Maintenance of Seasonal Differences in Reproductive Characteristics of Bank Voles After 30 Years Captive Breeding

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Summary
Bank vole, *Myodes (Clethrionomys) glareolus,* serves as an object of research in many scientific disciplines. Both wild and laboratory-reared animals are used in such studies but the latter, contrary to the wild individuals, reproduce throughout the year. The aim of this study was to investigate the effect of the season outside the breeding chambers on the reproductive characteristics of bank voles kept under constant, optimal breeding conditions for 30 years. The comparison was made between summer, a breeding season, and winter, a non-breeding season, in four consecutive years.
Significant differences in reproduction and development were observed. More females gave birth in summer than in winter. The number of pups born were similar in both seasons but more pups survived the first day of life in summer than in winter. Moreover, more young survived to the time of weaning (19 days) and reached higher level of body weight in summer than in winter. Season influenced the rate of morphological development of the reproductive tract of weaned males and those individuals born in summer had significantly heavier testes than those born in winter. Differences in sexual maturation were observed also in 6 week old males. Sperm concentration, as well as the proportion of viable sperm, motile sperm and not swollen sperm were higher in males born in summer. Our results may have important implications for bank vole breeders and scientists working on other captive bred rodents.

Introduction
Bank vole, *Myodes (Clethrionomys) glareolus* is one of the most widespread species of rodent in the Palearctic region. This species is often used as an object of research in behavioural, population, environmental protection, genetic or medical studies (*Włostowski et al., 2000; Marchlewska –Koj et al., 2003; Di Bari et al., 2008; Blixt, 2010; Kapusta & Pochroń, 2011; Schønecker et al., 2011*). Some of the experiments are performed on wild individuals, some of them on animals reared in the laboratory for different lengths of time.
In the wild, bank voles breed seasonally and the breeding period is regulated mainly by the photoperiod (*Clarke, 1981*). The reproductive season lasts from the beginning of March to the end of October and there are only very few observations of winter breeding (*Larsson et al., 1973; Ylönen & Viitala, 1985*) although this can be induced by accessible food (*Andrzejewski, 1975; Eccard & Ylönen, 2001*). Pregnancy in bank voles varies from 17.5 to 18.5 days and the average number of young in a litter is 4 – 6 (*Droździ, 1963; Gustafsson et al., 1980*). Newborn bank voles are naked, blind, helpless and do not leave their nest (*Gębczyński, 1983*). At the end of the third week (around 19 days of age) young rely on themselves for food and they are sufficiently independent to leave the nest (*Gębczyński, 1983*). Female bank voles become sexually mature at an age of 1-1.5 months (*Buchalczyk, 1970*). This sexual maturity varies with time of the year and is maximal for females born in spring and early summer (*Stenseth & Gustafsson, 1985*). It is further known

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that young females do not reach maximal fertility until after becoming physiologically capable of mating (*Westlin & Gustafsson, 1983*). Males are able to reach full sexual maturation at an age of about 2 months (*Buchalczyk, 1970*) although mature spermatozoa are observed in the seminiferous tubules already in 6-week-old males (*Kruczek, 1986*).

Constant and optimal laboratory conditions make bank voles able to reproduce throughout the year. However in laboratory animals, like mice and rats, seasonal variations in some aspects of the reproductive biology remain (*Drickamer, 1977; Ramaley & Bunn, 1972; Lee & McClintock, 1986; Drickamer, 1990; Kruczek & Gruca, 1990*). There is relatively little information about the effect of season on the reproduction of others, not typically laboratory rodents kept under constant laboratory conditions.

In the Institute of Environmental Sciences, Jagiellonian University, there is a laboratory colony of bank voles established more than 30 years ago. The rooms in which the animals are housed are windowless. Room temperature, lights and humidity are controlled. The animals are kept under optimal conditions for breeding and they reproduce throughout the year. In the early years of laboratory breeding; 10 years after introduction of animals to the laboratory, the influence of season was clearly observed. Males reared under constant laboratory conditions showed a significant relationship between the season and sexual maturation (*Kruczek, 1986*). The testes as well as the seminal vesicles and coagulating glands were heavier in males born in the reproduction season, lasting from mid-April to mid-October, than in the winter season, lasting from mid-October to mid-January (*Kruczek, 1986*). During many experiments conducted on bank voles from this laboratory colony, the seasonal differences in reproductive activity are still observed, even thirty years later. The present study examined the influence of outside season on reproductive activity of bank voles in laboratory conditions. Knowledge of the potential differences in reproductive activity and development of bank voles reared in the laboratory can be important for planning experiments and interpretation of the results of studies using bank voles as a model.

In the present study the following parameters of reproductive activity and development of bank voles depending on the season were analyzed: the number of pairs mated that produced litters; the quantity and quality of offspring born and weaned; the weight of gonads of weaned 19-days-old pups and additionally the sperm evaluation of 6-week-old males born in winter and in summer.

Material and Methods

Animals

The initial stock of bank voles (20 females and 20 males) were trapped near Hajnówka village in a protective zone of Białowieski National Park (north-eastern Poland) in 1975. The animals were maintained, as a closed colony, at Jagiellonian University (Kraków, Poland) without additional introductions of animals for 30 years. To ensure heterozygosity of the colony, all matings were between voles that did not have parents and grandparents in common (*Green, 1966*). Such a colony can be assumed to be comparable with wild living bank voles (*Bujalska, 1990; Kruczek, 1990*). All animals were housed in monogamous pairs and a continuous breeding system was used. Animals were kept in polyethylene cages (36 x 21 x 17cm) (Tecniplast Gazzada, Buguggiate, Italy) in artificially controlled laboratory conditions. The animal colony room was maintained at 18-20 °C under a 14 h-10 h photoperiod (light on at 07:00 h). The relative humidity was kept constant within a range of 50%. Soft wood shavings (PPH "Arab" pet products, Zabierzów, Poland) were provided as bedding material, which was changed once per week. No environmental enrichment was provided. Standard pelleted diet (Labofit H, Kcynia, Poland) was provided daily *ad libitum*. Makrolon bottles with stainless steel caps, ACCP 2511 (Tecniplast Gazzada, Buguggiate, Italy) were used and tap water, passing through the system of several ceramic filters, was available *ad libitum*.

Mating, litter size and body and organ weight

The data compiled for this part of experiment were collected over four years: 2004, 2005, 2006, 2007. Animals were paired twice a year: in summer (August) as breeding season and in winter (December) as nonbreeding season. Twenty to twenty two pairs were mated every time. The mating procedure involved pairing one mature virgin female with one mature male (80 – 120 days of age). Paired individuals were not related to each other two generations back. Starting from day 17 after mating, females were checked for the determination of parturition. The last day of checking was 40 days after mating. On the day of birth the pups were counted and left undisturbed. Next day (1-day old) pups were counted once again and they were weighed. The pups stayed with the mother to 19 days of age when the young were weaned, counted and weighed again.

Sexual maturation

The effect of season on sexual maturation of bank voles was studied in two consecutive years. Females and males born in summer and winter were killed by cervical dislocation at 19 days of life and weighed. The uterine area of females and testes of males were dissected out and weighed. The weight of gonads was used as an indicator of the rate of morphological development of the reproductive tract.

Sperm evaluation

Males at the age of 6 weeks were killed by cervical dislocation, testes dissected and weighed. Each cauda epididymis was gentle press with forceps, allowing sperm to pass to the vasa deferentia. The latter was dissected out and their content was expressed into 100 µl of M2 medium (Sigma) containing 2% albumin bovine fraction V, and allowed to disperse for a few minutes (sperm suspension). A 1:20 dilution of sperm suspension with M2 medium (Sigma) was prepared, and the number of spermatozoa in 100 squares of a hemocytometer were counted under a light microscope at 400x magnification. The average of two sperm counts was used to estimate the sperm concentration (*Styrna & Krzanowska 1995, Busso et al. 2005*). 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 was reported (*Seed et al., 1996*). The integrity of the sperm tail membrane was determined by the hypoosmotic swelling test (*Lomeo & Giambersio, 1991; Walczak et al., 1994; Styrna et al., 2003; Kruczek & Styrna, 2009*): 20 µl of sperm suspension 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 not showing swelling among 200 counted spermatozoa from each male was reported. To assess sperm viability, 20 µl of sperm suspension 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.

The experimental procedures for this study were approved by the Regional Committee on Animal Experimentation in Kraków, Poland (permits: No. 46/OP/2003, No.26/2007). The animals were cared for, and all procedures were performed, in accordance with National Legislation and The Council of European Convention ETS 123.

Statistical analysis

A two-tailed 2x2 Fisher's exact test was used to calculate association between the season and the number of females mated that produced litters. The influence of season and year on the time from mating to birth, on the number of young born, on the young mortality at age of day 1 and at age of day 18 as well as on the number and weight of newborn pups and 19-days old young bank voles was analyzed by a two-way ANOVA. The testes and uterine weight of 19-days old animals and testes weight and sperm evaluation variables of 6-weeks–old males were analyzed using a one-way ANOVA. For statistical treatment the relative weight of gonads was taken and percentages were treated appropriately. All re-

sults were expressed as means ± S.E., and $p<0.05$ was considered significant. All procedures used Statistica PL ver. 8.0.

Results

The number of mating pairs and pairs that produced litters during all tested years and both seasons are given in Table 1. The Fisher's exact test showed that the difference between the season is statistically significant ($p<0.05$). More mated females gave birth in summer (breeding season) than in winter (nonbreeding season). The two-way ANOVA showed that neither season nor year affected the time from mating to birth and that the number of pups born was not affected either (Table 1). However, the season influenced the number of 1-day old pups: more pups survived after birth to the next day during summer than during winter (Table 2). There were no effect of year on the mortality of 1-day old pups and the interaction between experimental factors was not statistically significant. As a consequence of greater survival, the number of pups at age of 1 day was higher in summer than in winter (Figure1A, Table 3). Influence of season was also observed in the weight of 1-day old pups. Young born in summer were significantly heavier than young born in winter (Figure 1B). There was no influence of year on the weight of 1-day old pups and the interaction between experimental factors was not statistically significant (Table 3). A similar pattern of significant differences between the seasons was observed for the mortality, number and weight of 19-days old bank voles. More young survived to the time of weaning (Table 2, Figure2A) and reached a higher level of body weight in summer than in winter (Figure B, Table 3). In all cases there was no influence of year and the interaction between experimental factors was not statistically significant (Table 2, Table 3).
Results of sexual maturation depending on the season in which the young were born are presented in Table 4. Season did not affect the sexual maturation of females at 19 days of age measured by

Table 1. Number of pairs producing pups, time from mating to birth and number of pups born in summer (breeding season) and in winter (nonbreeding season) by bank voles bred 30-years in laboratory. Mean ± SE.

year	season	number of		time from mating to birth (days)	number of pups born (litter size)
		mating pairs	pairs with pups		
2004	summer	20	20	20.80±0.64	4.20±0.36
	winter	20	18	20.78±0.71	4.80±0.29
2005	summer	20	20	21.75±0.92	4.15±0.26
	winter	20	18	21.33±1.02	4.11±0.25
2006	summer	20	19	20.68±0.50	4.16±0.16
	winter	20	18	20.05±0.43	3.78±0.24
2007	summer	22	22	19.64±0.33	4.27±0.25
	winter	22	20	21.00±1.01	3.70±0.19

Analysis of variance						
	Time from mating to birth			Number of young born		
	df	F	P	df	F	P
year	3	1.721	0.165	3	1.176	0.321
season	1	0.318	0.574	1	0.020	0.887
interaction	3	1.912	0.130	3	0.773	0.511

Table 2. Mortality of 1 day-old pups and 19 days-old pups born in summer and in winter by bank voles bred 30-years in laboratory. Values are shown as mean ± *SE*.

year	season	mortality of 1 day-old pups	mortality of 19 days-old pups
2004	summer	0.25±0.16	0.15±0.10
	winter	0.94±0.28	1.28±0.42
2005	summer	0.20±0.12	0.45±0.22
	winter	1.00±0.34	0.55±0.29
2006	summer	0.32±0.22	0.84±0.21
	winter	0.66±0.24	0.95±0.30
2007	summer	0.18±0.10	0.36±0.16
	winter	0.65±0.26	0.90±0.26

Analysis of variance						
Mortality of	1 day-old pups			19 days-old young		
	df	F	P	df	F	P
year	3	1.476	0.223	3	0.777	0.509
season	1	8.721	0.004	1	6.540	0.012
interaction	3	2.220	0.088	3	1.716	0.166

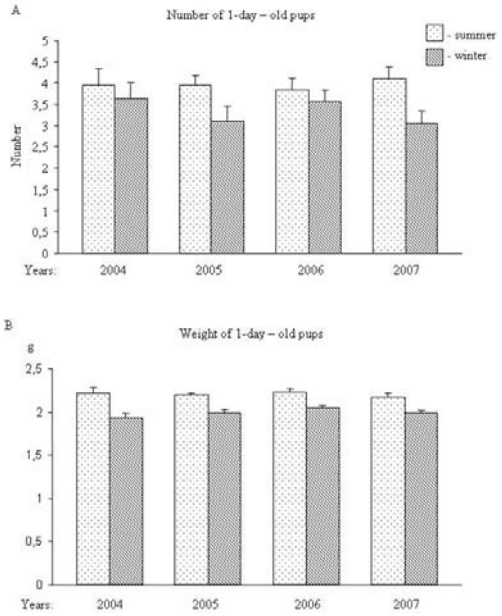

Figure 1. Number (A) and weight (B) of 1-day-old pups born in summer and in winter by bank voles bred 30-years in laboratory. Values are shown as mean ± *SE*.

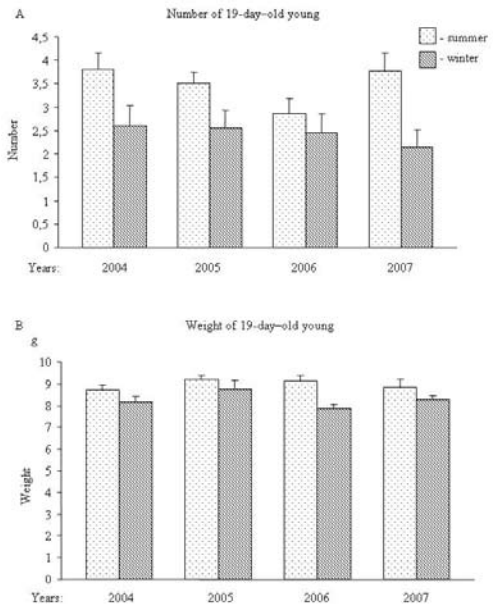

Figure 2. Number (A) and weight (B) of 19-days-old pups born in summer and in winter by bank voles bred 30-years in laboratory. Values are shown as mean ± *SE*.

Table 3. Results of two-way analysis of variance (ANOVAs) of number and weight of 1-day old pups and 19-days old young born in summer and in winter by bank voles bred 30-years in laboratory.

Analysis of variance						
1 day-old pups	Number			Weight		
	df	F	P	df	F	P
year	3	0.566	0.638	3	0.899	0.443
season	1	5.940	0.016	1	56.954	0.000
interaction	3	1.112	0.346	3	0.942	0.422
19 days-old young	Number			Weight		
	df	F	P	df	F	P
year	3	0.763	0.516	3	1.366	0.256
season	1	15.436	0.000	1	9.736	0.002
interaction	3	0.963	0.412	3	0.743	0.528

Table 4. Body weight, relative uterine weight and relative testes weight of 19-days-old young born in summer and in winter by bank voles bred 30-years in laboratory. Values are shown as mean ± *SE.*

season	no. of males	body weight (g)	relative testes weight (mg)
summer	19	10.34±0.4	73.55±2.85
winter	18	8.82±0.3	62.98±2.52
ANOVA		$F_{/1,35/}$=9,73 P < 0.01	$F_{/1,35/}$=7.66 P < 0.01
season	no. of females	body weight(g)	relative uterus weight (mg)
summer	19	10.45±0.38	10.15±0.52
winter	15	8.62±0.19	9.84±0.5
ANOVA		$F_{/1,32/}$=9.73 NS	$F_{/1,32/}$=0.18 NS

Table 5. Weights of testes, sperm concentration and proportion of viable, mobile and not swollen sperm of 6-weeks-old males of laboratory bred bank vole born in summer and in winter by bank voles bred 30-years in laboratory. Values are shown as mean ± *SE.*

season	no. of males	testes weight	sperm concentration (x10^4/ml)	sperm viability	sperm motility	not swollen sperm
summer	10	204.5±15.4	32.0±3.0	0.78±0.03	0.81±0.03	0.80±0.02
winter	10	219.6± 5.9	8.7±1.6	0.59±0.02	0.60±0.03	0.70±0.03
ANOVA		$F_{/1,18/}$=0.84 NS	$F_{/1,18/}$=46.42 P < 0.01	$F_{/1,18/}$=24.22 P < 0.01	$F_{/1,18/}$=19.96 P < 0.01	$F_{/1,18/}$=6.26 P < 0.05

uterus weight but influenced the sexual maturation of males measured by testes weight. 19-days old males born in summer had significantly heavier gonads than males of the same age but born in winter. These differences disappeared in older males and testis weight was similar at 6 weeks irrespective of the season (Table 5).

Data on sperm evaluation are shown in Table 5. There were significant differences in all tested sperm parameters between males born in winter and in summer. Sperm concentration and sperm viability were significantly higher in males born in summer than in males born in winter. Similarly males born in summer had higher proportion of motile sperm and not swollen sperm than males from winter season.

Discussion

Free-living bank voles are seasonal breeders (*Clarke, 1981*) with a reproduction period lasting from March to November. Animals kept in a constant laboratory environment under optimal breeding conditions reproduce throughout the year (Kapusta, personal observation). However, the results of our investigation showed that there are some important differences in reproduction of bank voles reared in the laboratory depending on the season outside. This means that after over thirty years of breeding in constant conditions, these animals maintain a seasonality in certain reproductive parameters. As showed by the results of this experiment, more mated pairs gave birth during summer than during winter. However, the season did not influence the length of time from mating to birth nor the number of pups born. Bank vole females are an induced ovulator (*Clarke et al., 1970*) and interactions with males influence the hormonal mechanisms controlling the reproduction of females (*Marchlewska et al., 2003*). Obtained results suggest that fewer pairs of laboratory-reared bank vole reproduce in winter. Seasonal variations were found in the quality of reared offspring born in the laboratory at different times of the year. It is known for wild living bank voles that there are some differences in the rate of physiological development depending on the season in which the young are born (*Gębczyński, 1983*). The autumn generation is physiologically younger than the spring generation and develop at a lower rate (*Gębczyński, 1977*). The lower weight of the body and higher mortality of young born in the laboratory in winter may reflect these seasonal differences visible in the wild. Increased mortality of 1-old pups as well as fewer weaned young (19 days-old) suggest also a weaker ability of females to nourish their offspring in winter despite optimal conditions in the laboratory.

The degree of sexual maturity of bank vole females and males at the age of weaning was also checked. Although the body weights were greater for both females and males born in the summer, the significant influence of the season on the gonads was seen only in males. This effect of season was visible also in the functions of the gonads. All parameters describing the quality of semen were higher for males born in summer. Summing up, the weight of the testes and sperm evaluation suggest that males reared under constant laboratory conditions reached maturity more quickly in what was summer outside than in winter. All theses results showed that bank voles reared more than thirty years under laboratory condition *maintained internal biological rhythms visible in reproduction*.

It is commonly known that many aspects of mammalian physiology and behaviour show circadian and seasonal rhythm (*Zucker et al., 1991*). The rhythm-generating mechanisms are located in suprachiasmatic nuclei (*Weaver, 1998*). They are controlled by the endogenous biological clock and synchronized by environmental stimuli, mainly lights (*Moore, 1996*). Maintenance of seasonal rhythms in reproduction of animals kept under stable laboratory condition, suggest that expression of these rhythms is not a passive response to the world around but it is pre-adapted, driven by an internal clock (*Hastings, 1998*). In the natural environment, seasonal changes regulate also several components of nonreproductive traits of rodents (*Prendergast et al., 2002*). The social strategy of many rodents is different depending on season (*Turner et al., 1975*). Body mass and food intake, as well as the brain

weight, changed in response to day lenghts (*Bartness & Wade, 1984; Yaskin, 1984; Loudon, 1994*). A seasonal shift is also visible in aggressivenes, odor preferences and ultrasonic vocalization of rodents (*Turner et al., 1975; Matochik et al., 1986; Ferkin & Gorman, 1992*). Our results indicate that the seasonal differences in reproductive activity and reproduction of bank voles reared under stable laboratory conditions can be maintained after a long period of captive breeding. It is possible that such seasonal differences can be observed also in others physiological and behavioural parameters thet can have significant influence on the results obtained in researches with bank voles as a model. The season outside the breeding chambers may affect test results and analysis. Our observations seem to have important implications for breeders and scientists working on bank voles and other rodents.

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