, 350, 400, and 450 eggs (until exhaustion). The numbers of eggs in the residue outside this scheme were counted separately.

The resulting samples were dried to constant weight in a drying oven at 90°C. Dry egg mass was measured on an electronic balance KERN ABT 120-5DM with an accuracy of 0.01 mg. The mean dry mass of a single egg was calculated from the mass of the whole sample divided by the number of eggs contained therein. The number of eggs per clutch was calculated as the ratio of the dry weight of the whole ovarian clutch size to the dry weight of a single egg.

The relative deviation (δ_N, %) of calculation was taken as a measure of the relation between N_{calc} and N_r, calculated after the formula:

$$\delta_n = \frac{N_{calc} - N_r}{N_r} \cdot 100$$

where N_{calc} is the calculated number of eggs per clutch, and N_r the number of eggs per clutch as obtained from a full count (the reference).

For each sample value, the mean relative error (the parameter values were taken without regard to sign character) and its minimum (δ_{min}) and maximum (δ_{max}) values (including sign character) were calculated. The normality of the distribution was verified by a Kolmogorov–Smirnov test. The hypothesis of normality was not rejected for all our samples. The equality of variances was verified by F-tests (the variances being unequal). Based on these properties of our sample population, the differences between the arithmetic means were evaluated by Satterthwaite's t-test. Differences were considered significant when $P < 0.05$. Statistical data were processed with the software packages PAST 2.17 (Hammer et al. 2001) and Attestat 12.5 (Gaydyshev 2001).

The sample of *Pelobates fuscus* females included the full range of size and body mass groups of mature individuals involved in reproduction. The average body length of the examined specimens (SVL) was 45.7 ± 4.1 mm (37.8–55.1 mm) and their live body mass was 12.2 ± 3.0 g (7.4–19.2 g). These parameters and the range of their variation do not differ substantially from those in the populations studied. The number of eggs in the ovaries of females (N_r) ranged from 400 to 1,989 (mean 1,051 ± 369, N = 32).

In general, the mean dry mass of an egg was 0.96 mg; this parameter is within the range of 0.95 to 0.99 mg when egg sample size is increased (Table 1). The smallest relative error of calculations was observed in the sample volume of 25. This indicator is significantly even if weakly correlated with the volume of the sample (Pearson's correlation coefficient: $r = 0.17$, $P \leq 0.01$). The error level was stable within a range of sample volumes from 25 to 250 eggs ($F = 0.69$, $df = 6$, $P = 0.66$) and then slightly increased to a value exceeding 2% ($F = 9.43$, $df = 1$, $P \leq 0.01$). Samples of 300 and 350 eggs characterized large females within the maximum fecundity category, and also included a slightly larger egg mass. At the same time, such variations were relatively small and statistically insignificant (Satterthwaite's t-test, $P > 0.35$).

A similar, but more clearly pronounced, pattern was observed when counting ovarian eggs in a relatively small sample and comparing the number with the results of a complete count. The minimum (about 1.5%) and maximum (about 2.5%) relative deviation of the calculated values from the reference results were found in the smallest and largest sample volumes, respectively (Table 1). The effect of the ovarian tissue mass on egg mass characteristics (their dry mass was negligible, i.e., less than 2–3% of that of gametes) can be regarded as a probable cause of the deviation of the calculated results from the known reference value. This effect increased with an increasing sample volume of 300 and 350 eggs.

Comparative analysis of the quality of the results of our calculation method for quantifying the fecundity of *Pelobates fuscus* indicated a fairly high level of agreement with the reference values obtained from complete counts of ovarian eggs. In all variants of the sample volume, the level of agreement was notably higher than 95% (the deviation from reference values ranged from 1.6 to 2.6% on average). Such an accuracy level of the calculated results is quite acceptable for most ecological studies.

However, it should be noted that our method is only applicable when using dry mass. Any attempt to express mass indicators for amphibians in any different form would make it impossible to standardize measurements and may lead to a significant decrease of data quality. Among the factors that could make a significant contribution to the total variance of the current data are, obviously, firstly weather conditions (especially temperature, ambient moisture, and relative humidity) on the day of the capture of female anurans and secondly, differences in the travelling distance from the hibernation to the spawning site. Thirdly, the duration of their stay in pitfall traps, and especially

Table 1. Convergence characteristics of the results of the calculated and reference dry egg weights and the number of eggs in the ovaries (fecundity) of female spadefoot toads, *Pelobates fuscus*. * – the mean in the numerator, the range of variation in the denominator; ** – the mean calculated by ignoring sign in the numerator, the range of variation involving sign in the denominator (negative and positive values are the deviation of calculated values in either direction from the reference).

Egg sample volume	Females number (N)	Dry weight of an egg			Deviation of the calculated number of eggs per clutch from the reference value	
		in the sample (mg)	Calculation–reference deviation			
			absolute (mg)	relative (%)	absolute	relative (%)
25	32	0.95±0.15*	0.02±0.01**	1.46±1.14**	15±12**	1.55±1.11**
		0.70–1.32	-0.03–0.04	-2.66–4.76	-52–33	-4.54–2.74
50	32	0.96±0.16	0.02±0.01	1.89±1.05	20±15	1.84±1.07
		0.67–1.31	-0.04–0.04	-3.99–3.71	-45–66	-3.58–4.16
75	32	0.96±0.16	0.02±0.01	1.80±1.11	20±16	1.84±1.08
		0.64–1.31	-0.03–0.04	-3.60–3.78	-45–74	-3.76–3.74
100	32	0.96±0.16	0.02±0.01	1.79±1.11	20±15	1.98±1.13
		0.68–1.30	-0.03–0.04	-3.18–4.18	-58–38	-4.08–2.93
150	31	0.95±0.15	0.02±0.01	1.69±1.15	20±18	1.74±1.11
		0.66–1.29	-0.04–0.03	-4.36–3.44	-39–78	-3.32–4.56
200	29	0.96±0.16	0.02±0.01	1.82±1.24	21±19	1.80±1.29
		0.65–1.29	-0.04–0.04	-4.14–3.54	-42–71	-3.42–4.32
250	21	0.96±0.12	0.02±0.01	1.96±1.34	25±17	1.87±1.14
		0.73–1.16	-0.04–0.04	-3.56–3.78	-44–52	-3.64–3.62
300	16	0.99±0.14	0.02±0.01	2.44±1.19	33±19	2.54±1.26
		0.73 –1.32	-0.03–0.04	-3.46–4.16	-66–52	-4.57–3.58
350	5	0.97±0.08	0.03±0.02	2.69±1.56	41±21	2.59±1.71
		0.84–1.06	-0.03–0.05	-2.95–4.79	-61–42	-2.61–2.77

the presence or absence of water therein after the passage of precipitation, or the degree of shading could exert an influence. Therefore, to completely eliminate the distorting nature of these factors on the water content of the amphibian body (including their sexual products), it is necessary to fix the body mass parameters only after drying the samples to constant mass.

It would be inappropriate to measure the mass characteristics of a clutch already laid at a spawning site. A number of factors difficult to individualise as to their effects and not subjectable to standardization would influence the water content as well. The hydrochemical parameters of a certain microhabitat in the spawning pond can be an example of such factors, which, on the one hand, determine the degree of diffusion through the egg membranes and, on the other, are very labile in shallower areas.

Spawning features of a particular anuran species should be regarded as a significant limitation to the applicability of our method. For species with fractional spawning, such as *Bombina bombina* or *Pelophylax ridibundus* and other green frogs, which may extend over a month for particular individuals, the females arriving at the spawning site carry oocytes at different stages of maturation in their ovaries (Bannikov & Denisova 1956, Kuzmin 1999). Moreover, egg maturation and oviposition continue for a long period of time. Therefore, the total number of eggs laid during the reproductive period (fecundity) is significantly higher than the number of oocytes in the ovaries of a female at the time of her arrival at the spawning site (Jørgensen 1992, Cogălniceanu & Miaud 2004). For example, the formation of oocytes in *B. bombina* obviously continues throughout the time of active feeding and during the female's stay in the pond, perhaps even as long as until the beginning of her migration to a terrestrial habitat for hibernating. As the number of eggs in the ovaries of the females of *B. bombina* and green frogs at the time of their arrival at the spawning site does not equate to "fecundity" at all, the application of the proposed method of calculation of this parameter to similar species may lead to significantly distorted (underestimated) results and is therefore inappropriate.

This calculation method is most applicable to amphibian species laying their eggs in a single clutch, i.e., explosive breeders. This type of spawning is typical of terrestrial species with a relatively restricted spawning period that leave their breeding sites immediately after spawning, such as *Rana arvalis*, *R. temporaria*, *Bufo bufo*, *B. viridis*, and *P. fuscus* (Bannikov & Denisova 1956, Kuzmin 2013, Yermokhin et al. 2013). The oocytes of a clutch of these temperate species are all at the same stage of formation and ready for ovulation at the end of hibernation and prior to arrival at the spawning site. Thus, the number of female ovarian eggs of these spring-breeding species corresponds well with the actual number of eggs laid during the spawning period, i.e., is equivalent to fecundity. We think that our proposed method can be applied with an acceptable accuracy level in ecological studies of these species.

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