Herpetologica, 60(3), 2004, 349–357

IDENTIFICATION OF REPRODUCTIVE STATUS IN FEMALE FROGS—A QUANTITATIVE COMPARISON OF NINE METHODS

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ABSTRACT: Many techniques commonly used by herpetologists for monitoring amphibian populations and communities yield censuses of the total adult population size (N). However, for many studies, e.g., of reproductive output, development of populations and potential for evolutionary changes, the effective population size (N_e) must be known. While modern molecular techniques make it possible to measure N_e, they are expensive, work-intensive and may not be possible or necessary for many questions on amphibian reproduction. For females of two species of water frog (*Rana lessonae* and *R. esculenta*), we investigate the effectiveness of several techniques to determine the presence or absence of eggs. The direct methods are (1) dissection of dead frogs and (2) a small skin incision into the abdominal side of live females. The indirect methods, all applied to live frogs, include (3) visual inspection of body shape, (4) tactile inspection of the epidermis, (5) transillumination with a strong cold light source, (6) ultrasound, (7) electromagnetic measurement of total body electrical conductivity (TOBEC), (8) calculation of body condition index and (9) analysis of blood plasma testosterone titers. Only two indirect methods were somewhat successful at predicting whether females were gravid. Testosterone titers (9) yielded the best results (ca. 80–90% effective). Body condition (8) was also significantly related to egg presence or absence, but predicted gravidity only weakly. We suggest that a combination of skin incision and hormone analysis provides a fairly good estimate of gravidity. When complemented by mark-recapture techniques and performed on the same individuals at different times of the season, this combination yields estimates not only of the reproductive output of the study population but also of the relative contribution of different females.

Key words: Anura; Effective population size; Eggs; Female reproduction; Monitoring; *Rana*

MOST techniques for monitoring amphibian populations and communities (summarized in Heyer et al., 1994) result in a census of the total number of adults (N), rather than of the effective population size (N_e), i.e., the number of individuals actually contributing to reproduction. From an evolutionary and conservation perspective, N_e is of central importance because it determines the amount of genetic variation and the net reproductive rate (R_0), which indicates whether a population will increase, decrease or remain stable over time (Begon et al., 1996; Jehle et al., 2001). N_e is usually much smaller than N (Frankham, 1995). In common toads (*Bufo bufo*), for instance, only 1% of the adult population successfully reproduced in a given year (Scribner et al., 1997); in European newts (*Triturus cristatus* and *T. marmoratus*) the corresponding figures were 10–20% (Jehle et al., 2001). In such situations the total adult population census (N) will grossly overestimate the extent of reproduction and the potential for adaptive evolutionary changes within populations.

Quantifying the number of eggs or larvae comes closer to the reproductive output than counting the number of adults, but the method faces various methodological difficulties. First, species often are difficult to identify at early stages (Duellman and Trueb, 1994). Second, eggs and larvae of some species are difficult to detect, e.g., in deeper water or vegetation, in terrestrial or arboreal nests, or for (ovo)viviparous species (Cooke, 1975; Duellman and Trueb, 1994). Third, even reliable estimates of the number of eggs and larvae do not reveal N_e, because we do not know how many and which females contributed to the reproduction.

Direct study of the genetic variation within and between populations through the application of molecular markers can yield much of the data needed to assess reproductive potential (review: Jehle and Arntzen, 2002). However, these techniques are expensive, time consuming and require sophisticated equipment. Accurate assessment of the reproductive condition of females (gravid or nongravid) could provide a useful estimator of N_e for females. Here, we compare the effectiveness of nine techniques for determining the presence or absence of eggs.

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As a model for our study we used two species of water frogs, the pool frog *Rana lessonae* (genotype LL) and the edible frog *R. esculenta* (LR), originally a hybrid between *R. lessonae* and the lake frog *R. ridibunda* (RR). The hybrid reproduces through hybridogenesis (Schultz, 1969), i.e., it eliminates one of its parental genomes (usually the L) prior to meiosis and regains it in the next generation by mating with the respective species. This makes the hybrid a sexual parasite that can persist only in sympatry with its sexual host species (usually *R. lessonae*). For further details about this LE-system see Graf and Polls Pelaz (1989).

Material and Methods

Study Area and Capture of Frogs

The study was performed in 1997 on a military training ground near Zurich airport (Switzerland) where several ponds lie within a few hundred meters. Details about the study area and characteristics of the ponds are given by Holenweg Peter et al. (2002).

Frogs were caught at night by hand. Collections were performed during three periods: breeding season (11 May–04 June), postbreeding season (28 July–02 August) and fall (02 October). All caught animals larger than 45 mm snout–vent length (SVL) were classified by sex and species. Males were distinguished from females through the presence of vocal sacs and large thumb pads which are particularly developed during the breeding season (D'Istria et al., 1972). The two species of water frogs were distinguished by the inner metatarsal tubercle (callus internus) which differs in size and form among species (Berger, 1990). In cases of uncertainty, 3–5 μl samples of lymph were taken with a glass capillary from an incision made into the web of a hind foot, and the lymph was later subjected to enzyme electrophoresis to determine the species based on albumin patterns (Uzzell and Berger, 1975). In females, the foot web incision was slightly lengthened and the emerging drop of blood was collected in a heparinised microvette (Microvette CB 300, Sarstedt, Germany) for later hormone analysis (see below). All frogs were weighed with a spring balance (Pesola AG, Baar, Switzerland) to the nearest 0.5 g and SVL was determined to the nearest 0.5 mm with a vernier caliper.

Identification of Reproductive Status

We compared the suitability of two direct methods (1–2) and seven indirect methods (3–9) for determining whether females contained eggs.

(1) *Dissection*: We euthanized seven females with 3-aminobenzoic-acid ethyl ester (MS-222; A-5040, Sigma Inc.) and then dissected them to check for the presence or absence of eggs.
(2) *Skin incision*: A ca. 2 mm long incision into the skin of the abdomen at the height of the ovary allowed direct visual detection of the eggs. From captive animals that were held over several months, we know that the incision closes within a few hours, and in the field we never found signs of infection in frogs that were caught repeatedly.
(3) *Visual inspection*: We classified females with rounded bellies as gravid and females with slender bellies as nongravid.
(4) *Tactile inspection*: We gently palpated the belly and classified females as gravid if we felt eggs and as nongravid if we did not feel eggs.
(5) *Transillumination*: Females were held with their backs against a cold, bundled fiberglass light source of a microscope lamp (Intralux 4000-1; Volpi AG, Schlieren, Switzerland). In some species this allows visual detection of the eggs through the skin (Gilette and Peterson, 2001; J. Pechman, personal communication).
(6) *Ultrasound*: We placed females on their dorsums, then slowly moved a 7.5 MHz-probe (UST-5512U) of an ultrasound unit (Aloka SSD-500) over their abdomens, and noted whether eggs were visible.
(7) *Electromagnetic scanning*: The total body electrical conductivity (TOBEC) correlates positively and highly with egg lean mass and the mass of the egg components albumen and lipids (Williams et al., 1997). Hence, TOBEC indices can potentially reveal the presence or absence of eggs. Animals were immobilized in a water bath containing a 0.1–0.2% solution of MS-222. Once they were motionless (usually after 5 min), the totally relaxed body was stretched out and placed on its ventral side in a Plexiglas halftube which then was

exposed to an electromagnetic oscillation field of 10 Mhz (EM-SCAN/TOBEC® Inc., Springfield IL, USA). The equipment consisted of a tunnel-like measuring chamber surrounded by a copper coil (SA-3114) which was inserted into a basic unit (SA-3000) where detectors recorded the energy absorbed from the electromagnetic field in proportion to the conductivity of the object that was measured. Each female was measured seven times over a period of a few minutes, and the values were averaged. After the measurements, each female was placed into fresh water with her head held above the surface until she had recovered from immobilization. Normal activity returned after about 20 min with no negative effects on subsequent condition and behavior in any of the females.

(8) *Body condition*: From body mass and SVL we calculated a body condition index ($BCI = 10^4 * mass/SVL^3$) (Green, 2001; Jakob et al., 1996). This index provides a potential method for identifying the presence or absence of eggs because gonads weigh up to 20% of the total body mass at the beginning of the breeding season (Redshaw, 1972) and females can lose ca. 30% of their mass due to spawning (Ryser, 1989).

(9) *Testosterone titer*: Testosterone titers are likely to reflect the presence or absence of mature follicles because oocytes and androgens develop synchronously (Follet and Redshaw, 1974; Rastogi et al., 1983). Blood for hormone analyses was taken soon after capture (see above) to insure that androgen levels did not drop due to the stress of captivity (Paolucci et al., 1990). Samples were immediately centrifuged at 1000 rpm for ten minutes in a mobile centrifuge (Microcentrifuge, Denver Instruments, Norfolk, UK) powered by a car battery. The plasma was transferred into Eppendorf tubes and kept on ice until it was stored in a deep freeze at –20 C before hormone analysis. Analysis of the testosterone titer was performed by radioimmunoassay (RIA). The samples were defrosted and the blood plasma transferred into Eppendorf tubes for analysis. Plasma testosterone was obtained by using column chromatography (Moreno et al., 1980) or ether extraction (Chard, 1990). Every sample was tested twice. The total activity, the unspecific binding and the specific binding were measured for every assay. The applied antibody AK 8/3 had a specific binding activity of 44.89% and an unspecific one of 2.83%. Depending on the amount of plasma available, the lowest testosterone concentration that could be interpreted with confidence ranged from 1.12 to 5.89 ng/ml.

Data Analyses

The two direct methods (1–2), which we considered to be the most accurate ones, categorized females as gravid or nongravid. Four of the indirect methods (3–6) also yielded dichotomous data (i.e., gravid, nongravid). For these four methods we calculated the percentage classifications that agreed with those from a direct method. For the three indirect methods yielding continuous variables (7–9) we performed logistic regression analyses to test whether categories obtained from a direct method (dependent variable) were significantly related to each of the continuous indirect parameters (independent variable). Agreement (in %) between classifications from direct methods and values from these three indirect methods was obtained from predictions made by the logistic model. Prior to these analyses, the continuous variables (conductivity, body condition index and testosterone titers) were ln-transformed to increase additivity of effects and equality of variance (Snedecor and Cochran, 1980). For methods where sample sizes were large enough (ultrasound, body condition index and testosterone titers), we compared agreement between direct and indirect methods for *R. lessonae* and *R. esculenta* separately; in all other cases we pooled results from the two species. With the exception of seven females that were euthanized and dissected (method 1), the direct reference against which all results from indirect methods were compared was the skin incision (method 2).

Results

For all seven females that were sacrificed, results from the dissection agreed with those

from the skin incision. None of the indirect methods resulted in classifications that totally agreed with those provided by the direct methods, but some proved to be more reliable than others in detecting the presence or absence of eggs (Fig. 1).

Results from visual and tactile inspection of body shape agreed with those from direct methods in only 60% and 56% of the cases, respectively. Transillumination was in agreement with the direct methods in only 36% of the comparisons. This method only produced a dark image with no clear boundaries. The image could not be equated to egg masses since it was also present in males. Similar to transillumination, ultrasound produced no consistently reliable results in *R. lessonae* (56% agreement) and *R. esculenta* (55% agreement). Figure 2 shows three examples to illustrate the difficulty of interpreting the images.

In females that were subjected to electromagnetic scanning, the highest and lowest values obtained from the same individual differed by almost the same factor as the highest and lowest means from different individuals (13.1 versus 15.2). With this large intra-individual variance, it is not surprising that median conductivity was similar for females classified as gravid or nongravid by a direct method (38.7 versus 34.7). According to the logistic regression analysis correct assignment was only 53% ($G = 0.422$, df = 1, $P = 0.516$).

Figure 1 shows that sample sizes for indirect methods (3), (4), (5) and (7) were quite small. Hence, percentage agreement with direct methods is likely to change with each additional female measured. But even when data from these four indirect methods were pooled, agreement did not differ significantly from randomness ($\chi^2 = 0.026$, df = 1, $P = 0.873$).

Although body condition was significantly related to direct gravidity classification ($P = 0.002$; Table 1a), the relationship was too weak for use as a reliable predictor of reproductive condition. On average, females with eggs had a higher condition index than females without, but the precise relationship varied with the condition index and between species. The proportion of females in which eggs were detected increased with body condition, but reached 100% only in the highest condition category of 1.35 (Fig. 3a), and this only in *R. lessonae*. Over an index range from 1.05 to 1.25 the proportion was fairly constant between 70% and 80%. In *R. esculenta* it was within 30–50%, with no obvious increase over the whole range of indices. Overall, classification agreement with the direct methods on the basis of the condition index was significantly lower for *R. lessonae* than for *R. esculenta* (56% versus 65%; $P < 0.001$, Table 1a).

Testosterone titers allowed the best discrimination between gravid and nongravid females ($P < 0.001$; Table 1b). Median plasma titers in females carrying eggs were 18.74 ng/ml, as opposed to only 0.137 ng/ml in those with no eggs. In both species, the proportion of females with eggs increased with increasing testosterone titers up to about 20 ng/ml blood plasma, but then leveled off to an average of 89% in *R. lessonae* and 77% in *R. esculenta* (Fig. 3b). Again, there was a significant species effect ($P = 0.013$; Table 1b) on classification agreement with the direct methods: agreement was with lower for *R. lessonae* (68%) than for *R. esculenta* (76%). Inclusion of both body condition and hormone titers in the logistic regression model yielded 73% for *R. lessonae* and 76% for *R. esculenta* and, hence, did not markedly improve the agreement based on testosterone alone (Fig. 1).

Discussion

None of the indirect methods that we tested was a reliable predictor of the presence or absence of eggs in *R. lessonae* and *R. esculenta*. Classifications of five of the seven indirect methods were correct (i.e., in agreement with those of the direct methods) only slightly better than the random expectation of 50%. Only testosterone titers and, to a lesser extent, body condition indices allowed a better than random classification. Below we discuss some reasons for these results.

Visual inspection of body shape was impaired by the fact that many animals inflated themselves when handled. Inflated and large animals more often gave the impression that they had eggs than slender and small ones. This may also explain the poor success with tactile investigation and transillumination. The relatively thick skin of water frogs does not allow one to feel the eggs when the abdomen is palpated, and it diffuses the light to create a blurred image of an amorphous mass of

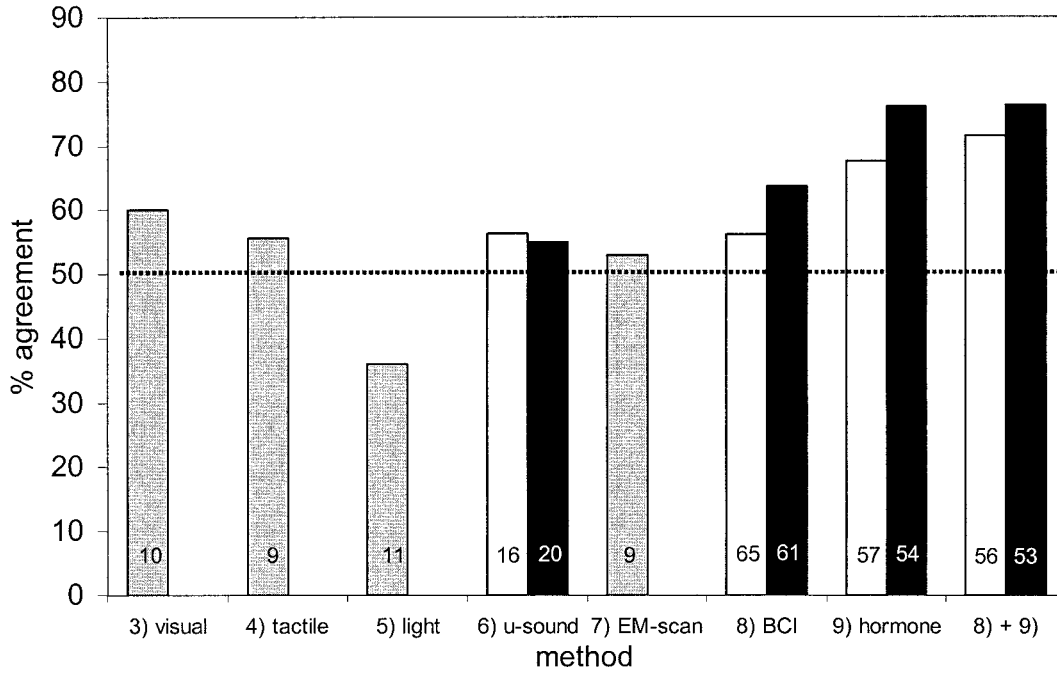

FIG. 1.—Agreement (%) between the presence of eggs found through the direct methods (1–2) and results from seven indirect methods (3–9) and one combination of indirect methods (8 + 9). The dotted horizontal line indicates the 50% chance agreement. White bars: *R. lessonae*; black bars: *R. esculenta*; gray bars: pooled over both species. Numbers at the bottom of the bars show total sample sizes, i.e., the number of frogs investigated with the respective indirect method, regardless of the agreement with the direct method. The total sample size of investigated females was 66 *R. lessonae* and 62 *R. esculenta*. With two exceptions, all females were subjected to method 8 (BCI), most of them also to method 9 (hormone analysis) and some to one or two additional methods (3–7). Hence, sample sizes represent multiple measures on the same individuals, rather than different frogs.

intestines, rather than of individual eggs. Successful application of transillumination in *Plethodon cinereus* (Gilette and Peterson, 2001) and *Ambystoma* (J. Pechman, personal communication), and results that we obtained in a few investigated *R. temporaria* and *Bufo bufo* (I. Bättig, unpublished data) indicate that the method can work in some species.

Ultrasound and electromagnetic scanning of total body electrical conductivity (TOBEC) provide uninvasive methods to determine ovarian development and chemical body composition, respectively, but reliability of both methods seems to decrease with decreasing size of the measured object (Asch and Roby, 1995; Melnychuk et al., 2002). For TOBEC, accuracy is highest for subjects that nearly fill the measuring chamber (Asch and Roby, 1995). The chamber available to us was designed for animals up to 1 kg and, hence, probably was not precise enough for our frogs that weighed only between 14 and 65 g. Technical modifications, including adequate chambers for TOBEC, smaller and higher resolution probes for ultrasound and the calculation of species- and sex-specific calibration curves are likely to improve the accuracy of egg detection in frogs. We feel, however, that at present the required high effort would not be rewarded by equally high benefits.

Some authors have used a decrease in body condition as an indicator of successful reproduction (e.g., Lüddecke, 1997). In our case, however, body condition was not a good predictor of reproductive condition. One potential problem is that the index is calculated as a ratio between mass and body length, and such ratios tend to increase with body size (Jakob et al., 1996). However, size-dependence of the index cannot explain the difference in agreement between the two species, because we controlled for body size by including SVL into the

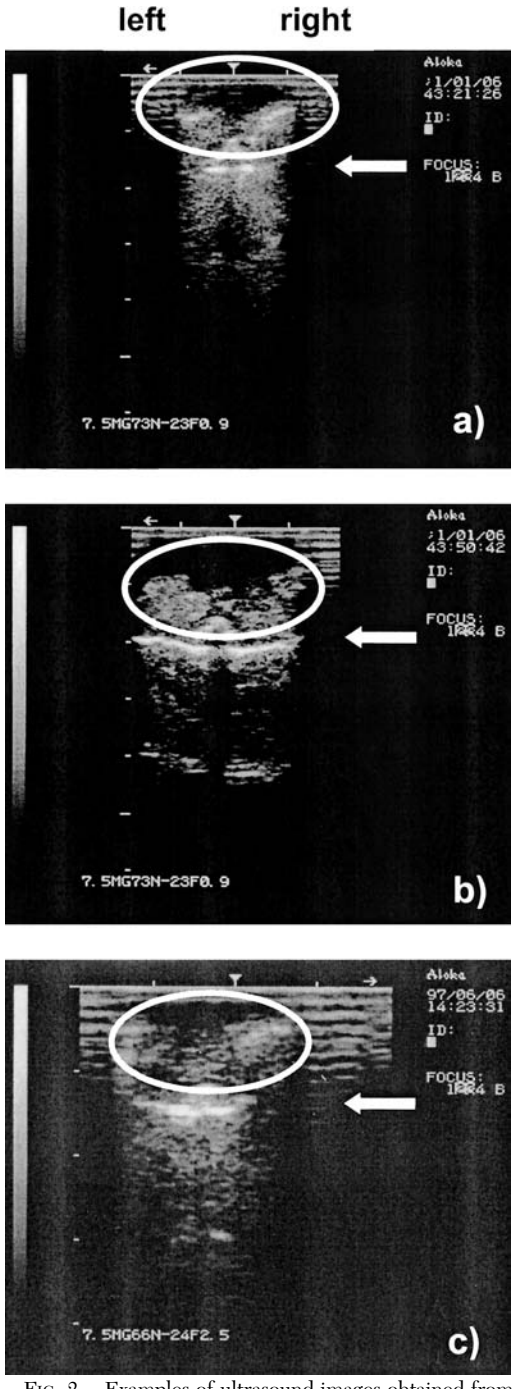

FIG. 2.—Examples of ultrasound images obtained from water frog females without eggs (a), with small eggs (b) and with larger eggs (c). In each Figure, the top represents the ventral side of the animal and the bright white areas, indicated by arrows, mark the dorsal side; the left and right flanks are also indicated. Ellipses show the areas of the eggs. Whitish areas outside the body boundaries result from noise, i.e., ultrasound reflections in the surrounding area.

TABLE 1.—Results from logistic regression analyses relating the presence or absence of eggs detected through direct methods to species (*R. lessonae*, *R. esculenta*) and body size (lnSVL) plus either (a) body condition (lnBCI) or (b) testosterone titers (lnHormone). *G* denotes the test statistics for the complete model.

	(a)			(b)		
Parameter	Estimate	*t*-ratio	*P*	Estimate	*t*-ratio	*P*
Constant	−8.34	−1.47	0.142	−6.34	−1.01	0.307
Species	0.98	3.91	<0.001	0.88	2.49	0.013
LnSVL	1.88	1.34	0.179	1.87	0.93	0.352
lnBCI	5.54	3.03	0.002			
lnHormone				0.52	4.80	<0.001
G	26.45	df = 3	<0.001	54.16	df = 3	<0.001

logistic regression. Size was not significant, but the species effect was. Although usefulness and reliability of the index also depend on a number of other key assumptions (Green, 2001), we feel that a more important reason for the poor success of the index is a biological one. Evaporative water loss and water intake can amount to as much as 20 percent of body mass (Sinsch, 1983) and, hence, be of the same magnitude as the ovary mass. G. Abt (unpublished data) found a decrease in mass of up to 13% within four hours after frogs left the water and a return to the original value within one hour after they returned to the water. Thus, the condition index of a female may be strongly confounded by when and where she was captured.

Testosterone titers were the best indirect indicators of the presence or absence of eggs. This is due to the clear annual cycle of reproduction in temperate zone amphibians, including water frogs. After breeding in April to early June and a postovulation phase with ovarian quiescence in June/July, reproductive condition recovers between July and October in those females that will breed again the next year. The recovery phase is characterized by vitellogenesis of oocytes and increasing titers of various sex hormones, especially androgens (Follet and Redshaw, 1974; Rastogi et al., 1983). This development is completed by late fall. During winter, ovaries are in a resting phase, but androgen titers and ovary mass/body mass ratios have similarly high values that are characteristic for the subsequent preovulation and ovulation periods in the next spring (Gobetti et al., 1990). Yet, in our study agreement between testosterone titers and presence

or absence of eggs detected with the direct methods was far from being perfect. Some gravid females had low testosterone values, probably because androgen titers can drop fairly rapidly (i.e., within a few days) between ovulation and spawning: *Rana lessonae*, *R. esculenta* and *R. catesbeiana* females caught in amplexus had androgen titers 2–10 times lower than those prior to ovulation (Gobetti et al., 1990; Licht et al., 1983; Reyer et al., 2004). Hence, for using testosterone as a good indicator for the presence or absence of eggs, the time course of its decrease and the date of spawning should be known. In contrast, some females with high testosterone titers (and high condition indices) were found not to have eggs. This possibly reflects an error in detection of existing eggs through the incision. The same conclusion emerges from the fact that agreement between results from the skin incision and those from testosterone titers or body condition was higher for females in which eggs were detected through the incision than for females in which they were not (testosterone: 74% versus 67%; body condition: 64% versus 53%; pooled over both species). Such a result is to be expected when hormone levels and body condition are high enough to suggest gravidity, but the (probably present) eggs are overlooked. The probability of overlooking existing eggs is influenced by a number of factors, including the precise location of the incision, the number and size of the eggs, and the species. For comparable testosterone titers and body condition indices, eggs were detected in a smaller proportion of *R. esculenta* than of *R. lessonae*. This could be due to the slightly thicker and less transparent skin of the hybrid.

Egg screening could probably be improved by increasing the length of the incision. This would also provide a better impression of the number of eggs. However, seeing eggs through the skin incision is no evidence that these will be released within the year of the study. Spawning anurans, including water frogs, carry several follicle generations and can even resorb eggs and invest them into next year's reproduction (Delgado et al., 1990; Reyer et al., 1999). Which of the follicles are ready for the present breeding season and which ones represent early stages for future reproduction can be determined through biopsy of the ovaries (Ritke and Lessman, 1994). These benefits, however, must be weighed against the risk that a longer incision and/or biopsy may decrease the frogs' survival through higher rates of inflammation, physical damage (e.g., during amplexus), reduced food intake and/or greater susceptibility to predators.

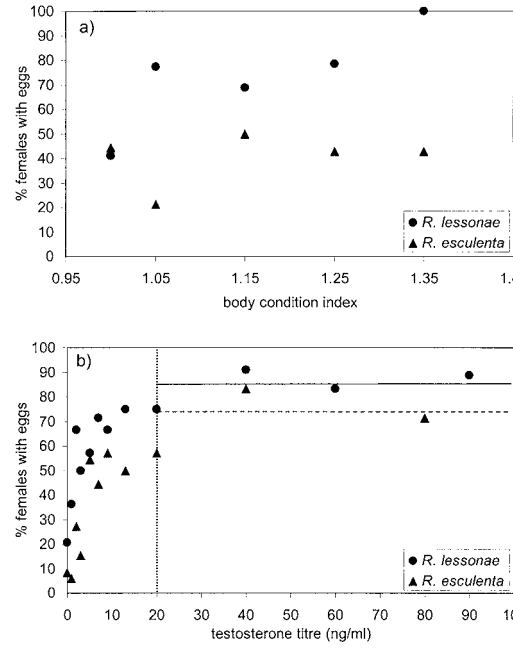

FIG. 3.—Percentages of *R. lessonae* and *R. esculenta* females with eggs in relation to (a) four classes of body condition indices (0.95–1.00, 1.01–1.15, 1.16–1.25 and 1.26–1.35) and (b) eight classes of testosterone titers (0–2, 3–5, 6–8, 9–13, 14–20, 21–40, 41–60, 61–80 and >80 ng/ml blood plasma). The symbols are located at the upper boundaries of the respective classes. In contrast to (a), where the four classes are evenly distributed over the total range of body conditions, the eight classes in (b) increase in width from the lower to the higher end of the hormone range. This uneven distribution was chosen to obtain about equal sample sizes for all eight categories. In (b) the horizontal lines indicate the mean percentage of gravid *R. lessonae* (solid) and *R. esculenta* (broken) for females with testosterone titers ≥20 ng/ml blood plasma. For *R. esculenta* there were no females with hormone titers in the 41–60 ng/ml range; hence, only seven data points.

The tiny skin incision as a direct method and the analysis of blood plasma hormones as an indirect method seem to identify the presence or absence of eggs fairly reliably. Since both methods are relatively uninvasive, they can be performed more than once during the same season. When combined with mark-recapture techniques and measurements of body size

(usually a good correlate of fecundity) they provide data on reproductive output of individuals and populations that cannot be obtained with other methods or only with much higher effort and costs.

Acknowledgments.—We are grateful to the many people who helped us catch frogs, to G. Abt and A.-K. Holenweg Peter for stimulating ideas and to M.-O. Wälti for helpful comments on the manuscript. D. von Holst enabled hormone analyses in his lab at the University of Bayreuth, (Germany) where I. Zerrenner-Fritsche competently analyzed all our blood samples. Ch. Lischer (Inst. Veterinary Pathology, Univ. Zuerich) gave us unlimited access to the ultrasound apparatus and Mr. Gasser and Mr. Pfeiffer (Novartis Pharma AG, Basel) provided us with the EM-Scanner. In our own lab, A. Schymainda and S. Röthlisberger kindly introduced us to and assisted with enzyme electrophoresis for species identification. The study was part of a project on behavior and population dynamics of water frogs funded by the Swiss National Science Foundation through a grant to H.-U. Reyer (No. 31-40688.94). Capture of frogs and all methods of screening for eggs conform to the ethical and animal care guidelines issued by the Swiss Academy of Natural Sciences (SANW) and were granted by the Veterinary Office of the Canton Zurich (permits no. 133/96 and 131/97).

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Accepted: 15 April 2004
Associate Editor: Susan Walls