



## Retrospective evaluation of the reproductive status of male grey wolves (*Canis lupus*) in Germany based on animals found dead: Influence of age, body weight and season

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### ABSTRACT

Since the return of the grey wolf (*Canis lupus*) to Germany in the late 1990s, the Federal Documentation and Consultation Centre on Wolves has been conducting nationwide population monitoring, with animals found dead representing an important source of information. In addition to the standardized necropsy at the Leibniz Institute for Zoo and Wildlife Research, 182 male wolves were examined for their reproductive status between 2020 and 2024. Testis mass was measured and spermatogenic activity was determined by flow cytometric ploidy analyses of dissociated testis tissue and the presence of epididymal sperm. These reproductive parameters were evaluated with regard to age class (juvenile, subadult, adult) and month of death (seasonality). Deciphering seasonality in subadult and adult males showed that haploid cells and epididymal sperm are not only present during the mating season (January to March) but also before (October to December), as meiotic activity already begins in late summer. Almost no spermatogenic activity was detected between May and August. While the body mass of (sub)adult males remained relatively constant throughout the year, testis/body mass ratio increased with spermatogenic activity. Spermatogenic activity already occurred in juvenile males, but it was delayed, and the average level of reproductive parameters increased from juvenile to subadult males, reaching their maximum in adult wolves. Sperm production varied greatly among juveniles and was related to their body and testis mass. Basic sexual maturity was only reached at a body mass of more than 25 kg and a testis mass of more than 5 g.

### 1. Introduction

Since the return of the grey wolf (*Canis lupus*) to Germany in the late 1990s, the population increased rapidly fostered by strict protection policies, abundant prey base and public attitudes in favor for wildlife conservation (Reinhardt et al., 2019). The population status and development is closely monitored according to the German national monitoring standards for large carnivores (Reinhardt et al., 2015). By August 2025, 209 wolf packs, 48 pairs and 18 lone individuals had been documented for 2023/24 and breeding has

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been reported in 196 of 275 territories (DBBW Federal Documentation and Consultation Centre on Wolves). The return of a large carnivore into a human dominated landscape is not only a conservation success but also harbors the potential for numerous human-wildlife conflicts. Science-based monitoring is an important prerequisite for evidence-based management measures to be taken when necessary. As part of the national wolf monitoring program, dead wolves are examined at the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin, which serves as the reference institute for monitoring dead wolves throughout Germany. The IZW has been using a standardized scheme for the examination of these wolves since 2006, which has been extended to analyze the condition and functionality of the reproductive organs of male wolves found dead between 2020 and 2024.

Characterizing the reproductive traits of individuals in a population makes it possible to assess the reproductive performance of the population and identify reproductive problems. Such problems can be caused by increasing isolation between populations, for example, when this leads to increased inbreeding and a loss of genetic diversity, which can affect health and/or reproduction (Ruiz-López et al., 2012; Marelli et al., 2020). In addition, climate change could have an impact on the reproduction of grey wolves as seasonal breeders (Asa and Valdespino, 1998; Nagashima and Songsasen, 2021). So far, little is known about whether and how wolves can respond to changing environmental conditions, such as climate change and the associated changes in food availability, by shifting their mating and birthing season. Mahoney et al. (Mahoney et al., 2020) reported that wolf reproductive success in western Canada, as defined by the presence of pups at the end of summer, appeared to be relatively insensitive to a two-week earlier onset of spring, defined by premature temperature warming and observed from 2000 to 2017. Dependence on the prey availability however, which was influenced by weather conditions during the summer rearing season, made wolves vulnerable to climate change and ultimately affected their reproductive success (Mahoney et al., 2020). No comparable studies have been conducted to date in Europe, which is one of the regions most severely affected by climate change (Copernicus Climate Change Service (C3S) and World Meteorological Organization (WMO)).

Understanding reproductive traits can also help to support species conservation management. For example, professionally managed programs were established for the Iberian lynx, one of the world's most endangered felids, or the Mexican grey wolf, a subspecies of grey wolf, that was almost extinct after around 1980, in order to restore populations by releasing captive-bred animals into the wild (Kleinman-Ruiz et al., 2019; Asa and Bauman, 2023). The management of *in situ* and *ex situ* populations through the exchange of males or sperm using assisted reproduction techniques contributes to the conservation of genetic diversity, but knowledge of the reproductive potential of males is essential for their selection as breeding males (Gañán et al., 2010).

Most of the knowledge about grey wolf reproduction has been gained by observing reproductive behavior and phenology. The strong seasonality is characterized by a monoestrus cycle of females within a mating period from late January to March in the temperate climate zones of the world (Asa et al., 1987; Asa and Valdespino, 1998). During the mating season, sperm were successfully collected from adult captive males using electroejaculation (Asa et al., 2006; Zindl et al., 2006; Christensen et al., 2011) and by preparation from the epididymis of adult dead wild or captive males (Muñoz-Fuentes et al., 2014) for the purpose of cryopreservation and artificial insemination.

In a recent study, male reproduction was examined in more detail using the reproductive organs of 215 male wild grey wolves that were legally hunted or found dead in Sweden between 2003 and 2018 (Petersen et al., 2021). Body and testis masses and the presence of sperm were analyzed in males of different age classes. The probability of potential fertility, defined by the presence of sperm, was correlated to the testis mass, which showed significant annual variations in subadult and adult males. Sperm were already present in the epididymis of 42 % of the juvenile males, and testis mass was also related to body mass.

Based on examination of samples from 182 grey wolves found dead in the wild in Germany between January 2020 and December 2024 we present data on age-related development and seasonal changes of spermatogenic activity in relation to individual testis and body mass. For the analysis of the timing and extent of spermatogenic activity with respect to age and season, the proportions of haploid, diploid or tetraploid cells in testis tissue were determined by nuclear staining and flow cytometry.

## 2. Material and methods

### 2.1. Study animals and scoring of reproductive age

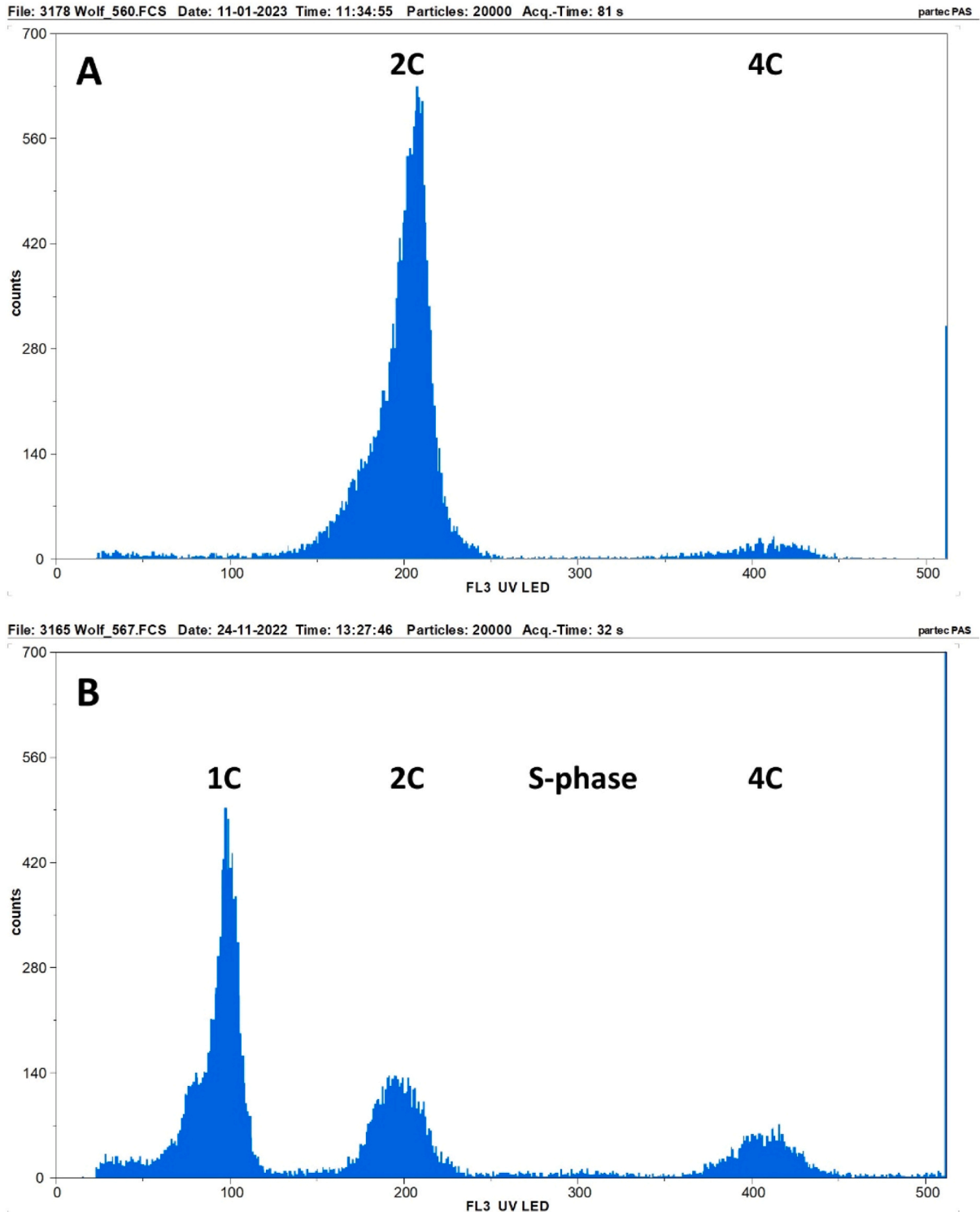
Wolf monitoring is under the jurisdiction of the federal states. Data are compiled once a year by the Federal Documentation and Consultation Centre on Wolves and made public available (<https://www.dbb-wolf.de>). Until 2022, all wolves found dead in Germany were routinely subjected to standardized examination and necropsy at the IZW. Due to the increasing number of animals found dead and limited research resources, only a representative sample of dead wolves has been examined at the IZW since 2023. After weighing and measuring body length and total length, the physical condition of the animals, including fur, teeth, thymus gland, and reproduction tract, was analyzed.

Before the necropsy, all wolves underwent computertomographic (CT) scans. Similar to the precise cutting of the root tip of the upper canine tooth with a fine saw (Landon et al., 1998), the closing of the canine tooth root and the size of the pulp cavity can be estimated via CT scans. Open roots with a wide pulp cavity represent juvenile wolves (< 1 year). Roots in the condition of closing or just closed with wide pulp cavity represent subadult wolves (1–2 years). Closed roots and dens pulp cavities were seen in adult wolves (> 2 years) (Goodwin and Ballard, 1985). The wolves were classified into three age classes accordingly: juvenile, subadult, adult.

Since 2020, males have also been examined for their reproductive status, and testes and epididymides were isolated and weighed during necropsy (Suppl. Fig. 1 A). The gonads from one side of each individual were frozen at  $-20^{\circ}\text{C}$  until analysis of spermatogenic activity (2.2.), while the gonads from the other side were fixed for histological analysis (2.3.).

Between January 2020 and December 2024, 359 male wolves were brought to the IZW. In 70 males, the body and tissue decay was

so far advanced that it was not possible to determine the age class and/or month of death and/or to distinguish testicular ploidy stages. In 17 males, testes were missing, one male had only one small testis, and one male had suffered from bilateral orchitis. Three other severely cachectic males and two males with incomplete carcasses also had to be excluded from the study. The very small (< 1 g)



**Fig. 1.** Examples of flow cytometric measurements of isolated and stained nuclei from a juvenile inactive (A) and an adult active testis (B) from grey wolves (*Canis lupus*) found dead in Germany. The histograms show haploid (1 C), diploid (2 C), tetraploid (4 C) and S-phase nuclei.

spermatogenically inactive testes of 83 juvenile male wolves were also not examined. Most of the 182 males examined (90.1 %) died in traffic accidents and therefore had typical injuries such as fractures, organ ruptures and internal bleeding. Other causes of death included territorial fights resulting in bite wounds, poaching, or the removal of individuals for management purposes and the associated gunshot wounds. We suspect that the sudden death of the animals had no influence on the current spermatogenesis status, and no clinical changes in the reproductive tract were observed.

## 2.2. Analysis of spermatogenic activity

If not stated otherwise, chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany). After thawing, male reproductive organs were freed from surrounding tissue. Testis and epididymis with a part of the vas deferens of the same length as the epididymis were separated (Fig. 1) and weighed. The testis tissue was longitudinally cut into quarters and the tunica albuginea as well as the rete testis region was removed.

To assess the spermatogenic activity, the cell cycle stages of testicular cells were determined by flow cytometry-based DNA content analysis (ploidy analysis) as described earlier by Blottner et al. (Blottner et al., 1996). Approximately 0.05 g of testis parenchyma was minced in 0.8 mL of 100 mM citric acid containing 0.5 % (v/v) Tween 20 using scissors and gently shaken for 20 min at room temperature to release the nuclei. The DNA in the isolated nuclei was stained with 5  $\mu$ M 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI) in 4 mL of a 400 mM  $\text{Na}_2\text{HPO}_4$  solution for 10 min in the dark. Analysis was performed using a CyFlow space flow cytometer (Sysmex Deutschland GmbH, Norderstedt, Germany) equipped with an UV LED and an appropriate filter set for excitation (360 nm) and emission (420 nm). For each sample, 15000 nuclei were measured, and the FlowMax software (Version 2.9, Sysmex Deutschland GmbH, Norderstedt, Germany) was used to determine haploid (spermatids and spermatozoa), diploid (spermatogonia, preleptotene and secondary spermatocytes and somatic cells) and tetraploid cells (all cells in the G2/M phase of the mitotic or meiotic cell cycles, mainly primary spermatocytes).

In Fig. 1, typical histograms of an inactive (Fig. 1A: no haploid (1 C) and very few tetraploid (4 C) cells) and an active (Fig. 1B: many haploid and significant amounts of tetraploid cells) testis are shown. Fluorescence intensities between the diploid (2 C) and tetraploid peak characterize cells in the S-phase. An increase in cell division at the beginning of mitosis and during the first phase of meiosis is characterized by an increase in the proportion of tetraploid cells. A further increase in meiotic activity and thus spermatid and sperm production is indicated by an increase in the proportion of haploid cells and the ratios 1 C/4 C and 1 C/2 C.

To assess the completion of spermatogenesis by the presence of sperm cells in the cauda epididymis, the caudal part of the epididymis was separated from the caput and corpus, and was minced with scissors in 500  $\mu$ L Dulbecco's PBS (D-8537). The presence of sperm cells was observed by the same person under the microscope with phase contrast optics and a 200  $\times$  magnification (Nikon, Tokyo, Japan). The sperm count was assigned to one of five classes: no (0), single (1), few (2), medium number (3) and large number (4) of sperm.

## 2.3. Histological evaluation of testis tissue

For histological examination, testis samples were fixed in 4 % buffered formalin. The organ samples were routinely processed in a tissue processing device (Excelsior AS, Thermo Fisher Scientific, Waltham, MA, USA), where the tissue pieces passed through an ascending alcohol series and xylene and were finally embedded in paraffin. After embedding, 3  $\mu$ m thin sections were cut and stained with hematoxylin and eosin (H&E).

The stained sections of testis tissue from a subset of samples were viewed under an inverted microscope IX-81 and a 20  $\times$  objective (Olympus, Tokyo, Japan). Images were taken by a DP72 camera (Suppl. Fig. 1B-D) and the diameters of 10 testicular tubuli were measured in each of two slices per animal using the CellSens Dimension V1 software (Olympus, Tokyo, Japan). The mean value of two perpendicular lines per tubule was determined as tubule diameter. The mean tubule diameter of all 20 values per animal was calculated.

## 2.4. Data presentation and statistical evaluation

Data analysis was performed using IBM SPSS Statistics 24 (SPSS Inc., IBM, NY, USA).

The Chi-Square test for homogeneity was used to test whether the year or month had an influence on the age class to which the analyzed animals belonged. A generalized linear model (GLM) was used to test, whether age class or month had a main or an interactive effect on the parameter body mass, and on reproductive parameters. Reproductive parameters include testis mass, epididymis mass, and relative testis mass (the ratio of testis mass to body mass, also known as the gonadosomatic index), spermatogenesis parameters (1 C, 4 C, 1 C/4 C, 1 C/2 C), and sperm count. Parameters were considered as dependent variables, age class and month were considered as main factors, and an interaction term (age class  $\times$  month) was included in the model. The model with the lowest value for the Akaike Information Criterion (AIC) was chosen (Akaike, 1973). The Kruskal-Wallis test was used to evaluate separately for each age class whether the month of death had an influence on the parameters. Selected parameters were directly correlated with each other using Spearman's rho. Effects and correlations were considered statistically significant if  $P < 0.05$ .

Visualization of data in scatter plots or box plots was performed by Sigma Plot 10.0 (Systat Software, Inc., CA, USA). Boxes show the median line and extend from the 25th to the 75th percentile, the whiskers from the 10th to the 90th percentile.

### 3. Results

#### 3.1. Analyzed male wolves as a function of year, season and age class

Overall, spermatogenic activity could be evaluated in 182 male wolves found dead in Germany within the study period (Table 1). Using the Chi-Square test for homogeneity, no statistically significant influence of the year in which the carcass was recovered was found on the proportion of examined individuals in the three age classes (Chi-square,  $P = 0.729$ ). Nevertheless, the number of juveniles and subadults examined was lowest in 2020, while the number of analyzed adults remained similar each year (Fig. 2A).

For the month of death (Table 1, Fig. 2B), a significant influence was found on the total number and the number per age group of the males examined (Chi-square,  $P > 0.001$ ). This was mainly due to the unbalanced number of juveniles. Note that juveniles are born in late April/early May and reach their subadult stage 12 months later. According to the national monitoring standard, May 1 was used to separate between juveniles (wolves in their first year of life) and subadults (wolves in their second year of life). All juveniles found dead between May and end of August can be considered spermatogenically inactive due to their small testis size ( $< 1$  g) and were excluded from the detailed measurement. An examination of juvenile testes with a testis mass between 1 and 3 g ( $n = 8$ ) revealed only a mean percentage of 2.8 % haploid cells and no sperm in the epididymis. The mean ratio of diploid to tetraploid cells (2 C/4 C) was 15.4, and a value  $> 10$  is characteristic of the absence of spermatogenesis in pre-pubertal individuals of the domestic cat (Braun et al., 2015) or of downregulated spermatogenesis in roe deer in December (Blottner et al., 1996). Juveniles that died between September and April and had a testis mass of  $> 1$  g were examined. Their proportion increased from 11 % in September to more than 70 % of juvenile males found in the breeding season (Table 1).

#### 3.2. Effects of age class and season on body mass and reproductive parameters of male wolves

The development of body mass and reproductive parameters over the course of the year is shown separately by age class in Fig. 3 and Fig. 4. The GzLM (Table 2) revealed a strong influence of age class on body mass and all reproductive parameters with the exception of the proportion of tetraploid cells in the testes. With the exception of the tetraploid cells in the testes (Fig. 4B), all other values were lower in juveniles than in adults and mostly also than in subadults. The difference depends on the month, which is reflected in the statistical significance of the interactive effect of age class and month. In particular, the four examined juveniles out of the 56 found in the fall between September and November had the lowest body mass, absolute and relative testis mass (Fig. 3A-C) and no spermatogenic activity (Fig. 4A, C), even though their testis mass exceeded the value of 1 g. The median values of all these parameters increased in juveniles found from December to April, and the differences to the level of adult and subadult males decreased. Note that juveniles, in which spermatogenesis was not expected due to their very small testes, were not included at all.

A strong significant effect of the month of death was found for all reproductive parameters, but was less pronounced for the body mass (Table 2). When applying the Kruskal-Wallis test to separately evaluate the influence of the month of death for each age class, no significance was observed for the body mass ( $P = 0.111, 0.121, 0.163$  in juveniles, subadults, adults, respectively), but significance was observed for all reproductive parameters in each age class ( $P \leq 0.020$ ). The annual development of body mass in each age class is shown in Fig. 3B. A relatively low body mass was only observed in adult males in June and in the four juveniles examined between September and November. As already mentioned in the previous paragraph, the interactive effect of age class and month therefore reaches statistical significance (Table 2).

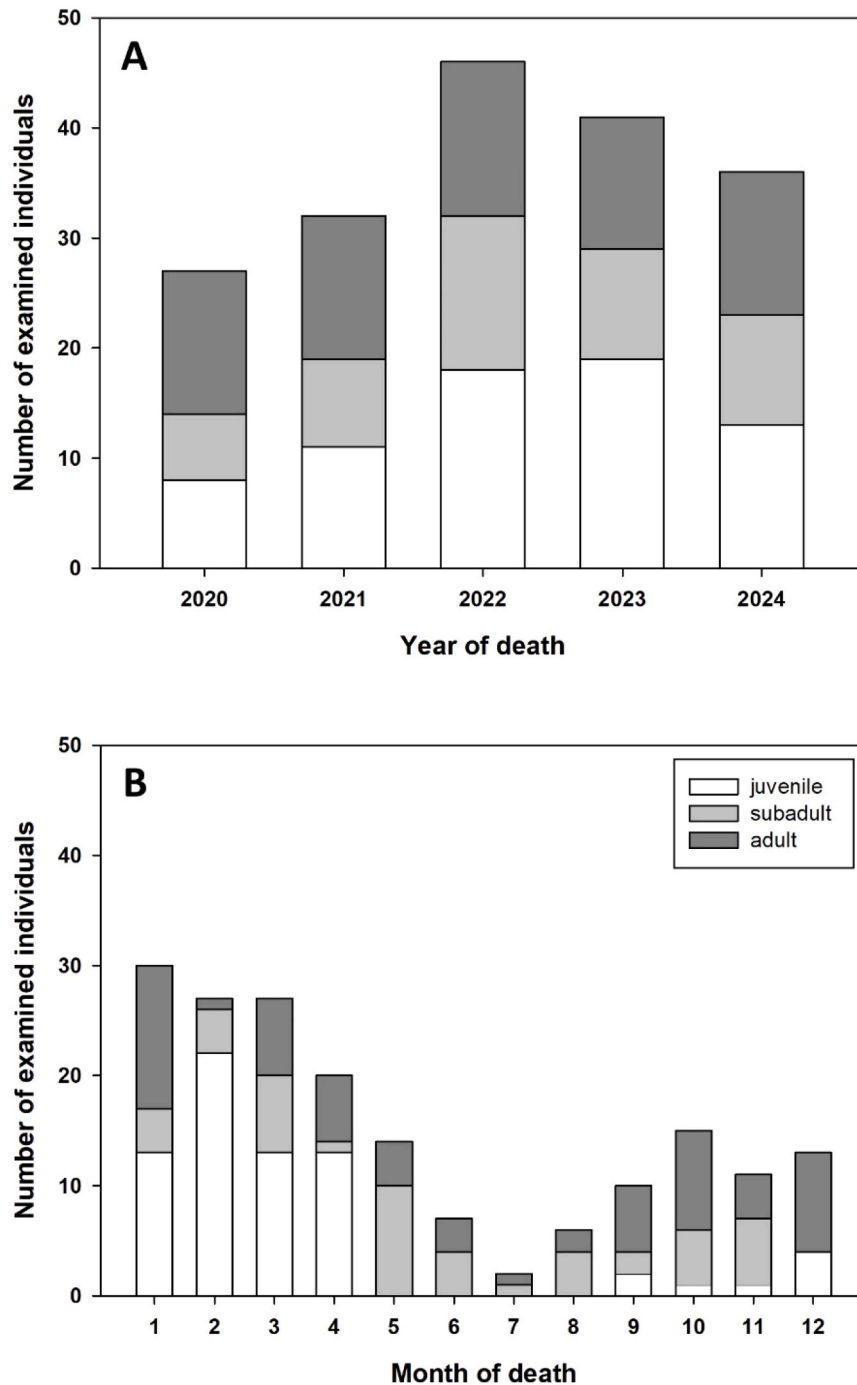
Although a significant influence of the month on the proportion of tetraploid cells in the testes was observed, it should be noted that the seasonal variation of this parameter and its variation within the respective age class were not very pronounced (Fig. 4B) compared to the changes in other reproductive parameters.

A detailed overview of the seasonal fluctuations in reproductive parameters is given in Suppl. table 1 for adult males. From June to September, no sperm were detected in the epididymides, and the mass of testes and epididymides was lower than in any other month of the year. Spermatogenic activity in the testes however, already began in September, as there was a slight increase in the percentage of tetraploid and also haploid cell stages. This increase continued in the fall, where highest values for the proportion of tetraploid cells were reached in October and November. The proportion of haploid cells and the yield of haploid cells in relation to tetraploid and diploid cells then culminated later from December to March when medium (score 3) and large (score 4) sperm numbers were present in

**Table 1**

Number of juvenile, subadult, and adult grey wolf (*Canis lupus*) individuals examined that were found in Germany in different months (1–12) between 2020 and 2024. The number of juvenile wolves examined is also given in relation to the total number of juveniles, as spermatogenically inactive juveniles (testis mass  $< 1$  g) that were brought to the IZW were not examined.

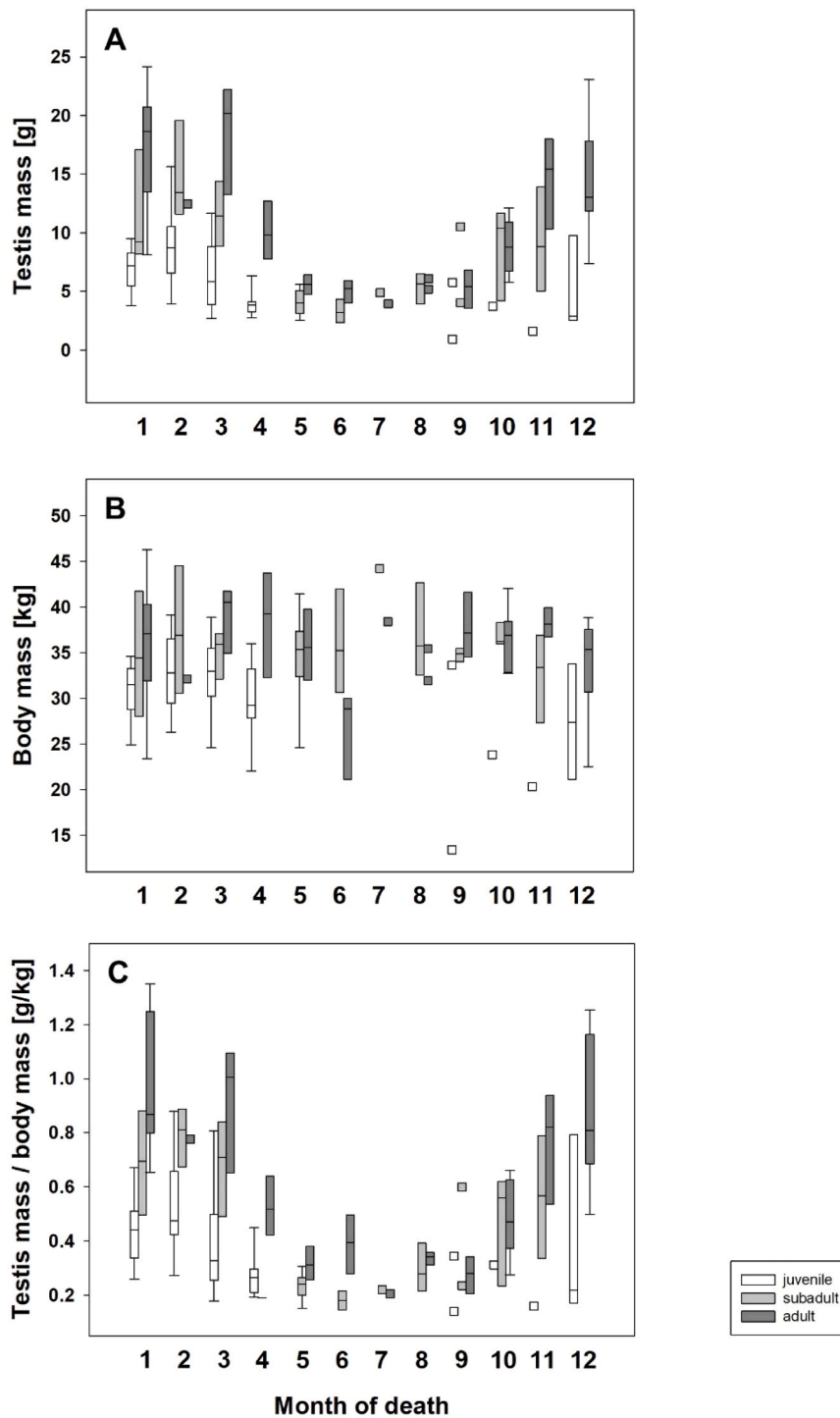
Month	1	2	3	4	5	6	7	8	9	10	11	12	
<b>total</b>													
adult	64	12	1	7	6	4	3	1	2	6	9	4	9
subadult	47	5	3	6	1	10	4	1	4	2	5	6	0
juvenile	71	13	22	14	14	0	0	0	2	1	1	4	
not examined	83	4	6	4	5	0	1	1	5	17	17	8	15
all	154	17	28	18	19	0	1	1	5	19	18	9	19
% examined	46	76	79	78	74	0	0	0	11	6	11	21	
<b>total</b>	<b>182</b>	<b>30</b>	<b>27</b>	<b>27</b>	<b>20</b>	<b>14</b>	<b>7</b>	<b>2</b>	<b>6</b>	<b>10</b>	<b>15</b>	<b>11</b>	<b>13</b>



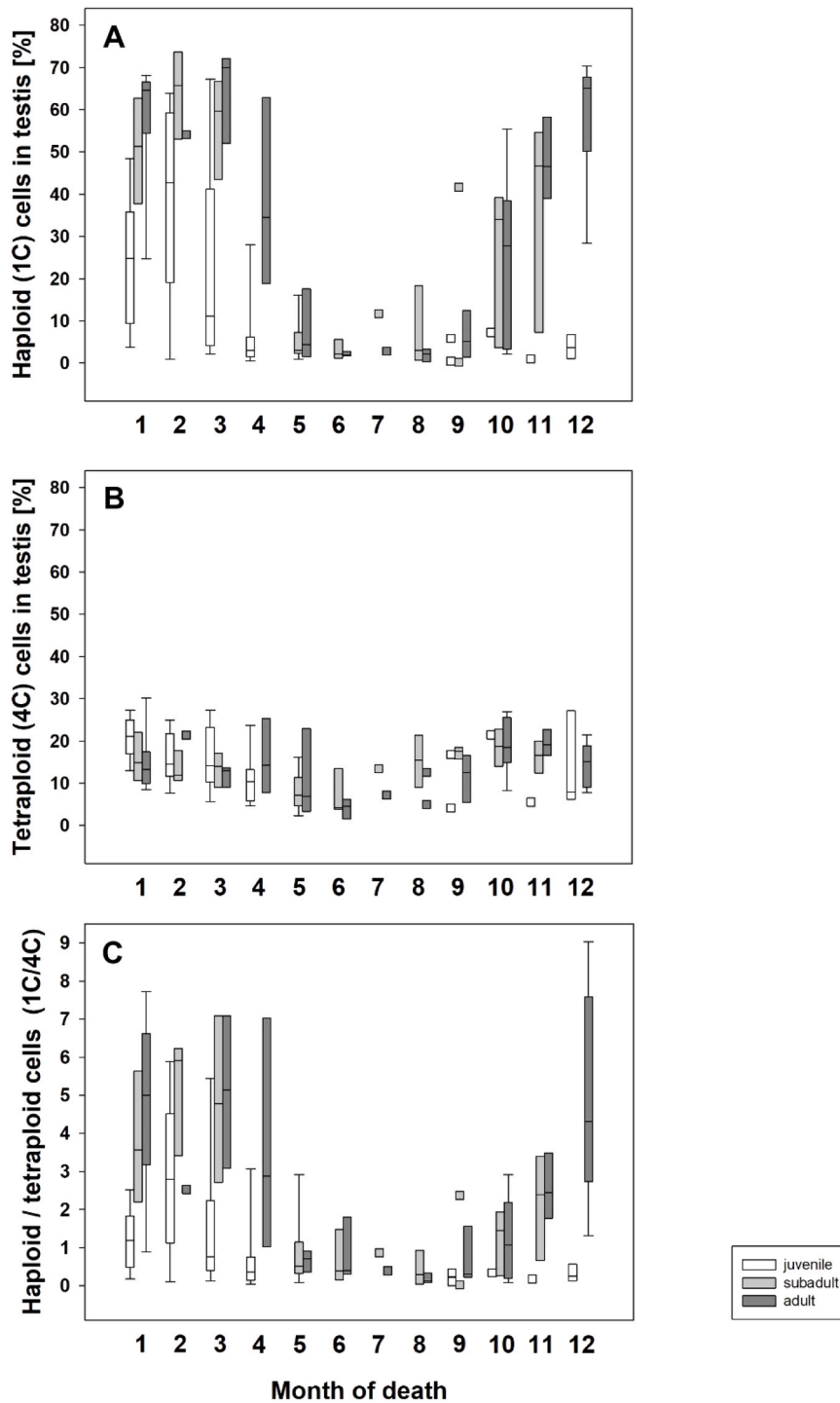
**Fig. 2.** Numbers of juvenile (white), subadult (light grey) and adult (dark grey) grey wolf (*Canis lupus*) individuals examined for spermatogenesis in different years (A) and months (B). The number of individuals was 27 (2020), 32 (2021), 46 (2022), 41 (2023) and 36 (2024). Note that spermatogenically inactive juveniles with a testis mass < 1 g were not examined. None of the juveniles found between May and the end of August had a testis mass > 1 g.

most males and the testes and epididymides had the highest masses. Only one adult male had no sperm in March and only 8 % haploid cells in his testis.

The seasonal fluctuations in subadult wolves were similar to those in adult wolves (Fig. 3, Fig. 4). Interestingly, while no sperm were detected in adult males from June to September, in five of eleven subadult males a few sperm were detected during the same period. Only one subadult male had no sperm in January and a low spermatogenic activity (1 C/4 C of 0.93). In the juvenile males in



**Fig. 3.** Seasonal changes of testis mass (A) and body mass (B) of juvenile (white), subadult (light grey) and adult (dark grey) grey wolf (*Canis lupus*) individuals found dead in Germany in the years 2020–2024. The ratios between testis and body mass (C) are also shown. The boxes with the median line extend from the 25th to the 75th percentile, the whiskers from the 10th to the 90th percentile. The number of animals examined per month is given in Table 1. Less than four data values in an age category are shown as individual data points (squares). Note that spermatogenically inactive juveniles with a testis mass < 1 g were not examined. None of the juveniles found between May and the end of August had a testis mass > 1 g.



**Fig. 4.** Seasonal changes of haploid (A) and tetraploid (B) cells in the testes of juvenile (white), subadult (light grey) and adult (dark grey) grey wolf (*Canis lupus*) individuals found dead in Germany in the years 2020–2024. The ratios between haploid and tetraploid cells in testes (C) are also shown. The boxes with the median line extend from the 25th to the 75th percentile, the whiskers from the 10th to the 90th percentile. The number of animals examined per month is given in Table 1. Less than four data values in an age category are shown as individual data points (squares). Note that spermatogenically inactive juveniles with a testis mass < 1 g were not examined. None of the juveniles found between May and the end of August had a testis mass > 1 g.

**Table 2**

Generalized linear model for the effects of age class and month on body mass and reproductive parameters of male grey wolves (*Canis lupus*) found in Germany between 2020 and 2024. Significant *P*-values are marked in bold. Note that spermatogenically inactive juveniles with a testis mass < 1 g were not examined. None of the juveniles found between May and the end of August had a testis mass > 1 g.

	Age class	Month	Age class × Month
<b>Body mass [kg]</b>			
Wald-Chi-Square	44.918	20.410	28.480
Sig.	<b>0.000</b>	<b>0.040</b>	<b>0.028</b>
<b>Testis mass [g]</b>			
Wald-Chi-Square	65.890	137.406	26.607
Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.046</b>
<b>Epididymis mass [g]</b>			
Wald-Chi-Square	117.665	110.096	33.832
Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.006</b>
<b>Relative testis mass [g/kg]</b>			
Wald-Chi-Square	82.357	182.809	37.359
Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.002</b>
<b>Haploid (1 C) cells in testis [%]</b>			
Wald-Chi-Square	66.908	172.131	45.815
Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>
<b>Tetraploid (4 C) cells in testis [%]</b>			
Wald-Chi-Square	1.172	68.194	28.549
Sig.	0.557	<b>0.000</b>	<b>0.027</b>
<b>Haploid/tetraploid cells (1 C/4 C)</b>			
Wald-Chi-Square	54.327	108.035	36.677
Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.002</b>
<b>Haploid/diploid cells (1 C/2 C)</b>			
Wald-Chi-Square	77.406	167.897	94.665
Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>
<b>Sperm count (score 0–4)</b>			
Wald-Chi-Square	8.647	52.785	2.314
Sig.	<b>0.013</b>	<b>0.000</b>	0.889

the fall, the proportion of tetraploid cells in the testes was relatively high and comparable to adult and subadult animals (Fig. 4B), while the testis mass (Fig. 3A) and the yield of haploid cells (Fig. 4A, C) remained low until January/February. Spermatogenic activity was then very variable, and sperm were observed in 23 % (3/13), 61 % (14/23), 50 % (7/14), and 15 % (2/13) of the examined juveniles found dead in January, February, March, and April. In February and March, six of the 37 juvenile males had already produced a large number (score 4) of sperm.

In order to investigate the histological changes in the testicular tissue in relation to the month of death, the diameter of the testicular tubules was measured in a subgroup of 17 adult wolves over the course of a year (Suppl. table 1). Table 3 shows the Spearman-Rho values for the respective correlations. The tubule diameter changed in line with the seasonal fluctuations in spermatogenic activity and testis mass from approx. 120 µm during summer to approx. 230 µm in winter and early spring. Images and data for three samples found in January, May, and October are shown as examples in the Suppl. Fig. 1B-E. The poor condition of the tissue however does not allow for clear identification of testicular cell types based on their morphology, as the animals were usually frozen after being found and prior to necropsy and preparation of the testes.

### 3.3. Spermatogenic activity of male wolves as a function of body and testis mass

The variability of spermatogenic activity, defined by the proportion of haploid cell types in the testis and the sperm count in the epididymis, was enormous in winter and early spring. In order to investigate whether – in addition to belonging to a certain age class – body mass influenced the spermatogenic activity, we determined the Spearman correlations between all measured parameters separately by age class (Table 4).

**Table 3**

Spearman correlations between testis mass, histology and ploidy analysis of adult grey wolf (*Canis lupus*) testes during the year (two samples each of January, March, April, May, September, October, November, December and one sample of July have been included). Significant correlations ( $P < 0.05$ ) are marked in bold.

		Testis mass	Haploid cells (1 C) in testis	Sperm count (score 0–4)
<b>Tubule diameter</b>	Rho	0.870	0.856	0.879
	Sig.	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>
<b>Testis mass</b>	Rho		0.934	0.874
	Sig.		<b>0.000</b>	<b>0.000</b>
<b>Haploid cells (1 C) in testis</b>	Rho			0.854
	Sig.			<b>0.000</b>

**Table 4**

Spearman correlations between body mass and reproductive parameters of male grey wolves (*Canis lupus*) found in Germany between 2020 and 2024. Juveniles (white,  $n = 71$ ), subadults (light grey,  $n = 47$ ) and adults (dark grey,  $n = 64$ ) were treated separately. Significant correlations ( $P < 0.05$ ) are marked in bold. Note that spermatogenically inactive juveniles with a testis mass  $< 1$  g were not examined. None of the juveniles found between May and the end of August had a testis mass  $> 1$  g.

		Testis mass	Epididymis mass	Relative testis mass	Haploid (1 C) cells in testis	Tetraploid (4 C) cells in testis	1 C/4 C
Body mass	<i>Rho</i>	0.412	0.448	0.206	0.361	0.237	0.281
	<i>P</i>	<b>0.000</b>	<b>0.000</b>	0.084	<b>0.002</b>	<b>0.047</b>	<b>0.017</b>
Testis mass	<i>Rho</i>		0.779	0.965	0.866	0.421	0.736
	<i>P</i>		<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>
Epididymis mass	<i>Rho</i>			0.747	0.727	0.399	0.618
	<i>P</i>			<b>0.000</b>	<b>0.000</b>	<b>0.001</b>	<b>0.000</b>
Relative testis mass	<i>Rho</i>				0.849	0.416	0.723
	<i>P</i>				<b>0.000</b>	<b>0.000</b>	<b>0.000</b>
Haploid (1 C) cells in testis	<i>Rho</i>					0.276	0.937
	<i>P</i>					<b>0.020</b>	<b>0.000</b>
Tetraploid (4 C) cells in testis	<i>Rho</i>						-0.030
	<i>P</i>						0.807
Body mass	<i>Rho</i>	0.240	0.178	-0.042	-0.015	0.047	-0.070
	<i>P</i>	0.104	0.232	0.781	0.922	0.754	0.638
Testis mass	<i>Rho</i>		0.884	0.940	0.827	0.413	0.745
	<i>P</i>		<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.004</b>	<b>0.000</b>
Epididymis mass	<i>Rho</i>			0.833	0.807	0.412	0.713
	<i>P</i>			<b>0.000</b>	<b>0.000</b>	<b>0.004</b>	<b>0.000</b>
Relative testis mass	<i>Rho</i>				0.847	0.385	0.769
	<i>P</i>				<b>0.000</b>	<b>0.008</b>	<b>0.000</b>
Haploid (1 C) cells in testis	<i>Rho</i>					0.203	0.947
	<i>P</i>					0.171	<b>0.000</b>
Tetraploid (4 C) cells in testis	<i>Rho</i>						-0.036
	<i>P</i>						0.809
Body mass	<i>Rho</i>	0.313	0.346	0.050	0.286	-0.077	0.296
	<i>P</i>	<b>0.012</b>	<b>0.005</b>	0.694	<b>0.022</b>	0.546	<b>0.018</b>
Testis mass	<i>Rho</i>		0.873	0.951	0.910	0.167	0.826
	<i>P</i>		<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	0.187	<b>0.000</b>
Epididymis mass	<i>Rho</i>			0.802	0.839	0.215	0.769
	<i>P</i>			<b>0.000</b>	<b>0.000</b>	0.088	<b>0.000</b>
Relative testis mass	<i>Rho</i>				0.854	0-216	0.760
	<i>P</i>				<b>0.000</b>	0.087	<b>0.000</b>
Haploid (1 C) cells in testis	<i>Rho</i>					0.086	0.933
	<i>P</i>					0.501	<b>0.000</b>
Tetraploid (4 C) cells in testis	<i>Rho</i>						-0.168
	<i>P</i>						0.186

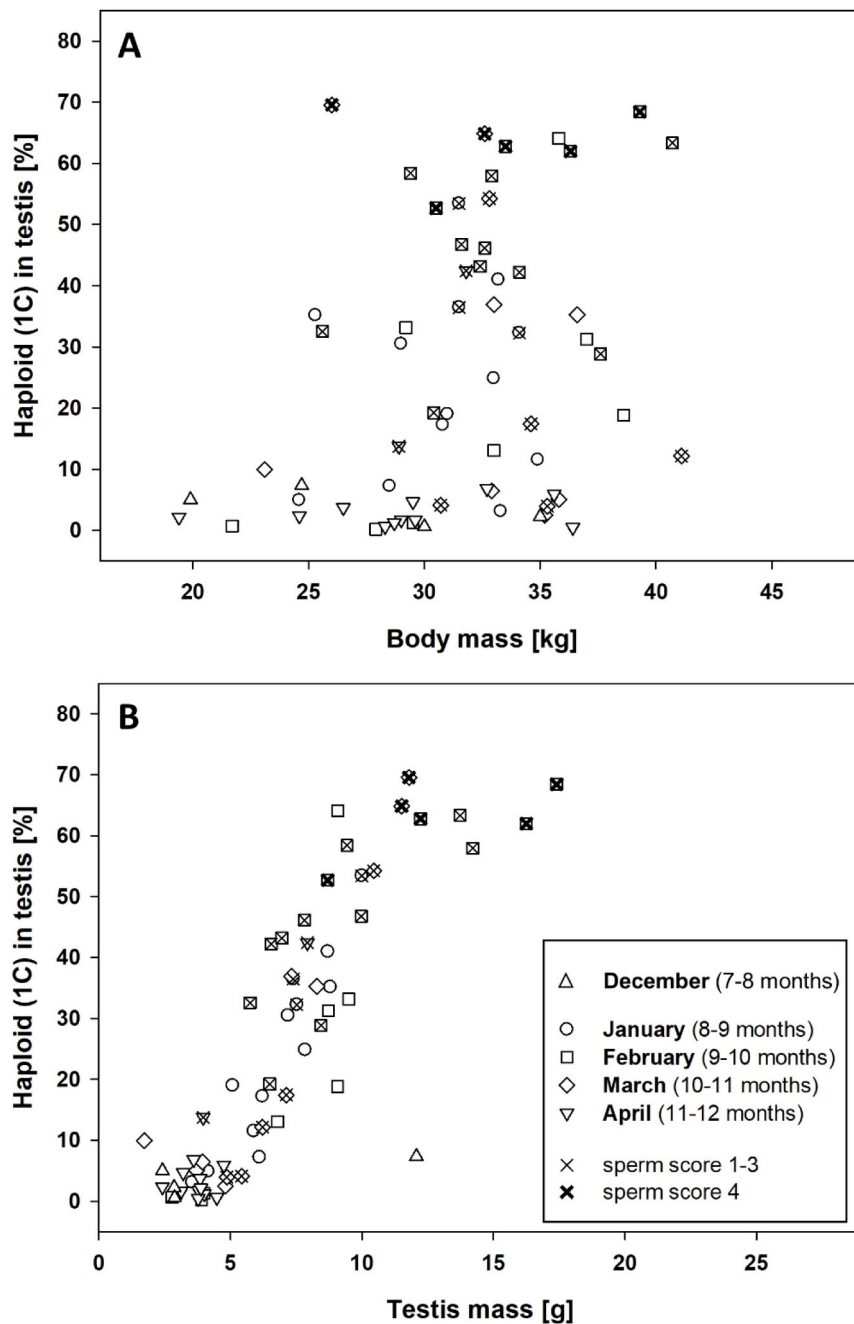
Body mass correlated with testis and epididymis mass in juveniles and adults, but the *Spearman-Rho* values were not high and even lower with spermatogenic activity (1 C and 1 C/4 C). The associations between spermatogenic activity (1 C and 1 C/4 C) and the absolute and relative testis mass and epididymis mass were more pronounced and were also observed in subadults. The relative testis mass as the ratio between testis mass and body mass was basically determined by the testis mass (*Spearman-Rho*  $> 0.9$ ).

Fig. 5 shows the proportion of haploid cells in testes as a function of body mass (Fig. 5A) and testis mass (Fig. 5B) using the example of juvenile wolves. A body mass of about 25 kg and a testis mass of about 4 g appeared to be threshold values for the ability to produce haploid cells. However, a body mass above the threshold value was not necessarily an indicator for the production of haploid cells and ultimately sperm. Testis masses above the threshold were proportionally associated with haploid cell and sperm yield, and juvenile males with the highest testis mass produced a medium (score 3) or large number (score 4) of sperm as early as nine to ten months of age in February. In total, sperm were present in 26 (39 %) of the 67 juvenile wolves examined between December and April. This corresponds to 26 % of all 101 juvenile males found during that period (Table 1).

Within each age class, the reproductive parameters were correlated with each other with one exception (Table 4). Compared to the situation in juvenile males, the proportion of tetraploid cells in the testes no longer correlated with the proportion of haploid cells in subadult and adult males, nor with the testis and epididymis mass in adult males. The efficiency of meiosis as the ratio of haploid to tetraploid cells was therefore high, and from December to April, 32 of the 35 adult (Suppl. table 1) and 14 of the 15 subadult males had sperm.

#### 4. Discussion

This is the first study investigating the reproductive status of male grey wolves in Central Europe. It was possible to analyze the reproductive status *post mortem* in 182 wild male grey wolves found dead in Germany and examined at IZW during a study period of four years. Based on 64 adult, 47 subadult and 71 juvenile wolves, we present data on age-related development and seasonal changes of



**Fig. 5.** Spermatogenic activity in juvenile grey wolf (*Canis lupus*) individuals from December to April of their first year of life ( $n = 65$ ). Only juveniles with a testis mass  $> 1$  g were examined. The percentage of haploid cells in testes and the presence of sperm in epididymides are shown as a function of body mass (A) or testis mass (B).

spermatogenic activity in relation to individual testis and body masses as measures of physical maturity.

#### 4.1. Sampling characteristics and limitations

While the composition of the age classes to which the males examined belonged was similar in each year, the composition of the age classes of examined males varied between individual months. In particular, the testes of most juveniles were still very small during their first summer and fall, and spermatogenesis had not yet begun. Valid conclusions could however be drawn about reproductive characteristics in older juveniles, as the spermatogenic activity of more than 70 % of the males that died in winter or during the mating

season was examined.

The unbalanced composition of samples also applies to the aspect of seasonality, as fewer individuals could be analyzed in summer overall. This was also the case in the study by (Petersen et al., 2021) on wolves recruited in Sweden. In this study, three times as many males were analysed in January as in any other month, as this is the main hunting season in Sweden. In comparison, our study from Germany shows a more balanced distribution of study individuals from fall to spring and provides a realistic assessment of seasonal changes of the spermatogenesis in adult male wolves.

In addition to the unbalanced composition of the samples, the unknown social background of the dead animals must also be taken into account. The resource allocation hypothesis predicts that investment in sperm production may vary depending on individual behavioural tactics for achieving reproductive success (Parker, 1990; Schradin et al., 2012). In wolves, however, it can be assumed that all (sub)adult males, regardless of whether they are in a breeding position or not, are spermatogenically active. This was confirmed by Seal et al. (Seal et al., 1979) and Asa et al. (Asa et al., 1986), who found that sperm production is not influenced by social status. Floaters (adult wolves roaming over and in between several pack territories) or dispersing subadults may suddenly be able to take over a breeding position when the breeding male of a pack dies. Consistent with this, only one of 26 adult males and only one of 15 subadult males had no sperm during the breeding season (January–April). The reason for the insufficient sperm production in these two males is unknown. Against the backdrop of fertility problems that can be caused by inbreeding or by the whole range of environmental stress factors, not only in human but also in wildlife species (Holt and Comizzoli, 2022), long-term monitoring of the reproductive status of German wolves could help to uncover such effects on these apex predators. The German wolf monitoring program (DBBW Federal Documentation and Consultation Centre on Wolves) has already recorded several cases in which a genetically known wolf pair lived in a territory for several years without producing offspring.

#### 4.2. Age related growth and increase in reproductive potential

As expected, an increase in body mass was observed with increasing age. While the difference between juveniles and the two older groups of males was clear in the fall (Fig. 4B), the body mass of some juvenile males approached that of older males during winter/spring. In contrary, testis mass of juvenile males (Fig. 3A) and the ratio of testes to body mass (Fig. 3C), defined as the gonadosomatic index, remained at a lower level compared to subadult and adult males.

In the wild, it was assumed that the first sperm production and the first ovulation, which define puberty, normally take place in grey wolves at the age of 22 months (Asa and Valdespino, 1998; Haase, 2000). In domestic dogs, sexual maturity and sperm production have been observed as early as eight months of age (Mialot et al., 1985). A few observations in captivity indicated that male wolves are also able to produce sperm at the age of ten months (Haase, 2000) and even may reproduce (Medjo and Mech, 1976). The authors assumed that the good nutrition and the removal of animals from their usual social setting led to an earlier onset of puberty. In the more recent studies by Petersen et al. (Petersen et al., 2021) and Asa and Bauman (Asa and Bauman, 2023), sperm were detected in juvenile wild Swedish and captive Mexican grey wolves as early as at seven and eight months of age, respectively. Cases of reproduction in juvenile males have also been genetically confirmed in Germany (Reinhardt and Kluth, personal communication). In the present study on German grey wolves, a few (score 1) sperm were detected in the cauda epididymis of three (23 %) out of thirteen examined juveniles in January, which corresponds to an age of 8–9 months. In February, sperm were present in the cauda epididymis of fourteen (61 %) out of 23 examined juveniles, which corresponds to an age of 9–10 months. Four of these males had already a large number (score 4) of sperm. At the same time, a high percentage of haploid cells was detected in the testes of many males. This confirms the basic reproductive capacity of wild juvenile males in their first season, although their testis mass is still smaller than in subadults and adults. As in our study, a high variation in sperm concentration was observed in captive juvenile Mexican grey wolves, as well as a high variation in sperm quality. Fifty-four percent of juvenile males were able to produce sperm in their first season, but only seven (23 %) of the 31 males with sperm were considered to be fully fertile, with motility and morphology values of  $\geq 60$  % (Asa and Bauman, 2023).

We assume that the sexual maturity, defined as the functional ability to produce sperm and reproduce, is reached in the first year in many wild and captive male juvenile wolves but not physical maturity with full body and organ size. Furthermore, juvenile male wolves in the wild rarely enter into a social constellation in which they become the breeder of a newly formed pair or even a pack. In African lions, another social carnivore species, sexual maturity also precedes physical maturity and we have defined a prime breeding age at which both sexual and physical maturity is reflected in full body weight, fully grown reproductive organs and high-quality ejaculates (Lueders et al., 2024). The prime breeding age of grey wolves is not reached before the age of two years, when both, the body mass and reproductive parameters, of many subadult males approach the values of adult males at the earliest. In a free-ranging population of grey wolves in Minnesota, the body mass of males and females only peaked at five or six years (Mech, 2006).

#### 4.3. Annual changes in reproductive characteristics

The determination of spermatogenic cell types in the testes revealed that there is almost no spermatogenic activity between May and August, the meiotic activity in subadult and adult wolves begins as early as late summer, and that sperm production peaks between December and March. Spermatogenic activity already occurred in juveniles, but it was delayed and less effective, and the amount of sperm present during the mating season varied greatly.

Grey wolves are strong seasonal breeders, as the time of rearing the young must be synchronized with optimal prey availability (Packard, 2003). The mating season in Europe and America lasts from January to March with moderate changes depending on latitude (Mech, 2002). Spermatogenesis must be initiated at least two months in advance, as studies in the domestic dog have shown that the

entire process takes 62 days (Linde-Forsberg, 2001). Accordingly, testosterone levels in wolves have been found to rise as early as the fall (Asa et al., 1990), which is also the case in other canids (Nagashima and Songsasen, 2021). In the adult males in our study, the percentage of tetraploid testicular cells, a measure of cell division activity, already began to increase in August/September, indicating increasing mitotic and meiotic activity. Five of the nine adult males found in October already had sperm in their cauda epididymis. The presence of sperm as early as October was confirmed in two out of six adult Mexican grey wolves (Asa and Bauman, 2023).

The percentage of haploid testicular cells, which are composed of postmeiotic early germ cell stages and later also sperm, began to increase in September and characterizes the maximum spermatogenic activity in adult males from December to March. The seasonal variations were accompanied by simultaneous mass changes in the gonads. The subadult males showed a similar temporal pattern of spermatogenic activity. Only the absolute and relative testis masses did not reach the maximum values of adult males during the mating season, which suggests a lower total sperm production in subadult males. This was in contrast to the situation described for Swedish wolves, where testis mass during the mating period was similar in subadult and adult males (Petersen et al., 2021), but on a lower level compared to adult wolves in Germany.

Wolves are monoestrous, i.e. the reproductive phase of females is limited to a single ovulation, which takes place between January and March after a long proestrous phase (Packard, 2003). The reproductive phase of males begins much earlier, and Asa and Valdespino (Asa and Valdespino, 1998) suggested that if the wolf pair spends time together during the proestrous to coordinate their activities and confirm their bond, the chances of successful mating are improved. A sufficient concentration of testosterone is presumably necessary to ensure appropriate behaviour such as urine-marking, as testosterone is a key mediator in the expression of numerous behavioural traits in mammals (Preston et al., 2012). The urination rate in male wolves correlates with seasonal changes in serum testosterone, appears to be related to the formation of pair bonds, and signals reproductive status (Harrington and Asa, 2003). In the European lynx, less pronounced synchronization was observed between the short period of reproductive activity in females (January to April) and the testicular function of the males, in which sperm production is maintained beyond the mating season (Müller et al., 2014). For these solitary living animals, a constant testosterone production is probably important to enable them to live in and defend their territories. The corresponding generation of testosterone maintains the spermatogenic activity, and testis mass and even the production of haploid spermatogenic cells are only moderately reduced in lynx during the non-mating period (May to December) (Müller et al., 2014). In grey wolves, which are social breeders, nearly no spermatogenic activity and sperm production has been observed between June and September, when both sexes contribute to the rearing of their litters (Mech, 1999).

Compared to the subadult and adult males, the seasonal onset of spermatogenic activity was delayed in juveniles. This is obvious from the later increase in the proportion of tetraploid and haploid testicular cells and the later appearance of sperm in the epididymis (see above 4.2). In agreement with this, (Haase, 2000) found a significant increase in plasma testosterone in young captive grey wolves born in May only in the following January. A similar observation was made in coyotes, whose juvenile males produced sperm later in their first season than their adult counterparts (Gier, 1975; Kennelly, 2001).

#### 4.4. The role of body and testis mass for the reproductive potential

Spermatogenic activity was related to body mass, with a body mass greater than 25 kg indicating the presence of sperm in juvenile males and thus their basic sexual maturity. Physical maturity is only reached at an average body mass of 35 kg, which is characteristic of (sub)adult male wolves in Germany, and a high gonadosomatic index during the reproductive season is the best parameter for predicting sperm production.

In our study, body mass increased from juvenile to (sub)adult males. In captive Mexican grey wolves, body mass was found to be a predictor of the onset of puberty, defined as successful sperm production in the first year. No male weighing less than 22.4 kg had sperm (Asa and Bauman, 2023). This approximate threshold was confirmed by the juveniles found in our study between December and April, in which only males with a body mass of over 25 kg had sperm (Fig. 5A). Nevertheless, one subadult and two adult males weighing slightly less than 25 kg during the mating season had a few (score 2) sperm.

Although a minimum body mass was required for sperm production, body mass in subadult and adult males was not closely related to spermatogenesis parameters (Table 4), and higher body mass during the mating season was not necessarily associated with higher sperm production. A comparison with the wolves in Sweden can be made in terms of average body mass (across all months), as body mass depended only to a very small extent on the month of death. Juveniles, but especially subadult and adult wolves in Sweden, were heavier ( $32.4 \pm 0.8$  kg,  $41.1 \pm 0.6$  kg and  $42.7 \pm 0.6$  kg (Petersen et al., 2021)) compared to their counterparts in Germany ( $30.9 \pm 5.3$  kg,  $35.2 \pm 5.4$  kg and  $35.8 \pm 5.4$  kg). Arnemo and Evans (Arnemo and Evans, 2017) reported even higher average body masses of 34 (24–44 kg) for juveniles and 48 (36–57 kg) for adult males in Norway and Sweden. This result is consistent with the finding that Palaearctic grey wolves follow Bergmann's rule and have a larger body size with increasing latitude (Meiri et al., 2004; Von Den Driesch, 1976), but larger body mass probably has no influence on the reproductive potential of males.

Like body mass, testis mass also increased from juvenile to (sub)adult males, and a minimum testis mass of about 5 g between December and April appeared to be crucial for minimal sperm production. Only two juveniles with a slightly lower testis mass of 4.0 and 4.9 g had produced single sperm. In line with this, Swedish wolf males with testes larger than about 6 g were classified as potentially fertile (Petersen et al., 2021). Looking at the period from December to April, the percentage of haploid cells in the juvenile testis increased linearly with increasing testis mass until a maximum of 60–70 % haploid cells was reached in males with more than 10 g testis mass (Fig. 5) and leads to high (score 3 and 4) sperm counts in the epididymis.

Absolute and relative testis mass were closely related to spermatogenesis parameters in every age class (Table 4) and are therefore better predictors of sperm production than body mass. Unlike body mass, testis mass was highly dependent on the season, but the average values reported for wolves in Sweden for absolute testis mass and gonadosomatic index tended to be at the low level

characteristic for wolves in Germany in summer and early fall. Genetic analyses would be necessary to evaluate inbreeding effects as a reason for the discrepancy between the Swedish and German wolf populations. A small relative testis size in combination with poor sperm quality has been discussed as consequence of a high inbreeding level in the Iberian lynx (Gañán et al., 2010). In fact, cases of reduced reproductive capacity, probably due to inbreeding depression, have already been described for the Scandinavian wolf population, which has descended from a small number of founding animals since 1983 (Liberg et al., 2005; Åkesson et al., 2016; Petersen et al., 2021).

## 5. Conclusions

Although physical maturity is only reached when the body and organs have reached their full size, this study has shown that sexual maturity can be reached as early as the first year of life in juvenile male wolves in Germany. If they get a chance to achieve a breeding position, some of them may make use of it. The basic importance of body mass to achieve sexual maturity and to secure reproductive capacity indicates the crucial role of nutrition and body condition of juvenile wolves for their reproductive potential. In addition to sufficient habitat and prey availability, health aspects are also important for maintaining reproductive competence of male grey wolves, as two of three cachectic adult males did not show active spermatogenesis. It is important to understand the physiological as well as social/behavioural factors that determine differences in reproductive potential between individuals. In view of the expected climate changes in Europe this can help to assess the adaptability of wolf populations in relation to rising temperature, reduced water availability, and changes in the seasonal availability of prey. The consideration of social factors and environmental stressors inevitably also requires an examination of the reproductive status of female wolves in future. Repeated hormone analyses in feces, urine, hair or blood would be useful for this purpose. Knowledge about the reproductive capacity of animals makes it also possible to estimate the impact of population management measures such as the removal of individual animals on changes in pack structure (Reinhardt et al., 2023).

## CRedit authorship contribution statement

**Claudia A Szentiks:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Karin Müller:** Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Ilka Reinhardt:** Writing – review & editing, Resources. **Alexandra Weber:** Investigation, Formal analysis. **Gesa Kluth:** Writing – review & editing, Resources. **Guido Fritsch:** Resources, Investigation.

## Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used the free software DeepL Translator (<https://www.deepl.com/de/translator>) in order to improve the language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.anireprosci.2025.108094](https://doi.org/10.1016/j.anireprosci.2025.108094).

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