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Allocation in reproduction is not tailored to the probable number of matings in common toad (*Bufo bufo*) males

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Abstract The theory of life history evolution assumes trade-offs between competing fitness traits such as reproduction, somatic growth, and maintenance. One prediction of this theory is that if large individuals have a higher reproductive success, small/young individuals should invest less in reproduction and allocate more resources in growth than large/old individuals. We tested this prediction using the common toad (*Bufo bufo*), a species where mating success of males is positively related to their body size. We measured testes mass, soma mass, and sperm stock size in males of varying sizes that were either (1) re-hibernated at the start of the breeding season, (2) kept without females throughout the breeding season, or (3) repeatedly provided with gravid females. In the latter group, we also estimated fertilization success and readiness to re-mate. Contrary to our predictions, the relationship between testes mass and soma mass was isometric, sperm stock size relative to testes mass was unrelated to male size, fertilization success was not higher in matings with larger males, and smaller males were not less likely to engage in repeated matings than larger males. These results consistently suggest that smaller males did not invest less in reproduction to be able to allocate more in growth than larger males. Causes for this unexpected result may include relatively low year-to-year survival, unpredictable between-year variation in the strength of sexual selection and low return rates of lowered reproductive investment.

Keywords Life history · Natural history · Potential reproductive rate · Repeated mating · Reproductive success · Resource allocation

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Introduction

Life history theory assumes that fitness traits cannot evolve independently from one another but are constrained by trade-offs originating from the allocation of limited resources to conflicting functions. Optimal resource allocation solutions should evolve to maximize fitness and should depend on the extrinsic environment and the intrinsic state of the organism (Williams 1966; Stearns 1992; Roff 2002). Trade-offs between reproductive investment and somatic growth have indeed been documented in empirical studies (Reznick 1985). Consequently, it may be expected that where large individuals have a higher reproductive success, small/young individuals should invest less in reproduction and allocate more resources in growth than large/old individuals (e.g., Warner 1984; Berglund 1991; Aday et al. 2003) as later revenues in the form of enhanced reproductive success may more than make up for lower current success. This may be

especially true for species where growth is indeterminate, lowering current reproductive investment can result in increased body size in adults, and where reproduction has large costs so that benefits from a lowered reproductive output may also be expected to be large (Kozlowski 1996; Heino and Kaitala 1999).

Anuran amphibians are ideal for testing whether small males trade off allocation in reproduction with growth as predicted by life history theory; as they exhibit indeterminate growth (Duellman and Trueb 1994), large males usually have a mating advantage (e.g., Davies and Halliday 1979; Ryan 1983; Tejedo 1992) and investment in reproduction is generally considered high in males as they lose around 20% of their body mass during the reproductive season (e.g., Arak 1983; Ryser 1989). In fact, there is suggestive evidence that anuran males may carefully optimize rather than maximize reproductive investment: males of some species seem to exhibit a limited reproductive potential, allowing them only a few successful matings (e.g., *Bufo bufo*: Hettyey et al. 2009; *Rana sylvatica*: Smith-Gill and Berven 1980; *Rana temporaria*: Gibbons and McCarthy 1986). However, we know of no direct and explicit test of the hypothesis that small anuran males invest less in reproduction and exhibit a lower reproductive potential compared to large males (e.g., in a crustacean: Hinojosa and Thiel 2003; in a fish: Howard et al. 1998); a decrease in reproductive investment that could be traded off for increased growth and survival (Taborsky 1994).

We hypothesized that small anuran males should carefully optimize investment in reproduction and trade it off against growth. Due to a widespread large-male mating advantage (Davies and Halliday 1979; Ryan 1983; Tejedo 1992), small males would benefit from a larger body size in terms of increased mating success in subsequent breeding seasons. Also, as small males generally have a low mating probability, they would only waste energy by investing in the production of multiple ejaculates. On the other hand, large males being able to secure several matings should invest less in growth and more in reproduction as they would most probably not be able to secure more matings if they grew even larger and also because they have to be able to fertilize several clutches of eggs. We tested the prediction arising from this hypothesis that smaller males would produce smaller testes containing lower numbers of sperm and that they would sooner become sexually exhausted over the course of repeated matings when compared to larger males. To test these predictions, we analyzed relative testis size in 46 common toad (*B. bufo*) males and performed an experiment where we could follow changes in the size of sperm stores, in fertilization success, and in the readiness to mate repeatedly over the course of repeated matings.

Material and methods

The study species

The common toad (*B. bufo*) is a large and widespread anuran native to the Palaearctic region (Nöllert and Nöllert 1992). Its reproductive season lasts several weeks, but most individuals mate within 1–2 weeks ("explosive breeder" sensu Wells 1977; Davies and Halliday 1979; Reading 1998). Males arrive first to the breeding ponds and stay there for most of the reproductive period, whereas females only stay for one or a few nights to deposit their eggs, which results in biased operational sex ratios with an excess of males over the entire reproductive season (Davies and Halliday 1977, 1978, 1979; Loman and Madsen 1986; Reading 1998). Direct female choice is overrun by scramble competition among males, with large males being more successful in acquiring and defending females (Davies and Halliday 1979; Loman and Madsen 1986; Höglund 1989; but see also Lengagne et al. 2007). Even though excessive multiple matings are rare, there is variation in male mating success, where large males are likely to mate repeatedly and small males often do not mate at all (Davies and Halliday 1979; Loman and Madsen 1986). Since there is no sperm production during the breeding season, males are unable to replenish sperm stocks after matings and so must use the sperm stocks already accumulated before the start of sexual activities (Lofts 1975; Hettyey et al. 2009). The intrinsically limited reproductive potential of males allows only a few successful matings (Lengagne et al. 2007; Hettyey et al. 2009). Male body size positively affects fertilization success but explains little of the variation (Davies and Halliday 1977; Lengagne et al. 2007).

Data collection

At the beginning of the breeding season in early April 2008, we hand collected 100 *B. bufo* males from a population totaling several hundred individuals in the Pilis Mountains, Hungary (47°42′N, 19°02′E) and transported them to a nearby pond, also sustaining a small breeding population of around a hundred individuals. We measured snout to vent length (SVL) with a plastic ruler (to the nearest 1 mm). To obtain a sample containing both small and large males while excluding extreme-sized individuals, we released the three largest, the three smallest, and 46 medium-sized males and used the remaining 48 males (mean±SE, 70.9±0.57 mm; range, 64–78 mm).

Out of the 48 males, we randomly assigned 16 to each one of three different treatments. Males in treatment 1 were immediately transported to the laboratory of the Department of Systematic Zoology and Ecology, Eötvös Loránd

University, Budapest, where they were stored individually in plastic boxes in a fridge at 5°C until dissection. Anuran amphibians are in an inactive, hibernation-like state when they are kept at 5°C in the dark. Males in this treatment provided baseline data on the size of testes and on the number of sperm stored in the testes after emergence from hibernation. We placed males assigned to treatments 2 and 3 individually into 32 large plastic containers (90 cm in diameter, 80-cm deep) positioned outdoors close to a breeding pond and containing 15 cm (100 l) of pond water. Males in treatment 2 were deprived of females. This group acted as a control, allowing us to study potential changes in sperm quantity due to sperm production, -degradation, or -leakage during the reproductive season. Males in treatment 3 were repeatedly provided with gravid females, allowing us to observe potential changes in sperm stock size, fertilization success, and readiness to re-mate over the course of repeated matings.

Females were collected from the same pond as well as from two other nearby ponds and were kept in large plastic boxes (1×1 m, 40-cm high) filled with moistened fallen leaves until they were used in the experiment. After placing females in boxes holding males, we monitored experimental pairs every 2 h day and night and every hour once egg laying had started. We approached containers quietly and used dim red headlights at night to minimize disturbance to the animals. As soon as a pair finished egg laying and the male released the female, we removed the female. One hour later, we moved the egg string to a shallow dish filled with 1 cm of pond water and counted the egg number. Subsequently, we cut four short sections off the egg string (one from the beginning, two from the middle part, and one from the end of the string, each containing around 200 eggs) and incubated these in plastic boxes filled with 1 cm of pond water. Three days later, we estimated fertilization success by counting developing embryos, exhibiting an elongated shape, and non-developing eggs in the four sections of the egg strings, and subsequently released all embryos in the pond where we performed experiments. Males in treatment 3 were provided with a new female 24 h after the end of the previous mating. The experiment lasted from April 4th to 16th, as long as we could find gravid females in the natural breeding ponds. Given the large variation in the time until females started egg deposition (range, 2–153 h), the long duration of egg deposition (2–54 h) and the common toad's relatively short breeding season, we could not provide all males with the same number of females. Gravid females were available until approximately 168 h after the start of the experiment, so that we could not enter further females after that time point. We terminated trials of treatments 2 and 3 approximately 300 h after we start, when only one female was still in amplexus (for 150 h) without having laid its eggs. The time

span of our experiment was, thus, ecologically relevant, since the peak reproductive activity also lasts about a week in natural populations, with a few more matings expanding the duration of the breeding season by another 1–2 weeks (Wells 1977; Davies and Halliday 1979; Reading 1998; Hettyey et al. 2009). At the end of the experiment, we released all females and embryos and transported the males in treatments 2 and 3 to the laboratory.

Within a week after termination of the field-based part of the study, we over anesthetized males of all three treatments with tricaine methanesulfonate (MS-222), dissected them, weighed their body mass and testes mass (to the nearest 0.1 mg), macerated testicles in ca. 10 ml of reconstituted soft water (RSW; Apha 1985) for 1 min, and weighed the sperm suspensions. We preserved two 1 ml samples from each suspension by adding 1 ml 96% ethanol and keeping the samples at 5°C. Sperm numbers were later estimated with a Bürker chamber at×200 magnification. We pipetted four 10 μl samples of the suspensions to the chamber and counted the sperm number in eight quadrates. Knowing the density and mass of sperm suspensions, we could subsequently calculate sperm numbers in the testes, assuming that 1 ml suspension equalled to 1 g.

Two males in treatment 1 died before dissection so that we had to remove these males from the analyses resulting in a sample comprising 46 males. The body size and body condition of males did not differ between treatments (one-way ANOVA; SVL: $F_{2, 43}=0.23$, $P=0.8$; body condition: $F_{2, 43}=1.02$, $P=0.37$) and their size distribution was continuous and did not deviate from normality (Kolmogorov–Smirnov test; all males: $Z=0.67$, $N=46$, $P=0.76$; treatment 1: $Z=0.21$, $df=14$, $P=0.084$; treatment 2: $Z=0.20$, $df=16$, $P=0.091$; treatment 3: $Z=0.15$, $df=16$, $P>0.20$).

Statistical analyses

We transformed the sperm count data using a square root transformation and data on fertilization success using an arcsine square root transformation to enhance the normality of the model residuals and the homogeneity of variances. To investigate variation in testes size, we built a general linear model (GLM) with log testes mass as the dependent variable, log soma mass (body mass–testes mass), and body condition (standardized residuals from a regression of body mass on SVL) as covariates and treatment as a fixed factor. We used ordinary log–log least squares regression (Bondurianskiy and Day 2003) and not major axis regression as we could assume a causal relationship between body mass and testis mass and as measurement error in body mass is likely to be smaller than measurement error in testes mass (Sokal and Rohlf 1981). To test if testes mass

was in positive or negative allometry with body mass, we used a Student's *t* test, evaluating whether the slope differed from one. To analyze sperm quantity, we entered the number of sperm stored in the testes of males as the dependent variable, treatment as a fixed factor, and body condition as a covariate into the GLM. To test if larger males invested more in sperm production than smaller males, while controlling for variation in testes size, we also entered testes mass and SVL into the model as covariates. To analyze patterns in fertilization success, we used a repeated measures GLM on data obtained from treatment 3. We entered fertilization success in the first three matings as the dependent variable and male SVL and male body condition as covariates. We excluded data on fourth matings from the analysis as only three males mated with four females. We analyzed among-male differences in the readiness to mate repeatedly in treatment 3 by categorizing males as ready to mate if they amplexed the provided female and maintained amplexus until the end of egg laying and as exhausted if they did not amplex the female anymore or did not maintain amplexus until the female had finished egg laying. We used generalized linear modeling procedures (GZLM) with binomial error distribution and logit link function. We entered sexual motivation of males as the response variable and SVL and body condition as covariates.

We included all possible two-way interactions into the initial models and applied a backward removal of terms with $P > 0.05$ to avoid problems potentially arising due to the inclusion of nonsignificant terms (Engqvist 2005) and re-entered removed variables one by one to the final model to obtain relevant statistics. All tests were two tailed. To provide more quantitative information on the importance of independent variables, we also present effect sizes (partial η^2). Statistics were calculated using SPSS 17.0 for Windows.

Results

Testes mass was in a positive relationship with soma mass and depended on the treatment (treatment 1 (mean±SE), 129.64±8.27 mg; treatment 2, 105.45±5.72 mg; treatment 3, 97.51±6.86 mg; Table 1). Males in treatment 1 had larger testes than similar-sized males in treatment 3, and males in treatment 2 were intermediate (Bonferroni-corrected pairwise comparisons of estimated marginal means; treatments 1–3, $P=0.028$; treatments 1–2, $P=0.79$; treatments 2–3, $P=0.32$; Fig. 1). We could not detect an effect of body condition on testes mass and interactions were nonsignificant (Table 1). The relationship between testes mass and soma mass did not differ from isometry (Student's *t* test; $t=-0.48$, $df=44$, $P>0.5$).

The number of sperm stored in the testes was positively related to testes mass and varied significantly between treatments (treatment 1 (mean±SE), $1.36\times10^8\pm1.13\times10^7$; treatment 2, $1.23\times10^8\pm8.74\times10^6$; treatment 3, $4.94\times10^7\pm6.83\times10^6$; Table 2), with males in treatments 1 and 2 not differing from each other but having significantly larger sperm stores than males in treatment 3 (Bonferroni-corrected pairwise comparisons of estimated marginal means; treatments 1–2, $P=1$; treatments 1–3, $P<0.001$; treatments 2–3, $P<0.001$). Snout to vent length, body condition, and interactions did not seem to have an effect on the relative number of sperm in the testes (Table 2).

Fertilization success did not depend on the main effect of male SVL or on the interactions involving male SVL (Table 3). Although we could not detect significant variation in fertilization success between matings, there was a significant linear decrease over repeated matings (first mating (mean±SE), 80.65±6.88%; second mating, 67.43±8.3%; third mating, 61.65±11.98%; Table 3). Body condition negatively affected fertilization success, but when tested separately its effect was only significant at the third mating (Table 3).

Out of the 16 repeatedly mated males, 1 male refused to amplex the second female and another male the third female. Further two males aborted amplexus while the female was still laying eggs (one male in the second and one in the third mating). These 4 males we categorized as being exhausted and the remaining 12 males as being ready to mate. The analysis on these data suggested that SVL did not affect readiness of males to re-mate (GZLM; Wald $\chi^2=0.02$, $B=0.02$, $SE=0.12$, $N=16$, $P=0.88$; Fig. 2). Males in better condition showed less interest in additional females after having mated already, but this correlation

Table 1 Effects of treatment, soma mass, and body condition on testes mass

Effect	df	B	SE	F	P	Partial η^2
Treatment	2, 42			3.814	0.030	0.154
Soma mass	1, 42	0.907	0.194	21.825	<0.001	0.342
Body condition	1, 41	−0.004	0.003	1.809	0.186	0.042
Treatment×soma mass	2, 40			0.021	0.980	0.001
Treatment×body condition	2, 39			0.031	0.970	0.002
Soma mass×body condition	1, 40	0.001	0.030	0.001	0.972	<0.001

Statistics were calculated using general linear modeling procedures
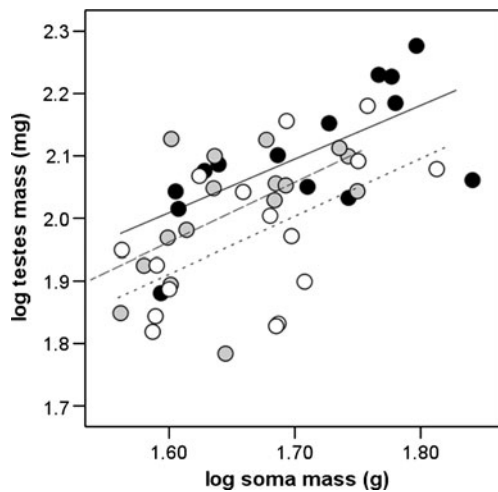
Fig. 1 The relationship between testes mass and soma mass. *Black dots and the solid regression line* represent males in treatment 1 (re-hibernated at the start of the breeding season), *gray dots and the broken line* represent males in treatment 2 (kept without potential mates throughout the breeding season), *white dots and the dotted line* represent males in treatment 3 (repeatedly provided with potential mates)

was not significant (Wald $\chi^2=3.33$, $B=0.56$, SE=0.31, $N=16$, $P=0.068$). The interaction between SVL and body condition was nonsignificant (Wald $\chi^2=0.27$, $B=0.29$, SE=0.56, $N=16$, $P=0.6$).

Discussion

Larger males had larger testes than smaller males in absolute terms, but testes mass varied isometrically with soma mass. Hence, small males did not invest relatively less in reproductive tissue than larger males. Similarly, the relative number of sperm produced in the testes was not related to male body size in either treatment. That is, testes of smaller males did not produce or store less sperm per gram of testes than those of larger males. Male body size also did not affect fertilization success or readiness of males

to re-mate. These results congruently suggest that smaller males do not invest less in reproduction and do not allocate more in growth than larger males.

It is possible that small males save resources for enhanced growth in other ways than by lowering investment in testicular tissue, sperm production, and the maintenance of high fertilization ability and of readiness to re-mate. For example, small males may use less energy-demanding mating tactics than large males. In *B. bufo*, large males tend to use the stationary calling tactic, whereas small males use the active searching tactic (Höglund and Robertson 1988). Nonetheless, active movement can be just as energy demanding as calling (Ryan et al. 1983), especially as searching for potential mates results in repeated fights in *B. bufo*. Furthermore, small males also call and all males use the active searching tactic over large parts of the breeding season, once densities have become high (Höglund and Robertson 1988). Consequently, it seems that small males do not use mating tactics that demand less energy. Small males may also spare resources for growth by being reproductively active only for a short time. *B. bufo* males, however, remain in or around the breeding pond for most of the reproductive season, and they do that irrespective of body size (Gittins et al. 1980; Loman and Madsen 1986; Arntzen 1999). Consequently, the relative energy expenditure related to reproduction may indeed be similar between different sized males.

Results suggesting that smaller males do not invest less in reproduction than larger males to save resources for growth have also been demonstrated by studies on other species (e.g., Rakitin et al. 1999; Kitchener 2000; Hettyey and Roberts 2007), but a positive allometry between body size and reproductive investment has more often been found (e.g., Andersson 1994; Simmons and Tomkins 1996; Emlen and Nijhout 2000). A positive allometry has been explained by larger males gaining more benefits from a higher reproductive investment (sensu Hosken et al. 2005) or paying relatively lower costs than smaller males (sensu Petrie 1992; but also see Bonduriansky and Day 2003).

Table 2 Effects of treatment, testes mass, male SVL, and male body condition on the number of sperm stored in the testes

Effect	df	B	SE	F	P	Partial η^2
Treatment	2, 42			31.581	<0.001	0.601
Testes mass	1, 42	9918.832	1965.220	25.474	<0.001	0.378
SVL	1, 41	68.328	63.145	1.171	0.286	0.028
Body condition	1, 41	−8.735	34.896	0.063	0.804	0.002
Treatment×testes mass	2, 40			0.059	0.943	0.003
Treatment×SVL	2, 39			0.359	0.701	0.018
Treatment×body condition	2, 39			0.495	0.613	0.025
Testes mass×SVL	1, 40	−478.667	546.137	0.768	0.386	0.019
Testes mass×body condition	1, 40	42.773	301.300	0.020	0.888	0.001
SVL×body condition	1, 39	−8.087	11.312	0.511	0.479	0.013

Statistics were calculated using general linear modeling procedures

Table 3 Effects of male SVL, male body condition, and mating number on fertilization success in treatment 3, where males were allowed to mate repeatedly

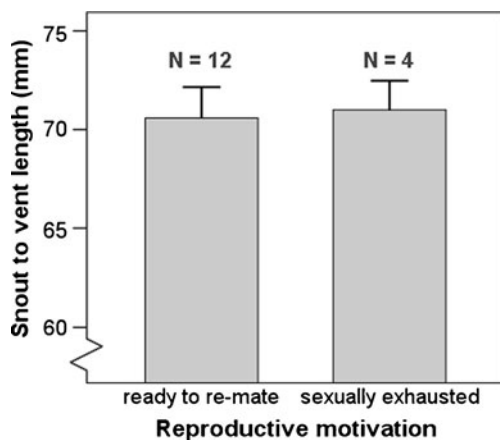| Repeated measures GLM			df	F	P	Partial η^2
Tests of between-subjects effects						
SVL			1, 7	0.112	0.748	0.016
Body condition			1, 8	13.645	0.006	0.630
SVL×body condition			1, 6	0.912	0.377	0.132
Tests of within-subjects effects						
Mating number			2, 16	1.954	0.174	0.196
Mating number×SVL			2, 14	0.011	0.989	0.002
Mating number×body condition			2, 16	2.274	0.135	0.221
Tests of within-subjects contrasts						
Mating number			1, 8	6.602	0.033	0.452
Mating number×SVL			1, 7	0.023	0.885	0.003
Mating number×body condition			1, 8	5.940	0.041	0.426
Separate GLMs	B	SE	df	F	P	Partial η^2
First mating						
Body condition	−0.017	0.014	1, 8	1.584	0.732	0.165
Second mating						
Body condition	−0.017	0.016	1, 8	1.109	0.969	0.122
Third mating						
Body condition	−0.059	0.016	1, 8	13.248	0.021	0.623

Statistics were calculated using repeated measures general linear modeling procedures. P values for the separate GLMs on the first three matings are Bonferroni corrected

Unfortunately, we have no direct estimates of how much a small male would gain in terms of increased growth by lowering reproductive investment or by skipping a reproductive season altogether. However, reproductive activities do bear significant energetic costs to male anurans (e.g., Grafe and Thein 2001; McLister 2003), so that a lowered reproductive investment in 1 year is likely to lead to increased growth and, hence, increased reproductive success in subsequent years, as it does in anuran females (Reyer et al.

Fig. 2 The relationship between readiness of males to re-mate and their SVL in treatment 3 (males repeatedly provided with potential mates). Males were categorized as ready to re-mate if they amplexed the provided female and maintained amplexus until the end of egg laying, and as exhausted if they refused to amplex the female or aborted amplexus while the female was still laying eggs. Bars represent±1 SE, sample sizes are indicated

1999; Waelti and Reyer 2007) and in individuals of other taxa (Berglund and Rosenqvist 1986; Taborsky 1994).

The question therefore arises: why did we not observe the predicted size-dependent investment in reproduction in *B. bufo* males? Allocation rules may be fixed, with all males allocating an equal part of their resources in growth and in reproduction each year, independently of their size (sensu Jokela and Mutikainen 1995). It is also possible that flexible allocation rules did evolve but went undetected at the population level due to large between-individual variation in male quality (van Noordwijk and de Jong 1986). Small, good quality males may have invested a lot in both growth and reproduction, creating such large variation in reproductive investment that size-dependent differences were swamped and went undetected. Finally, it is important to note that small males may use different strategies: some may not migrate to the breeding ponds in spring, skip the breeding season entirely (as reported for females; Loman and Madsen 2010), and save resources for growth, while others may invest much in reproduction to maximize potential reproductive success. As we collected males from a breeding population, our sample could only contain males using the second strategy.

The question nonetheless remains: Why do not all small males (of good and bad quality and using any life history strategy) invest as much in growth as possible to secure high reproductive success in the future? Fixed allocation rules or rules with low flexibility may have evolved due to a relatively low year-to-year survival of males of around 60% (Heusser 1968; Gittins 1983; Schmidt et al. 2002;

Loman and Madsen 2010). Males may not be selected for decreasing current reproductive effort and investing in an uncertain future (e.g., Schneider and Lubin 1997). Also, unpredictable between-year variation in the length of the breeding season, in sex ratio, and in density may result in variation in the strength of male–male competition (Gittins 1983; Höglund 1989; Loman and Madsen 2010), so that there are years when less competitive males may also obtain several matings. To be saving resources for the next season in such a year may be disadvantageous. Finally, a low return rate of lowered reproductive investment (for a similar result in female *R. temporaria*, see Lardner and Loman 2003) may make it unprofitable to forego possible mating opportunities. Small males that are generally unlikely to obtain matings but still allocate costly resources in reproduction may simply be making the best of a bad job.

In a previous study on *B. bufo* (Hettyey et al. 2009), we observed a qualitatively similar, but considerably larger decrease in fertilization success over the course of repeated matings than in the present study. Also, body condition of males was positively related to fertilization success and to readiness to re-mate in the previous study, whereas in the present study these relationships were negative. These discrepancies between the two studies in the outcomes on fertilization success could potentially be explained by between-year fluctuations in body condition and consequent differences in allocation in reproduction by males, but low sample sizes in relevant treatments of both studies and differences in experimental conditions and procedures (e.g., lab vs. field-based experiment, disparity in the timing of female addition) hinder any firm conclusions.

Our results stress the importance of a detailed knowledge of the natural history and reproductive biology of the study species for understanding the observed evolutionary outcomes regarding specific life history traits. We suggest that future studies that assess the energetic demand of different mating tactics and quantify the return rate of lowered reproductive investment (in terms of increased growth) and reproductive success in the following breeding season will significantly further the field of life history evolution.

Conflict of interest The authors declare that they have no conflict of interest.

Ethical standards We declare that the experiments comply with the current laws of Hungary, the country in which the experiments were performed.

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