Sexual Maturation in Common Vole (*Microtus arvalis)* Males Raised under Laboratory Conditions*

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The common vole is one of the most numerous rodents in Europe and Asia but its reproductive biology is not fully described. It is thought that females reach reproductive abilities at a very young age, however, there is no data concerning male sexual maturation. The aim of the present study was to determine the rate of sexual maturation of males of the common vole. Research was carried out on 4, 6, 8 and 10 week-old animals. Body, testes and accessory sex glands weights were compared to evaluate morphological development. Epididymal sperm quality was assessed by a motility test, sperm tail membrane integrity, viability, maturity and sperm head morphology. Moreover, the number of sperm cells in 1 mm^3 of semen was evaluated. The largest body weights were observed in 8 and 10 week-old males, which also possessed the highest relative weights of gonads and accessory sex glands. These groups of males produce about 2 times more gametes than 6 week-old individuals. The highest seamen quality was noted in 8 and 10 week-old males. Based on the obtained results, it is concluded that among the investigated age groups the most appropriate male age for fertile copulation is between 8 and 10 weeks.

Key words: Male sexual maturation, spermatozoa, reproductive abilities, common vole, *Microtus arvalis.*

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The common vole, *Microtus arvalis* (PALLAS 1779) is widespread all over Europe and Asia (WILSON & REEDER 2005). Originally, it inhabited grasslands and rural areas. Today, common voles as well as other small mammals are common in cultivated lands (FREEMARK 1995; SINGLETON & BROWN 1999). It is estimated that populations in cultivated areas constitute 60-90% of the total number of small rodent populations (STEIN 1955; MACKIN-ROGALSKA 1981; JACOB & TKADLEC 2010). The common vole is considered to be one of the most populous small mammals in Europe and every 3 to 5 years a population outbreak is observed (TKADLEC & STENSETH 2001; LAMBIN & BRETAGNOLE 2006; JACOB & TKADLEC 2010; LUQUE-LARENA *et al.* 2013).

From the economic point of view, the common vole is considered a pest. When the total number of individuals reaches 3000 per 1 ha, it is estimated that the possible damage may reach up to 90% of

the potential crop yield (TERTIL 1977; TRUSZKOWSKI 1982), especially if its diet is considered: mostly plant material, mainly *Monocotyledone*, and seeds (HOOGENBOOM *et al.* 1984). Moreover, like *M. oeconomus,* usually males have larger home ranges than adult females (TAST 1966). Better understanding of the reproduction biology of *M. arvalis* may improve methods of species control.

Presently, research concerning sexual maturation of the common vole is limited. The rate of female sexual maturation is a controversial subject. TKADLEC and ZEJDA (1995) claim that at the age of 12-13 days the vagina opening is developed, and BOYCE and BOYCE (1988) report a mean age of 13.7 days as early sexual maturation for daughters of solitary breeding females. Daughters from grouped breeding females reach sexual maturity at a mean age of 27.4 days (BOYCE & BOYCE 1988), nevertheless at the age of 14 days precocious

breeding occurs (TKADLEC & ZEJDA 1995). There is no data concerning the age of sexual maturation in males.

The aim of this study was to determine the age of sexual maturation in males and verify if it is comparable to the early sexual maturation of females.

Various fields of research use the common vole as a model species (KRÓL *et al.* 2012; LANTOVÁ *et al.* 2012; FISCHER *et al.* 2013). Its main attribute is that it is considered to be a wild living rodent. Only a well-known biology of this rodent will permit an explicit understanding of selected physiological mechanisms. Up till now, the age of sexual maturation in common vole males has been defined based on knowledge derived from other rodent species. For example, golden hamster males reach puberty at the age of 42.5 days (VANDENBERGH 1971), laboratory mice at 40-60 days – depending on the strain (BARTKE *et al.* 1974), bank voles at the age of 6-12 weeks (KRUCZEK 1986). Moreover, it has been shown that the odor of sexually mature males may accelerate female sexual maturation in mice (VANDENBERGH 1967, 1969) and also block pregnancy in newly conceived females (BRUCE 1959).

Male reproductive success and sperm quality and quantity are usually correlated with body and testes weights (KRUCZEK 1986; KLEMME *et al.* 2007; KRUCZEK & STYRNA 2009). To determine male sexual maturation in the common vole, the body, testes and accessory sex gland weights were compared in various age groups. Additionally, we examined epididymal sperm quantity and quality obtained from males of different ages. This method is widely considered a sufficient procedure to discriminate male reproductive abilities in rodents (KRUCZEK & STYRNA 2009).

Material and Methods

Animals

Common voles (*Microtus arvalis*) came from the laboratory colony of the Institute of Environmental Sciences, Jagiellonian University in Kraków. The original stock was obtained in 2006 from Magura National Park, and was maintained as an outbred stock colony according to the system described by GREEN (1966). Each generation consisted of at least 18 breeding pairs; the male and female in each mating pair did not have common parents or grandparents. This breeding system ensures the heterogeneity of the colony. Animals were housed in polyethylene cages (40 cm × 25 cm × 15 cm) under 14h of light (7am-9pm) and 10h of darkness (9pm-7am), in 21 ± 1°C, and 60% humidity. Wood chips were provided as bedding material, which was changed once a week. Standard pelleted chow for laboratory rodents (Labofeed H, Kcynia) and tap water were available ad libitum.

The described experiment was conducted in 2008 and 2009. Tested individuals came from the 3rd and 4th generation of animals bred in the Institute of Environmental Sciences, Jagiellonian University. To examine the rate of male sexual maturation, 4, 6, 8 and 10 weeks-old males were used in the experiment. Adult common vole males and females were mated; 1 female with 1 male in a cage. Following 19 to 21 days of pregnancy, the number of pups was noted and males were randomly assigned to four experimental groups. For the 4-week old age group, three week-old males were separated from their parents and placed into individual cages. For the 6, 8 and 10-week old groups, the males were kept in groups of 3 to 5 individuals per cage. Two weeks before the experiment, each individual assigned to 6-, 8- or 10-weeks old group had been placed into a separate cage.

Each experimental group consisted of 10 males. In order to avoid litter impact, a maximum of 2 males from 1 litter took part in the experiment. In addition, siblings were separated.

Experimental procedures

Body, testes and accessory sex glands weights

After cervical dislocation, males were weighed, next paired testes as well as seminal vesicles and coagulation glands were dissected and weighed (the latter two jointly).

Epididymal sperm evaluation

Preparation of the epididymal sperm suspension

After applying gentle pressure to each cauda epididymis with forceps, allowing epididymal sperm to press to the vasa deferentia, the content of the latter was suspended in 100 μl of M2 medium (Sigma-Aldrich, Germany), and allowed to disperse for 2 minutes.

Epididymal sperm concentration

A 1:20 dilution of epididymal sperm suspension with M2 medium was prepared, and the number of live spermatozoa in 100 squares of a hemocytometer (Bűrker chamber) was counted under a light microscope at 400x magnification. A coverslip was placed on the sample to restrict spermatozoa movements. The average of 2 sperm counts was used to estimate the sperm concentration as described by STYRNA and KRZANOWSKA (1995) and KRUCZEK and STYRNA (2009).

Epididymal sperm motility

Spermatozoa motility was assessed in a hemocytometer. The percentage of motile sperm, i.e. sperm showing progressive movement, among 200 counted spermatozoa from each male is reported (SEED *et al.* 1996).

Epididymal sperm tail membrane integrity – water test

The integrity of the epididymal sperm tail membrane was determined by the hypoosmotic swelling test. The procedure was the same as used for mice and bank voles (STYRNA & KRZANOWSKA 1995; STYRNA *et al.* 2003; KRUCZEK & STYRNA 2009): 20 μl of epididymal sperm suspension (as described above) was mixed with 120 μl distilled water on a clean glass slide, then the mixture was gently covered with a coverslip and incubated for 5 min at 37°C before it was examined. The percentage of spermatozoa showing swelling among 200 counted spermatozoa from each male is reported.

Epididymal sperm viability – eosin-Y test

The test reflects the structural and morphological integrity of the sperm membrane (WALCZAK *et al.* 1994; STYRNA & KRZANOWSKA 1995). To assess sperm viability, 20 μl of epididymal sperm suspension (as described above) was mixed with 20 μl of 0,2% eosin Y, incubated for 10 min at 37°C and smeared on a slide. The percentage of spermatozoa with unstained sperm heads (viable spermatozoa) among 200 counted spermatozoa from each male is reported.

Epididymal spermatozoa without a cytoplasmic droplet

Twenty μl of epididymal sperm suspension (as described above) was transferred to a slide and gently covered with a coverslip. The percentage of spermatozoa without a cytoplasmic droplet among 200 counted spermatozoa with progressive movements from each male is reported (KRUCZEK & STYRNA 2009)

Epididymal sperm morphology

For morphological examination, a small drop of the epididymal sperm suspension was smeared on a slide, air-dried, fixed in acetic-alcohol (absolute alcohol:glacial acetic acid, 3:1), and stained with Papanicolau to assess the proportions of different sperm head abnormalities.

This study discusses the following abnormalities (Fig. 1):
Normal – sperm with correct morphology;
Class 2 – lack of top part of hook and abnormalities in bottom part of the head;
Class 3 – lack of hook, serious abnormalities in the proximal part of sperm heads, possible changes in the bottom part of the head.

Individual counts and assessment of sperm were blind to the males age.

Statistical analysis

In order to obtain linearity, percentage outcomes were subjected to angular transformation. To compare means, a one-way ANOVA test was used. To show significant differences, a post hoc test (Tukey test) was used. All results were presented as means ± S.E. and P<0.05 was considered significant. To eliminate the impact of body mass on testes weights, the relative testes weights were compared. All calculations were made using Statistica PL ver. 10.0.

Ethical standards

The experimental procedures for this study were approved by the Regional Committee on Animal Experimentation in Kraków (Protocol No. 38/2008) acting in compliance with the European Communities Council Directive of 24 Nov 1986 (86/609/EEC).

Results

Male body, testes and accessory sex glands weights

The results summarized in Table 1 show that 10 week-old male body weights, testes relative weights and accessory sex gland relative weights were higher than in 4, 6, and 8 week-old males. There were no significant differences in body weights between 4, 6 and 8 week-old males (Table 1), whereas testes relative weight in 4 week-old males was significantly lower than in 6 week-old males as well as in 8 week-old males. Moreover, accessory sex gland weight was significantly lower in 4 week-old males as compared to older individuals. As reported in Table 1, there were no statistically important differences between 6, 8 and 10 week-old males in testes relative weight, nor in accessory sex gland weight. As shown in Table 1, relative weight differences amongst 6, 8 and 10 week-old males were significant in testes and accessory sex glands.

Epididymal sperm cells properties

Only in 1 of the 4 week-old males a single, non-motile spermatozoa was observed and that is why this data was excluded from further consideration. In 7 out of 10, 6 week-old individuals and in all 8 and 10 week-old males, epididymis sperm cells in semen were observed. Furthermore, sperm evaluation was made on 6, 8 and 10 week-old males. As demonstrated in Table 1, the highest number of spermatozoa occurred in 8 and 10 week-old males,

Table 1

Body weight, relative testes and accessory sex glands (seminal vesicles and coagulation glands) weights, and spermatozoa concentration in 4, 6, 8, 10 week-old common vole males; (Mean ± S.E.); Means marked by the same letters differ significantly at A, B – $P<0.01$ and a, b – $P<0.05$

	Males				F	P
	4 week-old	6 week-old	8 week-old	10 week-old		
body weight (g)	$19.21^A \pm 1.98$	$23.01^a \pm 1.64$	$22.87^b \pm 1.18$	$30.18^{ABa} \pm 1.61$	$F_{3.36} = 7.98$	<0.01
testes relative weight (mg/10g body weight)	$50.26^{ABa} \pm 9.69$	$97.84^a \pm 16.21$	$114.75^A \pm 5.63$	$124.80^B \pm 8.26$	$F_{3.36} = 9.56$	<0.01
accessory sex glands relative weight (mg/10g body weight)	$9.54^{ABa} \pm 4.25$	$41.34^a \pm 12.88$	$68.25^A \pm 9.98$	$92.14^B \pm 4.34$	$F_{3.36} = 16.71$	<0.01
spermatozoa concentration ($\times10^{-4}$/ml)		24.07 ± 8.05	42.78 ± 8.26	46.84 ± 8.13	$F_{2.36} = 2.22$	N.S.

a)

b)

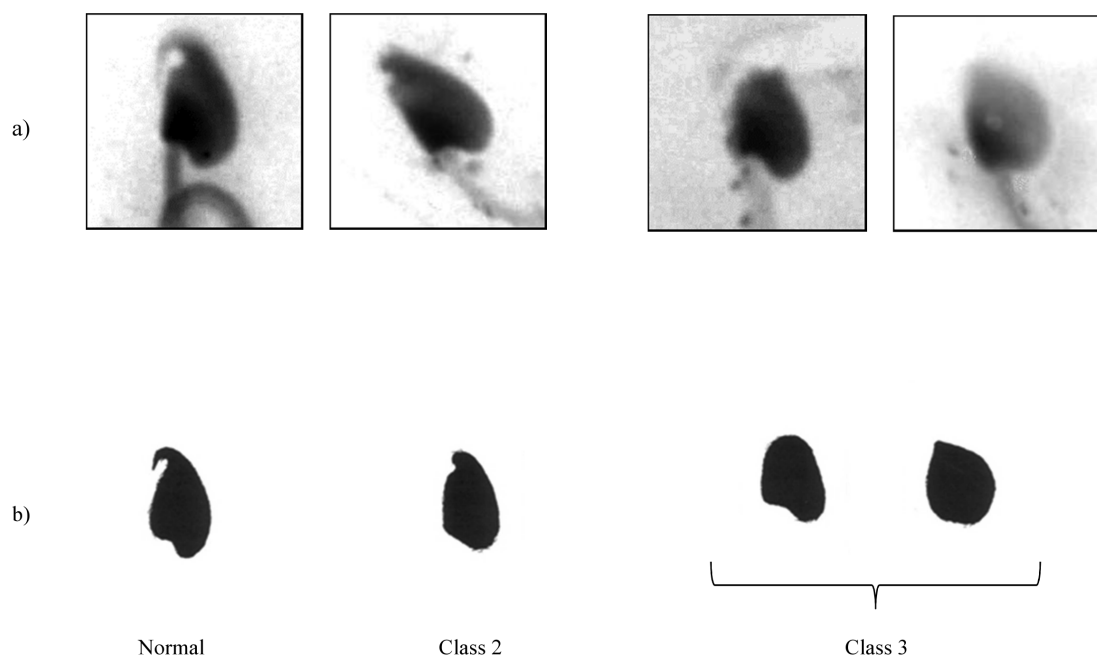Normal   Class 2   Class 3

Fig. 1. Photography (A) and diagram (B) presenting the morphology of common vole spermatozoa: Normal-sperm with correct morphology, class 2 and 3- sperm with head alterations.

while the lowest occurred in 6 week-old males. However, there were no significant differences among these 3 age groups.

Proportions of viable spermatozoa (Fig. 2) were almost the same in 8 and 10 week-old males. There were significant differences in the proportion of viable spermatozoa between the above 2 groups and 6 week-old males (P<0.01). The lowest proportion of viable sperm was observed in the 6 week-old group. Similarly, the proportion of motile spermatozoa (Fig. 3) was similar in the 8 and 10 week-old males and higher than in the 6 week-old males, although only differences between 6 week-old males and 10 week-old males reached a significant level (P<0.01).

The results obtained from the water test which presents the proportion of spermatozoa with correct form of sperm tail membrane (not swollen), are shown in Fig. 4. There were no significant differences between 8 and 10 week-old animals. In 6 week-old males, the proportion of sperm with the correct form of the tail membrane was significantly lower than in both 8 and 10 week-old males (P<0.01).

Data concerning sperm without a cytoplasmic droplet is shown in Fig. 5. The highest proportion of spermatozoa without a cytoplasmic droplet was
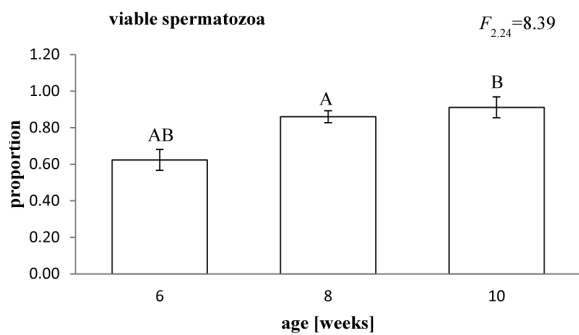
Fig. 2. Proportion of viable spermatozoa in 6, 8, 10 week-old common vole males; (Means ± S.E.); Means marked by the same letters differ significantly at A, B – P<0.01.
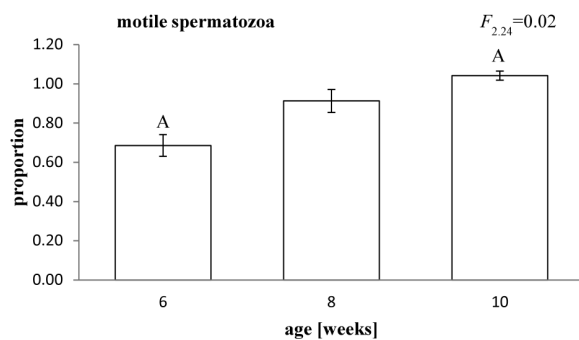

Fig. 3. Proportion of motile spermatozoa in 6, 8, 10 week-old common vole males; (Means ± S.E.); Means marked by the same letter differ significantly at A – P<0.01.
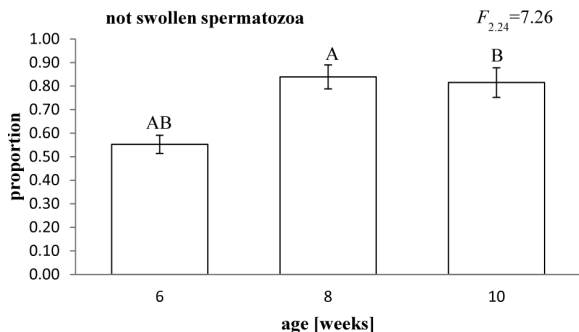

Fig. 4. Proportion of not swollen spermatozoa in 6, 8, 10 week-old common vole males; (Means ± S.E.); Means marked by the same letters differ significantly at A, B – P<0.01.
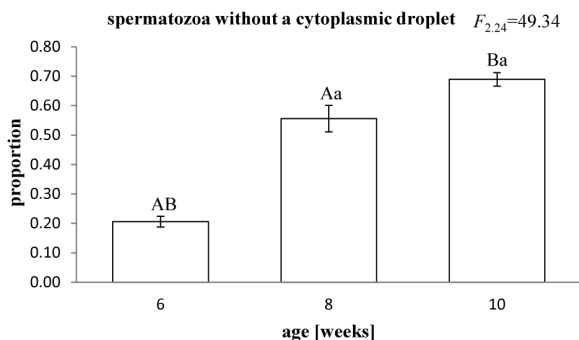

Fig. 5. Proportion of spermatozoa without a cytoplasmic droplet in 6, 8, 10 week-old common vole males; (Means ± S.E.); Means marked by the same letters differ significantly at A, B – P < 0.01 and a – P < 0.05.

noted in 10 week-old males, while the lowest proportion was in 6 week-old males (P<0.01). There were significant differences between 6 week-old males and both 8 and 10 week-old animals as well as between 8 and 10 week-old males.

Sperm head abnormalities are presented in Table 2. There were significant differences in abnormal sperm head proportions between 6 week-old males and both 8 and 10 week-old males. Six week-old animals had the highest proportion of abnormal sperm heads. There were no statistically important differences between 8 and 10 week-old males. The highest number of sperm heads with normal morphology was observed in those 2 age groups. Normal sperm head morphology was divided into 2 classes (Table 2 and Fig. 1). In each class, the same dependence as in the combined normal sperm head morphology group appeared.

Discussion

Our results have shown that common vole males reach sexual maturation at the age of 8-10 weeks. Maturing males in these age groups have the heaviest body, gonad and accessory sex gland relative weights and produce sperm cells of the best quality and quantity. This age is rather delayed when taking into consideration female age at sexual maturation. TKADLEC & ZEJDA (1995) indicate that for common vole females, the possible age for precocious breeding is 13-14 days, while BOYCE & BOYCE (1988) indicate a mean age of 13.7 days for female sexual maturation in daughters of solitary bred females, and 24.7 days as the mean age of sexual maturation for daughters of group-bred females. Our results contribute to the biology of this species, and at the same time facilitate further research especially in the area of neuro-hormonal activity regulation.

Morphological development is correlated with body weight. Research has demonstrated that bank vole (*Myodes glareolus*) males with higher body weight have heavier testes and accessory sex glands than males with lower body weight (KRU-CZEK 1986; KLEMME *et al.* 2007). Moreover, body mass is one of the factors which influences male reproductive success. The bigger and heavier the males, the more they are preferred by bank vole females (KRUCZEK & STYRNA 2009) and the higher the number of offspring they sire (KLEMME *et al.* 2007). In our experiment, the lowest body mass was observed in 4 week-old males, while the highest in 10 week-old males; 6 and 8 week-old male body weights were comparable. We assume that adult common vole body weight will not change with time, as in other mature rodents (HOFFMAN *et al.* 2002). Furthermore, in mammals, the quality and

Table 2

Proportion of spermatozoa head abnormalities in 6, 8, and 10 week-old common vole males; (Means ± S.E.); Means marked by the same letters differ significantly at A, B – P<0.01

Spermatozoa number	Males			F	P
	6 week-old	8 week-old	10 week-old		
normal	$0.600^{AB} \pm 0.03$	$1.142^{A} \pm 0.02$	$1.172^{B} \pm 0.03$	$F_{2.26} = 43.64$	0.01
abnormal – total	$0.76^{AB} \pm 0.1$	$0.18^{A} \pm 0.03$	$0.16^{B} \pm 0.03$	$F_{2.26} = 42.48$	0.01
abnormal – class 1	$0.488^{AB} \pm 0.08$	$0.242^{A} \pm 0.03$	$0.245^{B} \pm 0.05$	$F_{2.26} = 32.07$	0.01
abnormal – class 2	$0.729^{A,B} \pm 0.03$	$0.342^{A} \pm 0.02$	$0.304^{B} \pm 0.03$	$F_{2.26} = 38.82$	0.01

quantity of sperm depends on gonad size; bigger testes produce more spermatozoa that are of better quality (GOMENDIO et al. 1998; MONTOTO et al. 2011).

Testosterone is the main male androgen which controls male reproductive system development. Production of this hormone takes place in testes (SOLOMON et al. 2011). After conversion into dihydrotestosterone, testosterone controls secondary sexual trait development, accessory sex gland development and spermatogenesis (FRANÇA et al. 2006). Sperm production and sperm maturation is also regulated by testosterone (RACHMAN & CHRISTIAN 2007; SUN et al. 1990) and the number of sperm is an indicator of spermatogenesis effectiveness (RUIZ-PESINI et al. 2000). Considering these factors, we expected different quantities and qualities of spermatozoa in different male age groups. In our experiment, the youngest, i.e. the 4 week-old males, have the lowest testes weight and only in 1 out of 10 individuals, sperm cells were present in the visual field. Because there were only a few non-motile sperm cells in one individual, this age group was considered unable to reproduce. This data was excluded from further consideration. Therefore, only data concerning sperm cell quantity and quality obtained from 6, 8, and 10 week-old males was used in the statistical analysis. Although there were no statistically important differences in 6-, 8- and 10 week old animal body and testes weights, a tendency to increase with age was observed. In general, higher testes and accessory sex glands were possessed by older males. The probable interaction of accessory sex gland in Microtus is copulatory plug formation (TAMARIN 1985). Testes weight indicates accessory sex gland development in Yorkshire pigs (RAESIDE et al. 1997). Our data has shown that with an increase of testes weight, the accessory sex gland weight is increased in rodents as well. This relationship was clearly evident in the case of 8 and 10 week-old males. In addition, those 2 age groups produce the largest number of sperm cells. Although there were no important statistical differences in the number of sperm cells amongst 3 male age groups, 6 week-old males produce about 2 times less spermatozoa than 10 week-old males. With the increase in the number of spermatozoa, the possibility of fertilization becomes higher (SNOOK 2005). However, the effectiveness of fertilization also depends on sperm quality. Only sperm with highest parameters of motility, viability, sperm tail membrane integrity, sperm maturity, and sperm head morphology may survive in the female genital track and reach the ovum (LAMEO & GIAMBERSIO 1991; SOMFARI et al. 2002). In Microtine species (e.g. montane voles – M. montanus; GRAY et al. 1974, bank vole – M. glareolus; CLARK 1970, prairie voles – M. ochrogaster, RICHMOND & CONAWAY 1969) provoked estrus is presented and we assume that in the common vole the situation is similar. Generally, copulation takes place before ovulation and that is why sperm cells have to survive in the female genital track (SNOOK 2005). Only viable sperm may survive the capacitation process, acrosome reaction and finally may be capable of fertilizing the mammalian ovum (YANAGIMACHI 1981). The best sperm viability is observed in 8 and 10 week-old males. Multiple copulations in rodents (for example in bank voles – M. glareouls) are possible, which in consequence may cause sperm competition and hidden female choice (KLEMME et al. 2006; EBERHARD 1996). Moreover, recent research has shown strong evidence for multiple paternities in common voles: 50% of litters are sired by 2 or 3 males (BORKOWSKA & RATKIEWICZ 2010). In these situations, only sperm with the best properties: sperm tail membrane integrity and high number of mature sperm, can "win" by reaching the ovum (SNOOK 2005; STOCKLEY 2004). Our results show that the highest proportion of mature sperm is observed in 10 week-old males, while the best sperm tail membrane properties occur in the 8 and 10 week-old male sperm cells. Spermatozoa in these age groups probably have the best chance to "win the race" towards the ovum.

Sperm cell progressive movement is caused by the tail and is necessary to move towards the ovum for fertilization to take place (MORTIMER 1997). Sperm motility depends mainly on tail membrane properties (VETTER *et al.* 1998). In our experiment, the best semen quality was observed in 8 to 10 week old males. It can be therefore concluded that males of this age are considered best for mating, however 6-week old individuals are also capable of fertile copulations.

When spermatozoa reach the ovum, the correct form of sperm head is necessary. Hydrolytic enzymes present in the acrosome partake in an acrosomal reaction which allows sperm cells to fertilize the ovum (KRZANOWSKA *et al.* 1995). There is a positive relationship between a high amount of spermatozoa with head abnormalities and limited fertilization abilities in hamsters (WEISSENBERG *et al.* 1987) and mice (KRZANOWSKA *et al.* 1995). Our results show that the largest amount of abnormal sperm head morphology is present in the sperm of 6 week-old males, while the largest amount of normal morphology is prevalent in 8 and 10 week-old males. Results concerning sperm quality and quantity indicate that both 8 and 10 week-old males produce the best spermatozoa which may contribute to more successful reproduction. However, this conclusion should be confirmed with further research that would assess the number of successful breedings in different male age groups.

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