

# Testing the phenotype-linked fertility hypothesis in male Moor Frogs (*Rana arvalis*) exhibiting a conspicuous nuptial colouration

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**Abstract.** The conspicuous blue nuptial colouration of Moor Frog (*Rana arvalis*) males has been associated with sexual selection; it may provide females with information about benefits to be gained through mate choice. Here we investigated the phenotype-linked fertility hypothesis suggesting that exaggerated traits may advertise fertilization ability. We evaluated conspicuousness of males' colouration and related this to the number of sperm stored in their testes. Contrary to our expectation, we did not find a positive relationship between blueness of males and the number of sperm stored in the testes. We discuss this result in the light of alternative traits that may be advertised by colouration, such as sperm quality or good genes for offspring survival. We now need direct studies of female mate choice and the physiology of the blue nuptial colouration to clarify the evolutionary background of the striking temporal sexual dichromatism in the Moor Frog.

**Keywords:** fertilising ability, mate choice, mating success, phenotype-linked fertility hypothesis, sexual selection, sperm stock size.

Conspicuous phenotypic traits of males that are only expressed during the reproductive season are often targets of sexual selection through female mate choice (Andersson, 1994). In species where the female receives nothing but sperm from its reproductive partner, as in many fishes and amphibians, these male traits may signal genetic benefits (Trivers, 1972). However, phenotypic signals may also be associated with fertilising ability of males, as predicted by the phenotype-linked fertility hypothesis (Sheldon, 1994; Birkhead and Fletcher, 1995) and supported by an increasing number of empirical studies in a variety of animal taxa (e.g., fish: Matthews, Evans and Magurran, 1997; birds: Peters et al., 2004; mammals: Malo et al., 2005). Sperm quantity is an important determinant of fertilising ability (Birkhead and Møller, 1998). Consequently, the conspicuousness of the male

phenotype may signal the size of sperm stocks (e.g., Pitcher and Evans, 2001). On the other hand, if conspicuous males have a high mating success, their sperm reserves may become depleted over the course of repeated matings (Smith-Gill and Berven, 1980; Hettyey et al., 2009a). However, the reproductive potential of males and, thus, the size of their sperm stores may, as a result of selection, be tailored to the probable number of matings, resulting in a correlation between conspicuousness and sperm number. Finally, males of better quality may be able to produce and maintain a conspicuous phenotype and high numbers of good quality sperm simultaneously, despite related costs (Dewsbury, 1982; Olsson, Madsen and Shine, 1997; Huhta, Rytönen and Solonen, 2003). Irrespective of the evolutionary pathway, selection might lead to a positive relationship between the conspicuousness of the sexually selected phenotype and the size and quality of sperm stocks at the start of the reproductive season.

In the Moor Frog (*Rana arvalis*, Nilsson, 1842), males turn bright blue at the onset of the reproductive season and exhibit this conspicuous nuptial colouration only during the few days of peak reproductive activity (Hedengren, 1987;

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Ries et al., 2008; Hettyey et al., 2009b). Bluer males have been reported to have a higher mating success (Hedengren, 1987; Sheldon et al., 2003; Hettyey et al., 2009b). The mating advantage of bluer males may arise from (i) active female mate choice for bluer males, (ii) passive attraction of females to conspicuous males (*sensu* Arak, 1988) or (iii) male-male competition where colouration reveals fighting ability to other males (*sensu* Olsson, 1994; Ries et al., 2008). As more conspicuous males have higher mating success, several evolutionary pathways (see first paragraph) may lead to a positive correlation between blueness and initial sperm stocks of male Moor Frogs in accordance with the phenotype-linked fertility hypothesis (Birkhead and Fletcher, 1995). We tested this hypothesis and predicted that bluer males would have larger sperm stores than paler, more cryptic males.

The Moor Frog is a medium-sized (ca. 55 mm long) frog native to wide areas of the Palaearctic region (Gasc et al., 1997). Males and females both have a cryptic brown colouration with black and dark-brown stripes and blotches on the dorsal side and a light, whitish ventral side. Males develop a blue colouration during the reproductive period, which makes them highly conspicuous in front of the brownish background of the breeding pond (Ries et al., 2008). However, there is large among-male variation in the intensity of the nuptial colouration with some males remaining cryptic brown and others turning light blue (see Sheldon et al., 2003; Ries et al., 2008; Hettyey et al., 2009b). The breeding season usually lasts a few days in late March, when animals are active day and night (Nöllert and Nöllert, 1992; pers. obs.). As in many other frogs of the temperate zone, males arrive first to the breeding site and stay there throughout the reproductive period, whereas females arrive to the pond over the course of a few days and leave the water soon after egg-laying. Males form choruses and call to attract females, but when densities become high, the proportion of males actively searching

for mates strongly increases and the opportunities for female choice sharply decrease (Hedengren, 1987). Females generally get amplexed in the water and lay from several hundred to a few thousand eggs (Nöllert and Nöllert, 1992).

We captured male Moor Frogs at the beginning of the breeding season in 2005. The breeding site was a small, shallow pond of ca. 0.3 ha surrounded by woodland, located in the floodplains of the River Danube, approximately 20 km north of Vienna, Austria (48°22'N, 16°14'E). The study population consisted of a few hundred individuals. On 28 and 30 March, when males had become blue and egg-laying had just started, we caught 18 and 17 male Moor Frogs, respectively, from an active chorus by randomly moving around and opportunistically hand-collecting both mated and non-mated males. All individuals were sexually mature, exhibiting elaborate thumb-pads. Immediately after capture, we noted mating status and scored colouration of males. We scored the extent and intensity of the blue colouration of each male on a scale from one to five in nine pre-defined areas of the body surface (Sheldon et al., 2003). This method provides highly repeatable scores on colouration (Juha Merilä, unpublished data). Scoring was done by AH in all cases. The pre-defined areas were the (1) head; (2) back; (3) left flank; (4) right flank; (5) left foreleg; (6) right foreleg; (7) left hind leg; (8) right hind leg and (9) throat. These areas covered the body surface of frogs that can be seen and may be assessed by approaching females. Also, the colouration of these areas varies to some extent independently with some males exhibiting bright blue flanks but a cryptically coloured back, whereas others may be bright blue within all of these areas. We subsequently combined these scores into two measures of overall blueness in a Principal Components Analysis (PCA; see below).

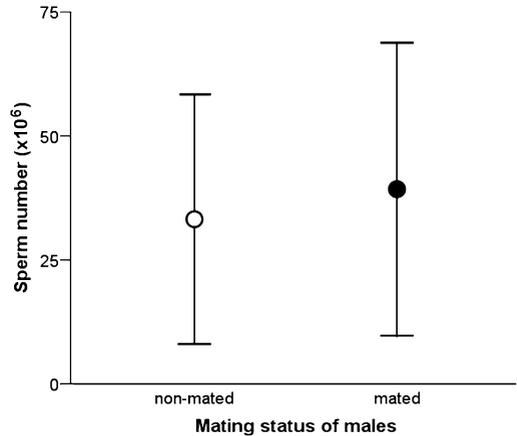
After scoring, we marked males individually by toe-clipping, placed them in covered plastic boxes (48 × 35 × 25 cm, containing ca. 15 litres of pond water) and transported them to the Konrad Lorenz Institute for Ethology, Vienna, Austria, where we kept them at ambient temperatures until further processing. One week later, we over-anaesthetized animals in 0.2% MS-222 (tricaine; ethyl 3-aminobenzoate methansulfonate) solution and measured their snout-vent length (SVL) with digital callipers (to the nearest 0.1 mm). Subsequently, to obtain sperm suspensions, we dissected the frogs and crushed their testes in 100% Holtfreter's solution (amphibian Ringer's). We estimated sperm concentrations using a Neubauer chamber by counting spermatozoa in eight quadrats for each of four samples per suspension (for further details see Hettyey and Roberts, 2006; Hettyey et al., 2009a). After measuring mass of the sperm suspensions with an analytical balance (to the nearest mg), we were able to calculate total number of sperm per suspension, assuming that 1 g of suspension equalled 1 cm<sup>3</sup>.

Before performing statistical analyses, we square-root-transformed sperm number to achieve normality and enhance homogeneity of variances. In one case we mistakenly

did not measure mass of the sperm suspension, reducing the sample size by one. To collapse measures of blueness into a few composite variables, we performed a PCA on the extent and intensity scores of blueness for the nine body areas. We obtained two principal components with eigenvalues greater than one: the first principal component (PC1) explained 72.2% and PC2 explained an additional 9.3% of the total variance in colouration. After Varimax rotation, extent and intensity scores for the first eight body areas all loaded strongly and positively only on PC1 (all  $r > 0.81$ ) whereas those for the throat loaded strongly and positively only on PC2 (both  $r > 0.79$ ). Consequently, we used PC1 scores as a measure of overall body colouration and PC2 scores as a measure of throat colouration in the following analyses. Throat colouration has been suggested to be potentially important for mate choice in anurans (Rosenthal, Rand and Ryan, 2004; Cummings et al., 2008).

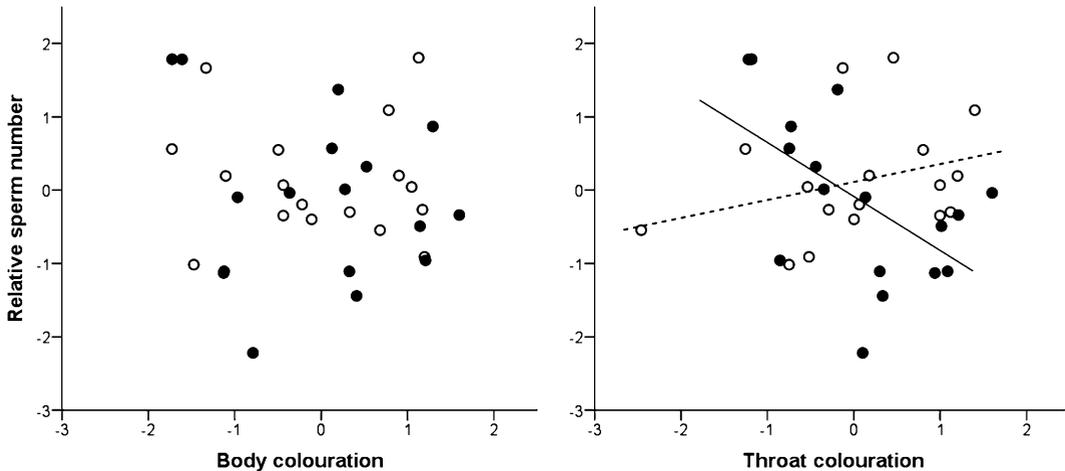
We tested whether bluer males have larger sperm reserves in a General Linear Model (GLM) with sperm number as the dependent variable, date code as a random factor, mating status (whether in amplexus or not at collection) as a fixed factor and SVL, body- and throat colouration as covariates. The scarcity of similar studies in amphibians prevented us from formulating reasonable hypotheses about what could influence the number of sperm stored at the onset of the reproductive period. We thus included all two-way interactions between the fixed factor and the covariates into the initial model and applied backward removal model selection to avoid potential problems caused by leaving non-significant terms in the model (Engqvist, 2005). The final model included SVL, mating status and throat colouration as main effects and the interaction between mating status and throat colouration. We calculated statistics using SPSS 15.0 for Windows.

The result from the GLM indicated that SVL had a highly significant effect on the number of sperm stored in the testes ( $F_{1,30} = 47.73$ ,  $P < 0.001$ ). Mating status ( $F_{1,30} = 0.85$ ,  $P = 0.36$ ; fig. 1), date code ( $F_{1,29} = 0.008$ ,  $P = 0.93$ ), body colouration ( $F_{1,29} = 0.2$ ,  $P = 0.66$ ; fig. 2) and throat colouration ( $F_{1,30} = 2.71$ ,  $P = 0.11$ ; fig. 2) were not related to sperm number. Interestingly, however, the interaction between mating status and throat colouration was significantly related to sperm number ( $F_{1,30} = 11.47$ ,  $P = 0.002$ ; fig. 2). When testing mated and non-mated males separately, we observed a significant negative relationship between throat colouration and sperm number in mated males ( $F_{1,14} = 9.08$ ,  $P = 0.009$ ; fig. 2), but there was no similar relationship in non-mated males ( $F_{1,15} = 2.5$ ,  $P = 0.14$ ; fig. 2). Effects of the other two-way interactions were not significant (both  $P > 0.3$ ).



**Figure 1.** The relationship between mating status of males and the number of sperm stored in their testes. For the ease of interpretation and to show that there was considerable variation in sperm numbers, means  $\pm$  SD of untransformed data are shown. There was no significant difference in the number of sperm stored in the testes of mated and non-mated males.

We predicted more conspicuous males to have larger sperm stocks. Contrary to our expectations, relative sperm number was not higher in bluer than in paler males. Moreover, mated males exhibiting a more conspicuous throat colouration had smaller sperm stores rendering the phenotype-linked fertility hypothesis unlikely to apply to male Moor Frog colouration. It is not known if *R. arvalis* males produce significant numbers of sperm during the breeding season, and if they are able to replenish their sperm stores after matings. Consequently, our results may have decisively been influenced by some collected males already having mated before collection. This is, however, rather unlikely as (1) according to our observations, no clutches had been laid before the first sampling and only very few clutches had been laid before the second sampling in the breeding population consisting of several hundred males. Consequently, chances are low for collected males having mated before collection. Also, (2) sperm number corrected for body size was normally distributed and there were no extreme values on the lower end of the distribution (see fig. 2). Finally, (3) sperm numbers did not differ between



**Figure 2.** The effect of body and throat colouration and mating status of males on the number of sperm stored in the testes relative to body size (filled circles: mated males; empty circles: non-mated males; solid line belongs to mated males; dotted line belongs to non-mated males). Body colouration is represented by PC1 scores and throat colouration by PC2 scores from a PCA on male colouration; relative sperm number was only calculated for the purpose of illustration and is represented by residuals from a regression of sperm number on SVL. Higher scores represent more intensive blue colouration, low scores represent paler or brown colouration. We found no relationship between body colouration and sperm store size in males, but the interaction between throat colouration and mating status of males had a significant effect on the number of sperm stored in the testes (see text).

males collected during the first and the second sampling.

One explanation for the lack of a positive relationship between colouration and sperm number could be that brighter colouration is just a temporary side-effect of elevated sexual hormone-levels during in amplexus (as suggested by Hettyey et al., 2009b). In this case, the causal relationship between mating success and blueness would be reversed; instead of blueness enhancing mating success of males, as suggested by previous studies (Hedengren, 1987; Sheldon et al., 2003; Hettyey et al., 2009b), amplexed males may be bluer than others because they are in amplexus. In such a scenario, blueness cannot function as a signal revealing male quality. Ries et al. (2008), however, showed that close contact with females does not affect colouration. Thus, blueness may not be affected by mating status, and may signal male quality.

Instead of sperm quantity, blueness of males may also advertise sperm quality (e.g., Locatello et al., 2006), another trait contributing to fertilising ability (e.g., Peters et al., 2004), but we did not measure sperm quality traits

and, thus, cannot conclude on this possibility. Male colouration may, however, also signal other traits than fertilising ability. Sheldon et al. (2003) showed experimentally that offspring sired by bluer males are more likely to survive when exposed to free-ranging predators than offspring sired by dull brown males. Offspring of more conspicuous males will thus have higher survival probabilities than offspring of dull brown males under natural conditions where predators are usually present. Consequently, similarly to acoustic traits (e.g., Welch, Semlitsch and Gerhardt, 1998; Forsman and Hagman, 2006), colouration may advertise genetic benefits that can be gained through mate choice in anurans.

In summary, we did not find any evidence for the phenotype-linked fertility hypothesis (Sheldon, 1994; Birkhead and Fletcher, 1995) in Moor Frogs as more conspicuous males did not exhibit larger sperm numbers at the beginning of the breeding season than their dull counterparts. Further studies on potential colour-linked differences in sperm quality and experiments directly comparing fertilising ability of con-

spicuously and cryptically coloured male Moor Frogs in repeated matings are needed as well as more studies testing for the potential indirect benefits of mating with colourful males. Also, the presence of active female mate choice and the physiological background of developing and maintaining blue colouration will have to be investigated directly in order to clarify the evolutionary origin and role of the blue nuptial colouration of male Moor Frogs.

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