Changes in sperm stores, ejaculate size, fertilization success, and sexual motivation over repeated matings in the common toad, *Bufo bufo* (Anura: Bufonidae)

ATTILA HETTYEY*, BALÁZS VÁGI, GERGELY HÉVIZI and JÁNOS TÖRÖK

Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, 1117 Budapest, Hungary

Fertilization is of central importance in the determination of reproductive success for both males and females. In species where males have the chance to mate repeatedly within a short period of time, sperm stocks may become depleted and males may have to carefully economize on their sperm reserves. Also, intensive intrasexual competition for females and repeated matings may lead to exhaustion on the behavioural level. To determine whether the reproductive potential of males is limited and if such a limitation is due to behavioural exhaustion or sperm depletion, we experimentally investigated changes in sperm stores, sperm expenditure, fertilization success, and sexual motivation over three repeated matings in the common toad, *Bufo bufo*, where the breeding season is short and sequential polygyny occurs. At the end of the breeding season, the number of sperm stored in the testes of males mated repeatedly was close to 50% lower than in testes of unmated males. Ejaculate size, which was estimated by applying a novel method allowing direct quantification, decreased by 88% from first to third matings. We also observed a drop in fertilization success from the first two to third matings by 65%, which was largest in males that had started the reproductive season in bad body condition. Some of these males also showed a decreased interest in females in the third mating round. Our results suggest that sperm depletion and loss of sexual motivation may together set a limit to the reproductive potential of common toad males. The present study draws attention to a limitation in reproductive potential, which may occur more often than currently anticipated and has the potential to strongly influence several aspects of reproductive behaviour. © 2009 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2009, **96**, 361–371.

ADDITIONAL KEYWORDS: ejaculate collection – exhaustion – natural history – reproduction – sexual conflict – sperm depletion.

INTRODUCTION

In non-resource based mating systems, where males contribute nothing more to offspring than their sperm, males have traditionally been viewed as exhibiting an almost limitless reproductive potential, only restricted by mate availability (Wedell, Gage & Parker, 2002). It has recently become clear that, even in such species, males may suffer severe energetic and mortality costs due to signalling, searching for mates, and fights over territories or females (Andersson & Iwasa, 1996); however, our knowledge of the costs and limitations related to the production of the ejaculate and the consequences for the mating system has remained limited (Wedell *et al.*, 2002). Nonetheless, it has been shown that sperm production may have significant costs (Dewsbury, 1982; Van Voorhies, 1992; Olsson, Madsen & Shine, 1997; Sella & Lorenzi, 2003), which may lead to limited sperm supplies and sperm depletion (Nakatsuru & Kramer, 1982; Preston *et al.*, 2001; Sato & Goshima, 2006). Consequently, it has been proposed that males may be forced to economize on their sperm reserves (Pitnick, 1993; Shapiro,

*Corresponding author. Current address: Konrad Lorenz-Institute for Ethology, Austrian Academy of Sciences, Savoyenstrasse 1a, 1160 Vienna, Austria. E-mail: hettyeyattila@yahoo.de

361

Marconato & Yoshikawa, 1994; Gómez & Serra, 1996), female choice may be biased towards previously unmated males (Dewsbury, 1982; Nakatsuru & Kramer, 1982; Harris & Moore, 2005; Sato & Goshima, 2007), and intersexual conflict may arise over sperm allocation into a given mating (Warner *et al.*, 1995; Jones, 2001; Preston *et al.*, 2001). How generally and to what extent the costs and limitations to male gamete production, and, more generally, to reproductive potential, may affect animal mating systems remains unknown.

Anuran amphibians have been popular model organisms in studies of sexual selection (Arak, 1983; Halliday, 1998; Zug, Vitt & Caldwell, 2001). Interestingly, however, the effects of repeated matings on the reproductive potential of male anurans have remained largely unstudied (for the few exceptions, see Kruse & Mounce, 1982; Gibbons & McCarthy, 1986; Elmberg, 1991; Lengagne *et al.*, 2007) and the potential importance of sperm depletion has been investigated directly only once (Smith-Gill & Berven, 1980). It is hard to understand why this question has not yet got into the focus of experimental investigations because: (1) fertilization success is of central importance in the determination of reproductive success (Andersson, 1994); (2) observations coming from several anuran species have reported that successful males can acquire repeated matings within one breeding season (*Rana catesbeiana*: Howard, 1978; *Bufo bufo*: Davies & Halliday, 1979; *Bufo rangeri*: Cherry, 1993) raising the possibility for sperm depletion and decreasing fertilization ability of males; and (3) depletion of sperm reserves has been proposed as a factor contributing to decreased fertilization success even over the course of single matings (Bourne, 1993). Ignoring potential limitations in maximum reproductive rates of males may lead to inexplicable results, wrong predictions on the reproductive behaviour, and false conclusions regarding the mating system of the studied species (Clutton-Brock & Parker, 1992).

Previous studies on the reproductive potential of anuran males have delivered equivocal results. Smith-Gill & Berven (1980) were the first to study sperm depletion in anurans and they demonstrated a decline in fertilization ability of males when they repeatedly induced spermiation with hormone injections in *Rana sylvatica*. Although they studied the effects of multiple matings in *Bufo americanus*, Kruse & Mounce (1982) found no decrease in fertilization success over repeated matings. However, some males mated five times and others only two-to-three times within the timeframe of the experiment, which could still be interpreted as between-male variation in reproductive potential with males becoming sperm depleted being the ones that refuse to mate again.
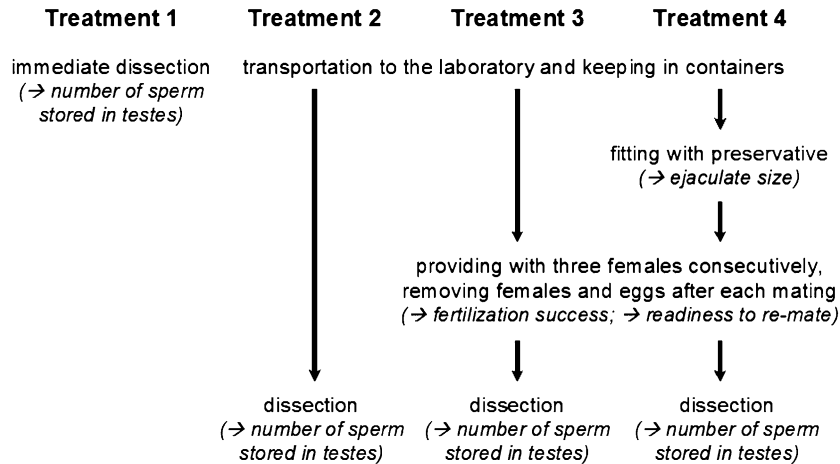
Furthermore, Gibbons & McCarthy (1986) reported a decline in fertilization ability and a decrease in the readiness for re-mating in *Rana temporaria*. Finally, Elmberg (1991) reported no significant decrease in fertilization success in a similar study on the same species and argued that physical or behavioural exhaustion rather than sperm depletion may cause declining fertility of males. It has thus remained unclear as to what extent the reproductive potential of anuran males is restricted and what intrinsic factors constrain the number of potentially successful matings.

To determine whether behavioural exhaustion alone is responsible for a limitation to reproductive potential or if sperm depletion may also play a role, one needs to simultaneously assess changes in the behaviour, sperm stocks, sperm expenditure, and the resulting fertilization success over a series of matings. There have been no attempts to perform such an integrated study in externally fertilizing taxa as yet. We experimentally investigated the reproductive potential of common toad (*Bufo bufo* Linnaeus, 1758) males by studying changes in fertilization success and in sexual motivation over the course of repeated matings. To determine whether a potential decrease in fertilization ability may be due to sperm depletion, we also examined changes in the number of stored sperm and in ejaculate sizes. We performed the latter by testing and applying a novel method of ejaculate collection. We also aimed to determine whether sperm production during the reproductive period is able to restock depleted sperm reserves between successive matings.

MATERIAL AND METHODS

THE STUDY SPECIES

The common toad (*Bufo bufo*) is a large [approximately 90 mm snout–vent length (SVL)], widespread anuran native to large areas of the Palaearctic region (Nöllert & Nöllert, 1992). The reproductive season starts around the end of March in Central Europe and lasts several weeks but, as it is an explosive breeder (*sensu* Wells, 1977), most individuals mate within 1–2 weeks (Wells, 1977; Davies & Halliday, 1979; Hemelaar, 1983; Reading, 1998). Female common toads arrive simultaneously at the breeding pond on a few distinct nights and spend only a few hours up to a few days in the breeding pond. Males are in or around the breeding site for a large part of the reproductive season (Davies & Halliday, 1979; Loman & Madsen, 1986). Males call to attract females at lower densities but switch to active mate searching and intensively fight for females at high densities (Gittins, Parker & Slater, 1980; Höglund & Robertson, 1988).

Treatment 1   **Treatment 2**   **Treatment 3**   **Treatment 4**

immediate dissection   transportation to the laboratory and keeping in containers
(→ *number of sperm*
stored in testes)

fitting with preservative
(→ *ejaculate size*)

providing with three females consecutively,
removing females and eggs after each mating
(→ *fertilization success*; → *readiness to re-mate*)

dissection   dissection   dissection
(→ *number of sperm*   (→ *number of sperm*   (→ *number of sperm*
stored in testes)   *stored in testes*)   *stored in testes*)

Figure 1. A summary of the four treatments. The goal of each step is written in parentheses.

Take-overs sometimes occur with large males being more successful in acquiring and defending females (Davies & Halliday, 1979; Loman & Madsen, 1986; Höglund, 1989, but see also Lengagne *et al.*, 2007). Consequently, there is variation in the mating success of males: some males engage in repeated matings, whereas others do not mate at all, but there is little opportunity for extensive multiple matings (Davies & Halliday, 1979; Loman & Madsen, 1986). Sperm competition has been shown to be present, leading to multiple paternity within clutches (Sztatecsny *et al.*, 2006).

COLLECTION AND MAINTENANCE OF EXPERIMENTAL ANIMALS

In April 2006, we hand collected 83 males and 40 females from a population (47°43′N, 19°00′E) counting several thousand individuals (Hettyey, Török & Kovács, 2003) in the Pilis Mountains, Hungary, at the onset of the breeding season and transported them to the laboratory of the Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest, where we conducted the experiments. We transported males and females individually in slightly wet 20×30 cm canvas sacks. After measuring SVL with a plastic ruler (to the nearest 1 mm) of all individuals, we selected 40 males of intermediate size (mean ± SD = 71.1 ± 1.15 mm) and returned the others to the breeding pond. We also measured body mass of selected males with a digital scale (to the nearest 0.1 g). We kept males and females in a fridge set to 5 °C during the selection of males, until the start of the experiment and prior to returning them to the breeding pond. Anurans kept at 5 °C in the dark are inactive and consequently may experience low levels of stress. Males spent a maximum of 10 h in the fridge before being returned to the pond, whereas females were kept for up to 1 week before being used in the experiment. Animals were not fed during the experiment because explosively breeding anurans are generally believed to be capital breeders. Limited sample sizes due to logistic constraints forced us to concentrate on average sized males to minimize confounding effects of male size which otherwise could have swamped the results.

EXPERIMENTAL DESIGN

We assigned males randomly to four experimental treatments (Fig. 1), with each treatment receiving ten individuals. To determine the number of sperm in the testes of males at the start of the breeding season, males in treatment 1 were over-anaesthetized with tricaine (MS-222) as soon as they were brought in from the field and, after dissection, their testes were preserved in 50% ethanol for later processing. All other males were placed individually into large plastic containers (90 cm in diameter), filled to a depth of 10 cm with tap water (approximately 65 litres), 2 days prior to experiments. We maintained a water temperature of 19 °C and a light/dark cycle of 10–14 h over the course of the experiments which lasted 12 days. For assessing changes in the number of stored sperm over the breeding season attributable to sperm production, sperm degradation, or sperm leakage, we kept males assigned to treatment 2 deprived from females until the termination of the experiment, when they were killed and their testes were preserved in 50% ethanol. To investigate changes in fertilization success with repeated matings, we provided males in treatment 3 with females three times, always adding new, gravid females simultaneously to all males once all males had finished mating in the previous mating round. Finally, for estimating changes in ejaculate sizes with

repeated matings, we subjected males assigned to treatment 4 to the same procedure as those in treatment 3, but also fitted them with non-lubricated condoms for the purpose of ejaculate collection and quantification. Two holes were cut in the condom and the male's hind legs were pulled through them. The condom was then rolled out on the abdomen of the male and was fixed with a piece of yarn around the waist. For a detailed description of the applied method of sperm collection, see Hettyey & Török (2005).

We put the first group of females into males' containers on 4 April, the second group on 8 April and the third group on 11 April. Mating experiments were terminated on 12 April, after all amplexed females had finished laying their eggs. Thus, there were 4 days between the initiation of the first and the second mating and 3 days between the second and the third mating, leaving an ecologically relevant amount of time for males to recover from previous matings as the peak of reproductive activites, when most matings occur, usually lasts approximately 1 week in general (Wells, 1977; Davies & Halliday, 1979; Hemelaar, 1983; Reading, 1998) and in the study population as well (A.H. pers. observ.).

We monitored experimental animals in treatments 3 and 4 once every hour day and night and, as soon as egg-deposition commenced, twice an hour. When a female had finished egg-deposition and the male had subsequently released it, we removed the female from the container. If the male was fitted with a condom, we removed it, washed the contents into a glass jar with a few millilitres of distilled water, added 95% ethanol (to achieve a final alcohol concentration of approximately 25%) and preserved four samples of 0.5 mL each. We removed and counted the eggs, and kept four sections of the egg-string, each containing approximately 200 eggs (one section from the beginning, two from the middle part, and one from the end of the string) in dishpans and covered eggs with 1 cm of tap water. We estimated fertilization success three days later by counting dead eggs and developing embryos. After each mating round, we returned eggs and used females to the site of collection. Once all pairs had finished egg-deposition and all females and eggs had been removed from the containers, we equipped males in treatment 4 with a new condom and placed a female into the containers in treatments 3 and 4 for a second mating then repeated the procedure for a third round of matings. After the third mating, we over-anaesthetized and dissected males and preserved their testes in 50% ethanol.

For the estimation of the number of sperm stored in the testes, we macerated testes of all males in distilled water, weighed sperm suspensions and estimated sperm concentrations from eight sub-samples by counting sperm in four quadrates of a Bürker chamber for each sub-sample at ×200 magnification. Similarly, to estimate the number of sperm in the ejaculates, we took two sub-samples from each of the four samples preserved from each ejaculate, and estimated sperm concentrations based on sperm numbers in four quadrates at ×200 magnification.

In treatment 3, for one male the number of stored sperm was by mistake not estimated. In treatment 4, ejaculate sizes could not be evaluated for three males in both the first and second mating rounds and for two males in the third mating round because those males removed condoms before we could obtain ejaculate samples from them. We had to completely exclude data on two males from the analyses as one male from treatment 2 received a female by mistake in the second and third mating rounds and another male in treatment 4 did not mate in the first mating round because the female it was mated to did not lay any eggs despite extended amplexus.

TESTING THE EJACULATE COLLECTION TECHNIQUE

We performed a test to estimate the accuracy of the method applied for quantifying sperm numbers in the ejaculates. After dissecting three males, macerating their testes and determining sperm concentrations to obtain three sperm suspensions of varying but known concentrations, we fitted nine males with a condom and placed them individually into plastic containers which were identical with the ones used in the experiment. One hour later, we injected 1 mL of sperm suspension of known concentration into the condom of each male with a pipette to imitate the presence of ejaculates with suspensions assigned to males randomly. Five minutes later, we removed the condoms and estimated sperm numbers in the 'ejaculates'. In one case, by mistake, we did not note the code of the original sperm suspension, and, in another case, a male removed the condom leaving us with a sample size of seven. This test of the applied ejaculate collection technique showed that it underestimates sperm numbers in the 'ejaculates' by approximately 55% (mean ± SE = 54.67 ± 6.49%). However, a strong correlation between the number of injected sperm and sperm numbers estimated from 'ejaculates' (Pearson correlation; $R = 0.935$; $N = 7$; $P = 0.002$) indicated a high consistency in the magnitude of sperm-loss caused by the methodology. Therefore, we accepted the method to be applicable to quantitative ejaculate collection.

STATISTICAL ANALYSIS

To enhance normality and homogeneity of variances, we transformed count data using square-root trans-

formation and percentage data using arcsine-square-root transformation as recommended by Précsényi *et al.* (1995). We regressed body mass measured immediately after capture on SVL length and used standardized residuals as measures of initial body condition. We compared the number of sperm found in the testes of males using general linear model (GLM) analysis of covariance (ANCOVA) by entering treatment-type as a fixed factor, sperm number as the dependent variable, and initial body condition as a covariate. We investigated ejaculate sizes of males in treatment 4 over repeated matings using a GLM ANCOVA with ejaculate size entered as the dependent variable, mating number as a fixed factor, male identity as a random factor and egg number, fertilization success and initial body condition as covariates. We used fertilization success as an estimate of sperm leakage and entered it into the model to control for it. To detect a potential decrease in fertilization success in sequential matings and to test the hypothesis that sperm depletion may occur and become visible over the course of a mating as proposed by Bourne (1993), we performed a linear mixed model (LMM) ANCOVA on data obtained from treatment 3. We entered fertilization success in different parts of the egg strings as the dependent variable, location on the egg string and mating number as fixed factors, male identity as a random factor, and the total number of eggs deposited in the given mating as a covariate. We analysed changes in sexual motivation in treatments 3 and 4 with a LMM ANCOVA by entering male behaviour as the dependent variable, mating number and treatment as fixed factors, male identity as a random factor, and initial body condition as a covariate. In all tests, we applied a backward stepwise removal procedure and re-entered the removed variables one by one to the final model to obtain relevant statistics. All tests were two tailed. Statistical tests were calculated using SPSS, version 12.0.1.

RESULTS

NUMBER OF STORED SPERM

Repeated mating had a significant effect on the number of sperm stored in the testes: mated males had, on average, approximately half the number of sperm found in unmated males [unmated males (treatments 1 and 2): mean $\pm$ SE $= 38.92 \times 10^6 \pm 4.07 \times 10^6$, $N = 19$; repeatedly mated males (treatments 3 and 4): mean $\pm$ SE $= 21.26 \times 10^6 \pm 2.8 \times 10^6$, $N = 18$]. This difference was significant (GLM ANCOVA: $F_{1,35} = 12.87$; $P = 0.001$). When analysed separately, we also found variation between the number of sperm stored by males exposed to the four
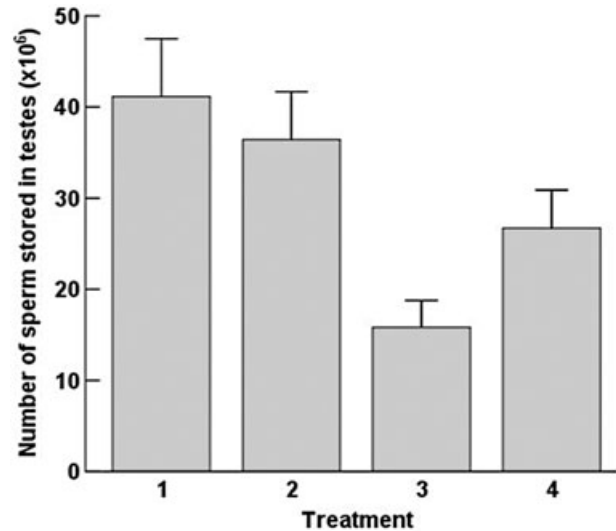

Figure 2. The number of sperm stored in the testes of males in the four treatments. Treatments: 1, preserved at the beginning of the breeding season; 2, preserved at the end of the breeding season, deprived from matings, not fitted with a condom; 3, repeatedly mated, not fitted with a condom; 4, repeatedly mated, fitted with a condom. For the ease of interpretation, untransformed data are shown, bars represent one standard error.

treatments (GLM ANCOVA: $F_{3,33} = 5.81$; $P = 0.003$). Post-hoc tests indicated that the amount of stored sperm was lower in treatment 3 than in treatments 1 and 2 [Tukey honestly significant difference (HSD) tests: $P = 0.003$ and $P = 0.013$, respectively; Fig. 2]. All other pairwise comparisons yielded nonsignificant results (all $P > 0.24$). Initial body condition did not have a significant effect in either analysis (both $P > 0.3$).

EJACULATE SIZE

As estimated from the sperm counted in the condoms of males in treatment 4, ejaculate size decreased over the course of repeated matings (GLM ANCOVA: $F_{2,16} = 9.28$; $P = 0.002$). We observed a large, significant drop in ejaculate size between the first and the second mating by close to 80% (first mating: mean $\pm$ SE $= 15.82 \times 10^5 \pm 5.2 \times 10^5$, $N = 6$; second mating: mean $\pm$ SE $= 3.15 \times 10^5 \pm 1.73 \times 10^5$, $N = 6$; Tukey HSD test: $P = 0.011$; Fig. 3). No difference could be detected between the second and the third mating (Tukey HSD test: $P = 0.812$; third mating: mean $\pm$ SE $= 1.87 \times 10^5 \pm 0.85 \times 10^5$, $N = 7$; Fig. 3). Neither male identity (GLM ANCOVA: $F_{8,8} = 0.64$; $P = 0.73$), nor egg number ($F_{1,15} = 0.03$; $P = 0.86$), nor initial body condition ($F_{1,15} = 0.29$; $P = 0.6$) influenced the number of ejaculated sperm. Sperm leakage esti-
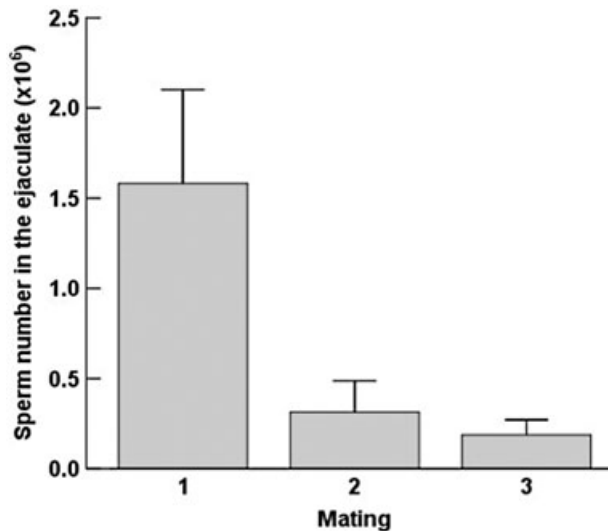
Figure 3. Sperm numbers collected by condoms fitted to males in treatment 4 to estimate ejaculate sizes over the course of three consecutive matings. Because some males did not mate in all rounds and/or the condom got displaced before the ejaculate could be retrieved, sample sizes were 6 for the first and the second mating and 7 in the third mating. Mating rounds were started on 4, 8 and 11 April by adding one female to each male. Note that the applied technique underestimates actual ejaculate sizes by approximately 55%. Untransformed data are shown, bars represent one standard error.

mated from fertilization success did not seem to affect sperm counts ($F_{1,15} = 0.09$; $P = 0.77$).

FERTILIZATION SUCCESS

We observed a decrease in fertilization success over the three consecutive matings (LMM ANCOVA: $F_{2,111} = 61.13$; $P < 0.001$). Fertilization success was high in the first and the second matings (first mating: mean ± SE = 74.4 ± 4.3%; second mating: mean ± SE = 86.6 ± 3.5%), but decreased significantly in the third mating (mean ± SE = 28.5 ± 5.7%; Fig. 4). Post-hoc tests based on Bonferroni adjusted comparisons of estimated marginal means revealed that fertilization success differed significantly in all pairwise comparisons (all $P > 0.045$). Location on the egg-string had a significant effect ($F_{3,111} = 3.68$; $P = 0.014$), indicating an increase in fertilization efficiency towards the end of a mating (Fig. 4). Initial body condition ($F_{1,111} = 4.88$; $P = 0.029$) and the interaction between mating number and initial body condition also had significant effects ($F_{2,111} = 11.53$; $P < 0.001$; Fig. 5). Male identity ($Z = 1.41$; $P = 0.16$) egg number ($F_{1,111} = 0.43$; $P = 0.51$), and all other two-way interactions (all $P > 0.05$) did not seem to affect fertilization success.
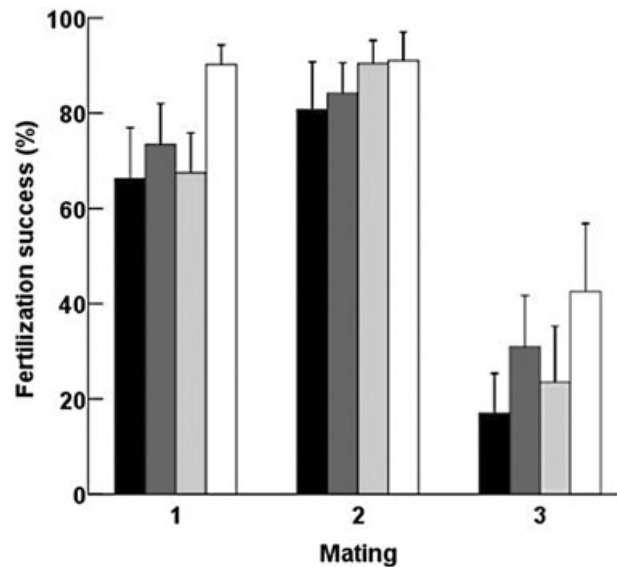

Figure 4. Changes in fertilization success over the course of repeated matings and within matings in treatment 3. Black bars represent the first part, dark grey bars the second part, light grey bars the third part, and white bars the last part of the egg strings. Untransformed data are shown, bars represent one standard error.

SEXUAL MOTIVATION

Out of the ten males in treatment 3 and nine males in treatment 4, all males mated in the first round, one male in treatment 4 refused to mate in the second and another one in the third round, and three males in treatment 3 abandoned the female when it was still laying eggs in the third round. Considering males that either did not form amplexus at all or abandoned the female prematurely as having a decreased level of sexual motivation, the interaction between initial body condition and mating number had a significant effect on sexual motivation (LMM ANCOVA: $F_{2,51} = 8.77$; $P = 0.001$; Fig. 6). The effects of initial body condition ($F_{1,51} = 7.85$; $P = 0.007$) and mating number ($F_{2,51} = 7.21$; $P = 0.002$) had significant effects on sexual motivation by themselves as well. We did not find a difference in sexual motivation between males that were fitted with a condom or not ($F_{1,50} = 0.08$; $P = 0.78$). Effects of male identity ($F_{17,34} = 0.83$; $P = 0.65$) and all other two-way interactions (all $P > 0.2$) were nonsignificant.
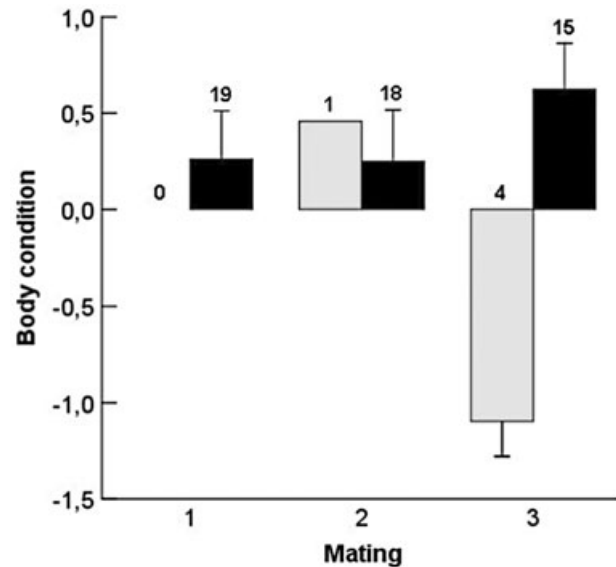
DISCUSSION

The results obtained in the present study suggest that the reproductive potential of common toad males may be limited to a very few successful matings within a breeding season. This may especially be true for males that start the reproductive season in bad
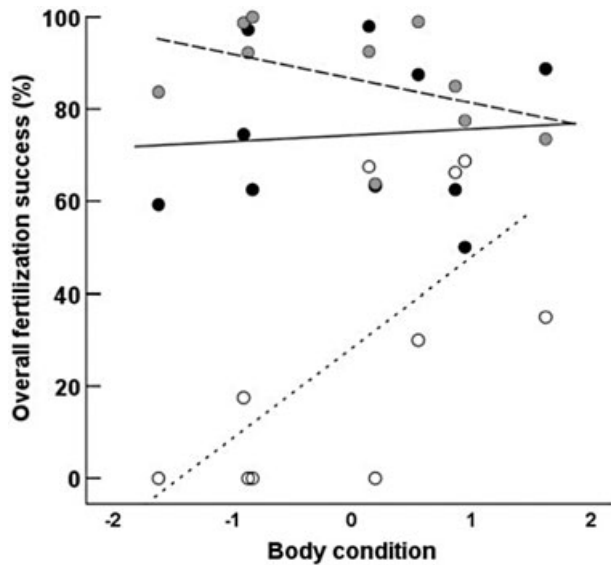
Figure 5. The relationship between initial body condition and fertilization success in the three repeated matings in treatment 3 (black dots: first matings, grey dots: second matings, empty dots: third matings). Separate regression lines are drawn for each mating; the solid line represents the first mating ($R^2 = 0.01$), the intermittent line represents the second mating ($R^2 = 0.2$), and the dotted line represents the third mating ($R^2 = 0.46$). Overall fertilization success is the average fertilization success over all four parts of the egg-strings resulting from a given mating event.

Figure 6. The relationship between sexual motivation and initial body condition over repeated matings in treatments 3 and 4. Body condition was calculated as the residuals from the regression of body mass on snout–vent length. Grey bars represent males showing decreased levels of sexual motivation (not amplexing the female or leaving amplexus before the end of egg-laying), black bars represent males with high sexual motivation. Number of individuals are indicated, error bars represent one standard error.

body condition. The number of sperm stored in the testes did not significantly change from the onset to the end of the breeding season in female-deprived males, but decreased in males mated repeatedly. Correspondingly, ejaculate sizes decreased over the course of successive matings, which is likely to have contributed to the observed drop in fertilization success in third matings. Consequently, it appears that males use the sperm they produced before the onset of the breeding season and are not able to replenish their sperm reserves from one mating to the other. Finally, we observed exhaustion in the individuals' behaviour because several males in bad initial body condition lost interest in females after having mated twice.

Males are generally still viewed as exhibiting a more or less limitless reproductive potential and as being always on the search for mating opportunities (Wedell *et al.*, 2002). Our results contribute to the growing body of research that challenges this view (Nakatsuru & Kramer, 1982; Preston *et al.*, 2001; Sato & Goshima, 2006) and that stresses the importance of body condition in the determination of reproductive potential of males (Smith-Gill & Berven,

1980; Rakitin, Ferguson & Trippel, 1999; Rowe & Hutchings, 2004; Trippel & Neil, 2004). However, the question arises if the limited reproductive potential of medium-sized common toad males may actually constrain the number of successful breeding events under natural conditions. We do not know how many matings males of different size-classes can achieve within one breeding season. There are reports on relatively low standing genetic diversity in some populations suggesting large among-male variation in reproductive success (Scribner, Arntzen & Burke, 1997; Brede & Beebee, 2004). Also, Davies & Halliday (1979) evaluated the percentage of males successfully mating in an English population to be only approximately 20%, although it has to be noted that they may have underestimated the number of mating males due to the applied methodology. Furthermore, larger males may sometimes overtake the place of mated smaller males, and are thus somewhat more likely to achieve repeated matings, but smaller males are not excluded from matings (Davies & Halliday, 1978, 1979; Reading & Clarke, 1983; Loman & Madsen, 1986; Reading, 2001; Lengagne *et al.*, 2007). Finally, the operational sex ratio at breeding ponds varies considerably both within and between years

(Reading, 2001). Consequently, small and especially medium sized males may have some chances to mate repeatedly, particularly during periods of relaxed intrasexual competition and in years when the sex ratio is less biased towards males. The limited reproductive potential allowing only two matings with high fertilization success in some intermediate sized males may thus sometimes impose a constraint on the number of offspring sired by them. Due to their superior competitive ability, large anuran males may be exposed to stronger selection for enduring sexual stamina (e.g. *Rana sylvatica*: Smith-Gill & Berven, 1980). If large common toad males indeed have a higher reproductive potential than smaller males remains to be tested, but it would provide an interesting case where the reproductive potential of males is tuned to their mating prospects.

Although the results of the present study coherently point towards a fast decrease in the reproductive potential of common toad males, there are some curious inconsistencies between some of our measures. Males mating three times appear to expend approximately 50% of sperm stored in their testes, leaving them with approximately 20 million sperm after the third mating. Why do males not use this large number of sperm in matings to maintain ejaculate sizes and avoid a drop in fertilization success? One possible explanation would be that repeatedly mated males losing interest in matings due to exhaustion may as a side-effect also deliver smaller ejaculates. However, as ejaculate sizes drop earlier than when signs of fatigue appear, it is more likely that sperm depletion contributes to the loss of sexual motivation rather than the other way around. By contrast, Montrose, Harris & Moore (2004) found in the cockroach *Nauphoeta cinerea* that males mate with females even after their sperm reserves had been depleted to increase their relative fitness. This discrepancy may be explained by the fact that *N. cinerea* males are able to pervasively manipulate female mating behaviour simply by copulating with them (Roth, 1964), whereas *B. bufo* males have no control over the behaviour of their mates and have to engage in tedious and prolonged fights over the possession of a female.

The question, however, remains: why do males not use their entire sperm stocks? Spermatozoa stored in the testes may not all be fully mature or repeated mobilization of sperm and production of other components of the ejaculate may be physiologically constrained. Over the course of repeated matings, when fully mature, good-quality sperm become depleted, and the ejaculate may increasingly contain non-mature, poor-quality sperm or insufficient quantities or qualities of accessory ejaculate-components, resulting in the observed lowered fertilization success

(Bissoondath & Wiklund, 1996; Strzeżek *et al.*, 2000). In a study on *Rana sylvatica*, Smith-Gill & Berven (1980) induced spermiation repeatedly and fertilized eggs artificially, that is they excluded effects of exhaustion on the behavioural level. Even though males did not deliver full ejaculates at any spermiation, the number of released sperm and resulting fertilization success decreased over only three matings. This result also points towards the possibility that the number of stored sperm does not have to drop low before sperm depletion or, more generally speaking, ejaculate depletion becomes apparent through decreased fertilization success. Although it is known that sperm are produced in surplus by anuran males (Sasso-Cerri *et al.*, 2006), it remains unclear why they produce that large numbers that appear not to be used and what constraint causes sperm retention.

If sperm depletion occurs, one would expect fertilization success to decrease with the progress of egg-deposition (Bourne, 1993). We observed the opposite. Fertilization success was lowest at the beginning of egg-strings and highest at their ends. It is possible that physiological and behavioural preparation for ejaculation constrains sperm expenditure at the beginning of mating, but it is more likely that the observed pattern in fertilization success is caused by the decreasing rate of egg-release. At the beginning of egg-deposition, the number of sperm released by males or the accuracy of synchronization of gamete release, both decisive factors in the determination of fertilization success in externally fertilizing species (Davies & Halliday, 1977; Geffen, 1999), may not be sufficiently high to fertilize the large number of eggs deposited within a short time. Subsequently, when egg-release slows down, it may become easier for the male to release a sufficient number of sperm at the right time. Temporal changes in the rate of egg-release may thus explain why fertilization success increased within matings, whereas it decreased between matings.

In species where repeatedly mated males become sperm-depleted, are not able to achieve high fertilization success, and ultimately lose their sexual interest, several aspects of the mating system may uniquely differ from that of species where males have a practically limitless reproductive potential. If sperm depletion is linked with fatigue appearing on the behavioural level, as it appears to be the case in the common toad, sexual conflict (*sensu* Parker, 1979) arising over matings between exhausted males and gravid females may be relaxed even in species where sexual coercion by males is ubiquitous or where females are unable to assess the mating status of males. Males may not be able to obtain extensive repeated matings in the face of intensive fights with

more vigorous non-mated males and may even terminate sexual activities. This may explain why common toad males are leaving the breeding pond, whereas gravid females are still arriving in numbers towards the end of the reproductive period (A.H. pers. observ.). Ultimately, if males have a limited reproductive potential, they may benefit from carefully choosing their mates and male mate choice may evolve (Clutton-Brock & Parker, 1992) even in the presence of intensive intrasexual competition for females (Owens & Thompson, 1994).

Females mating with males that have already mated and have thus become sexually exhausted, may lose out in terms of fertilization success (Warner *et al.*, 1995; Preston *et al.*, 2001; Sato & Goshima, 2006). Consequently, females may benefit from avoiding previously mated males (Gross, 1996; Wedell *et al.*, 2002; Montrose *et al.*, 2004). This may lead to the evolution of female mate choice based on the reproductive potential of males or to temporally varying mate preference patterns (Harris & Moore, 2005; Sato & Goshima, 2007). Females may also solicit male–male contests or induce polyandry when they are paired up with an exhausted male (Olsson & Shine, 1997; Smith & Reichard, 2005). At the same time, however, engaging in sperm-demanding matings with apparent sperm competition may be avoided by males that face the threat of sperm depletion, creating a possible scenario for sexual conflict.

We conclude that intermediate-sized common toad males, especially those that start the breeding season in bad body condition, have a limited reproductive potential that is manifested in fast-declining fertilization success and fatigue on the behavioural level accompanied by signs of sperm depletion. A limited reproductive potential of males may in turn affect the effective population size, variation in reproductive success, and the strength of sexual selection through a variety of mechanisms. The frequency of polygamy and the resulting risk and intensity of sperm competition may also be influenced by sperm limitation. Further investigations are required to help clarify the role of limitations in the reproductive potential of males in the evolution of reproductive behaviour.

ACKNOWLEDGEMENTS

REFERENCES

Andersson M. 1994. *Sexual selection*. New York, NY: Princeton University Press.

Andersson M, Iwasa Y. 1996. Sexual selection. *Trends in ecology and Evolution* **11:** 53–58.

Arak A. 1983. Male-male competition and mate choice in anuran amphibians. In: Bateson P, ed. *Mate choice*. Cambridge: University Press, 181–210.

Bissoondath CJ, Wiklund C. 1996. Effect of male mating history and body size on ejaculate size and quality in two polyandrous butterflies, *Pieris napi* and *Pieris rapae* (Lepidoptera: Pieridae). *Functional Ecology* **10:** 457–464.

Bourne GR. 1993. Proximate costs and benefits of mate acquisition at leks of the frog *Ololygon rubra*. *Animal Behaviour* **45:** 1051–1059.

Brede EG, Beebee TJC. 2004. Contrasting population structures in two sympatric anurans: implications for species conservation. *Heredity* **92:** 110–117.

Cherry MI. 1993. Sexual selection in the raucous toad, *Bufo rangeri*. *Animal Behaviour* **45:** 359–373.

Clutton-Brock TH, Parker GA. 1992. Potential reproductive rates and the operation of sexual selection. *Quarterly Review of Biology* **67:** 437–456.

Davies NB, Halliday TR. 1977. Optimal mate selection in the toad *Bufo bufo*. *Nature (London)* **269:** 56–58.

Davies NB, Halliday TR. 1978. Deep croaks and fighting assessment in toads *Bufo bufo*. *Nature (London)* **274:** 683–685.

Davies NB, Halliday TR. 1979. Competitive mate searching in male common toads, *Bufo bufo*. *Animal Behaviour* **27:** 1253–1267.

Dewsbury DA. 1982. Ejaculate cost and male choice. *American Naturalist* **119:** 601–610.

Elmberg J. 1991. Factors affecting male yearly mating success in the common frog, *Rana temporaria*. *Behavioral Ecology and Sociobiology* **28:** 125–131.

Geffen AJ. 1999. Variations in sperm motility of the Atlantic herring *Clupea harengus*. *Marine Biology* **134:** 637–643.

Gibbons MM, McCarthy TK. 1986. The reproductive output of frogs *Rana temporaria* (L.) with particular reference to body size and age. *Journal of Zoology (London)* **209:** 579–593.

Gittins SP, Parker GA, Slater FM. 1980. Mate assortment in the common toad (*Bufo bufo*). *Journal of Natural History* **14:** 663–668.

Gross M. 1996. Alternative reproductive strategies and tactics: diversity within the sexes. *Trends in Ecology and Evolution* **11:** 92–98.

Gómez A, Serra M. 1996. Mate choice in male *Brachionus plicatilis* rotifers. *Functional Ecology* **10:** 681–687.

Halliday T. 1998. Sperm competition in amphibians. In: Birkhead TR, Møller AP, eds. *Sperm competition and sexual selection*. London: Academic Press, 465–502.

Harris WE, Moore PJ. 2005. Female mate preference and sexual conflict: females prefer males that have had fewer consorts. *American Naturalist* **165:** 64–71.

Hemelaar ASM. 1983. Age of *Bufo bufo* in amplexus over the spawning period. *Oikos* **40:** 1–5.

Hettyey A, Török J. 2005. In situ' prevention of anuran fertilization – a simple method for the detection of sperm competition with potential for other applications. *Herpetological Review* **36:** 33–36.

Hettyey A, Török J, Kovács T. 2003. Breeding biology and habitat use of seven amphibian species in a hilly woodland (Pilis Mountains, Hungary). *Állattani Közlemények* **88:** 41–55.

Howard RD. 1978. The evolution of mating strategies in bullfrogs, *Rana catesbeiana*. *Evolution* **32:** 850–871.

Höglund J. 1989. Pairing and spawning patterns in the common toad, *Bufo bufo*: the effects of sex ratios and the time available for male-male competition. *Animal Behaviour* **38:** 423–429.

Höglund J, Robertson JGM. 1988. Chorusing behavior, a density-dependent alternative mating strategy in male common toads (*Bufo bufo*). *Ethology* **79:** 324–332.

Jones TM. 2001. A potential cost of monandry in the lekking sandfly *Lutzomyia longipalpis*. *Journal of Insect Behaviour* **14:** 385–399.

Kruse KC, Mounce M. 1982. The effects of multiple matings on fertilization capability in male american toads (*Bufo americanus*). *Journal of Herpetology* **16:** 410–412.

Lengagne T, Arthaud F, Cornier M, Joly P. 2007. Cost of sexually embracing a large female offset by the number of eggs fertilized for small male *Bufo bufo* L. *Biological Journal of the Linnean Society* **92:** 755–762.

Loman J, Madsen T. 1986. Reproductive tactics of large and small male toads *Bufo bufo*. *Oikos* **46:** 57–61.

Montrose VT, Harris WE, Moore PJ. 2004. Sexual conflict and cooperation under naturally occurring male enforced monogamy. *Journal of Evolutionary Biology* **17:** 443–452.

Nakatsuru K, Kramer DL. 1982. Is sperm cheap? Limited male fertility and female choice in the lemon tetra (Pisces, Characidae). *Science* **216:** 753–755.

Nöllert A, Nöllert C. 1992. *Die Amphibien Europas. Bestimmung-Gefährdung-Schutz*. Stuttgart: Franckh-Kosmos Verlag.

Olsson M, Madsen T, Shine R. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proceedings of the Royal Society of London Series B, Biological Sciences* **264:** 455–459.

Olsson M, Shine R. 1997. Advantages of multiple matings to females: a test of the infertility hypothesis using lizards. *Evolution* **51:** 1684–1688.

Owens IPF, Thompson DBA. 1994. Sex differences, sex ratios and sex roles. *Proceedings of the Royal Society of London Series B, Biological Sciences* **258:** 93–99.

Parker GA. 1979. Sexual selection and sexual conflict. In: Blum MS, Blum NA, eds. *Sexual selection and reproductive competition in insects*. Princeton, NJ: Academic Press, 123–166.

Pitnick S. 1993. Operational sex ratios and sperm limitation in populations of *Drosophila pachea*. *Behavioral Ecology and Sociobiology* **33:** 383–391.

Preston BT, Stevenson IR, Pemberton JM, Wilson K. 2001. Dominant rams lose out by sperm depletion. *Nature* **409:** 681–682.

Précsényi I, Barta Z, Karsai I, Székely T. 1995. *Alapvető kutatástervezési, statisztikai és projektértékelési módszerek a szupraindividuális biológiában*. Debrecen: Kossuth Lajos University.

Rakitin A, Ferguson MM, Trippel EA. 1999. Sperm competition and fertilization success in Atlantic cod (*Gadus morhua*): effect of sire size and condition factor on gamete quality. *Canadian Journal of Fisheries and Aquatic Sciences* **56:** 2315–2323.

Reading CJ. 1998. The effect of winter temperatures on the timing of breeding activity in the common toad *Bufo bufo*. *Oecologia* **117:** 469–475.

Reading CJ. 2001. Non-random pairing with respect to past breeding experience in the common toad (*Bufo bufo*). *Journal of Zoology (London)* **255:** 511–518.

Reading CJ, Clarke RT. 1983. Male breeding behaviour and mate acquisition in the common toad, *Bufo bufo*. *Journal of Zoology (London)* **201:** 237–246.

Roth LM. 1964. Control of reproduction in female cockroaches with special reference to *Nauphoeta cinerea*. I. First preoviposition period. *Journal of Insect Physiology* **10:** 915–945.

Rowe S, Hutchings JA. 2004. The function of sound production by Atlantic cod as inferred from patterns of variation in drumming muscle mass. *Canadian Journal of Zoology* **82:** 1391–1398.

Sasso-Cerri E, Cerri PS, Freymüller E, Miraglia SM. 2006. Apoptosis during the seasonal spermatogenic cycle of *Rana catesbeiana*. *Journal of Anatomy* **209:** 21–29.

Sato T, Goshima S. 2006. Impacts of male-only fishing and sperm limitation in manipulated populations of an unfished crab, *Hapalogaster dentata*. *Marine Ecology Progress Series* **313:** 193–204.

Sato T, Goshima S. 2007. Female choice in response to risk of sperm limitation by the stone crab, *Hapalogaster dentata*. *Animal Behaviour* **73:** 331–338.

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

Sella G, Lorenzi MC. 2003. Increased sperm allocation delays body growth in a protandrous simultaneous hermaphrodite. *Biological Journal of the Linnean Society* **78:** 149–154.

Shapiro DY, Marconato A, Yoshikawa T. 1994. Sperm economy in a coral-reef fish, *Thalassoma bifasciatum*. *Ecology* **75:** 1334–1344.

Smith C, Reichard M. 2005. Females solicit sneakers to improve fertilisation success in the bitterling (*Rhodeus sericeus*). *Proceedings of the Royal Society of London Series B, Biological Sciences* **272:** 1683–1688.

Smith-Gill SJ, Berven KA. 1980. In vitro fertilization and assessment of male reproductive potential using mammalian gonadotropin-releasing hormone to induce spermiation in *Rana sylvatica*. *Copeia* **1980:** 723–728.

Strzezek J, Fraser L, Demianowicz W, Kordan W, Wysocki P, Holody D. 2000. Effect of depletion tests (DT) on the composition of boar semen. *Theriogenology* **54:** 949–963.

Sztatecsny M, Jehle R, Burke T, Hödl W. 2006. Female polyandry under male harassment: the case of the common toad (*Bufo bufo*). *Journal of Zoology (London)* **270:** 517–522.

Trippel EA, Neil SRE. 2004. Maternal and seasonal differences in egg sizes and spawning activity of northwest Atlantic haddock (*Melanogrammus aeglefinus*) in relation to body size and condition. *Canadian Journal of Fisheries and Aquatic Sciences* **61:** 2097–2110.

Van Voorhies WA. 1992. Production of sperm reduces nematode life-span. *Nature* **360:** 456–458.

Warner RR, Shapiro DY, Marconato A, Petersen CW. 1995. Sexual conflict – males with the highest mating success convey the lowest fertilization benefits to females. *Proceedings of the Royal Society of London Series B, Biological Sciences* **262:** 135–139.

Wedell N, Gage MJG, Parker GA. 2002. Sperm competition, male prudence and sperm-limited females. *Trends in Ecology and Evolution* **17:** 313–320.

Wells KD. 1977. The social behaviour of anuran amphibians. *Animal Behaviour* **25:** 666–693.

Zug GR, Vitt LJ, Caldwell JP. 2001. *Herpetology: an introductory biology of amphibians and reptiles*. London: Academic Press.