University of Veterinary Medicine Hannover

Analysis of breed effects and genetic parameters of semen quality traits for frozen-thawed semen in stallions

Thesis

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To my children

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1. Theriogenology
2. Animal Reproduction Science

List of abbreviations

AI	Artificial Insemination
ADD	Additive genetic variance
CASA	Computer-Assisted Sperm Analysis
DFI	DNA fragmentation index
DNA	Deoxyribonucleic acid
EBV	Estimated breeding values
EBV-PAT	Estimated breeding values for the paternal component of the pregnancy rate per estrus cycle
FCP	First cycle pregnancy rate
FN	Fédération Équestre Nationale
GLM	General linear model
h^2	Heritability
IMV	Instruments de Médecine Vétérinaire
INRA	LÍnstitut Nationao de la Recherche Agronomique
KWPN	Koninklijk Warmbloed Paardenstamboek Nederland
LSM	Least-squares means
M	Molar
NaCl	Sodium chloride
Na_2HPO_4	Sodium hydrogen phosphate
PBS	Phosphate buffer saline
PC	Pregnancy rate per cycle
PERM	Permanent environmental variance
PEST	Prediction and Estimation
PEV	Prediction error variance
PI	Propidium iodide
REML	Restricted Maximum Likelihood
SAS	Statistical Analysis System
SCSA	Sperm chromatin structure assay
TNE	Tris-NaCl-EDT buffer

TNMS	Total number of progressively motile sperm
TNS	Total number of sperm
Tris-HCl	Tris(hydroxymethyl)-aminomethane- hydrochloride
Triton-X	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether

Chapter 1

Introduction

Introduction

Artificial insemination (AI) is mostly practiced in the equine reproduction, as it provides advantages compared to natural service, like reduced risks for stallions and mares and an advanced offer of various stallions. In 2018, a total of 7.544 breeding stallions were registered and 31.864 breedings were performed in Germany, according to the FN (Fédération Équestre Nationale) annual report. Over the last years, genetic selection on breeding horses mainly was based on pedigree and genetic potential as sport horses. But, to produce stallions, which have the abilities to fulfil the minimum requirements established for equine semen quality, selection on stallion fertility traits have to be taken into consideration. Semen characteristics are often used to approximate stallions' fertility. Correlations between the fertility of the semen of stallions and semen parameters are influenced by environment, management of the stallions and genetic influences. Therefore, population genetic analysis concerning stallion and breed effects on semen traits and a genetic study assessing genetic parameters and estimated breeding values for semen traits have to be performed.

For artificial insemination in mares, fresh, cooled and frozen semen is available, obtainable during breeding and non-breeding season. Frozen semen is mainly produced in the non-breeding season and enables the insemination of mares all over the world independent of the time of the year. Nevertheless, insemination of frozen-thawed semen often yields in low pregnancy rates. Quality of both fresh and frozen-thawed semen differs between breed, stallion age and month of semen collection when compared under similar environmental conditions. The ability of stallions' semen to endure freezing and thawing stress varies a lot between individual stallions.

To achieve insights into the influence and variability of breeds, stallions within a breed, age of the stallions and month of semen collection on fresh and frozen-thawed semen, reports on semen traits of a sufficiently large number of breeding stallions are analysed. Further studies focus on the question, if selection on high post-thaw semen quality in stallions is possible and if genetic correlation between fresh and frozen-thawed semen traits exists. To achieve genetic progress, knowledge of genetic parameters of semen quality traits and estimated breeding values (EBVs) with a sufficient accuracy is needed. Furthermore, the direction of selective breeding on fresh and frozen-thawed semen with regard to stallion fertility is important to know. Therefore, the study is extended to estimate the relationship among EBVs for frozen-thawed

semen traits and fertility of stallions, indicated by EBVs for the paternal component of the pregnancy rate per estrus cycle (EBV-PAT). The EBVs and their reliabilities of frozen-thawed semen quality traits for genetic selection should allow to evaluate if the freezing capacity of stallion semen can be improved through use of breeding value information and which relationships to EBV-PAT may be expected.

Chapter contents

The content of the thesis is presented in two papers according to § 8 of the Doctoral Regulations of the University of Veterinary Medicine Hannover for awarding the degree of Doctor medicinae veterinariae

Chapter 2 contains a population genetic analysis concerning stallion and breed effects on frozen-thawed semen traits in warmblood, light and quarter horses.

Chapter 3 offers a genetic study assessing genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German warmblood stallions.

Chapter 4 is a general discussion about the foregoing chapters.

Chapter 5 provides a summary of this thesis.

Chapter 6 is a detailed German summary.

Chapter 2

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

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Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

2.1 Abstract

The objectives of the present study were to analyze systematic effects on semen quality traits from fresh and frozen-thawed semen collected in and outside season. A total of 4,681 reports on semen traits of 121 stallions representing Arabian, Thoroughbred, Quarter Horse and four warmblood breeds used for artificial insemination at the Lower Saxon National stud Celle were edited for analysis of gel-free volume, sperm concentration, total number of sperm and total number of motile sperm in fresh semen and in frozen-thawed semen progressive motility, DNA fragmentation index and non-viable sperm. Month, year, age, breed and stallions' effects were analyzed with a linear mixed model procedure. Breed differences were significant for sperm concentration, total number of sperm, total motile sperm number and DNA fragmentation index. Hanoverian stallions showed significant higher least squares means for sperm concentration, total number of sperm and total motile sperm number in fresh semen, whereas Thoroughbred had significant higher least squares means for DNA fragmentation index. Stallions with an age of 2-6 years had significant lower least squares means in sperm concentration, total number of sperm and total motile sperm number and progressive motile sperm post-thawing than the other age groups. Month was significant for all semen traits but progressive motile sperm post-thawing. Month by age class interaction showed a significant influence. Inter-stallion variance accounted for 27-71% and the two-way stallion by month interaction for 2-7% of the total variance of semen quality traits. The largest proportion of inter-individual variance among stallions was explained by the DNA fragmentation index.

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

2.2 Introduction

Semen quality traits of fresh and frozen-thawed semen show broad variation among horse breeds under similar environmental conditions. Breed or breed type may cause significant differences in semen traits [1-13]. Ponys, Shetland ponys and Miniature ponys have lower number of total sperm in comparison to other breeds [2,5,6]. Previous studies on draught horse breeds showed higher gel-free volumes than warmblood and light horse breeds [7,8,11,13], whereas a lower sperm motility seems specific for Dutch draught horses. Despite of improvements in freezing equine semen in recent years still a considerable proportion of stallions are still not suitable for providing frozen-thawed semen fulfilling minimum requirements of quality. The proportion among acceptable semen samples differed among breeds, amounting to only 31% in purebred Icelandic horses [14]. A considerable source of variation in semen traits is due to stallions [2,4,13,15-17]. Inter-stallion variance accounted in fresh semen traits for 37 to 69% in a French study with Thoroughbred, French trotter, Selle Francais and Breton stallion [1] as well as in a study with Hanoverian stallions [17] and 40 to 59% in stallions from nine different warmblood/light horse breeds [13]. Cryopreservation enables international se-men trade throughout the year and long-term availability of semen from stallions [18-21]. Equine semen is mainly cryopreserved during non-breeding season. Freezing results may differ among individual stallions and breeds as well as due to changes of semen quality over the course of the year [19-21].

The objectives of the present study were to analyze breed and stallion effects on semen quality traits from fresh semen and frozen-thawed semen collected in and outside the breeding season. In addition, interactions between breed, stallion within breed, age of the stallion and month are studied. All stallions included in the present study were routinely used for artificial insemination (AI) on the Lower Saxon National stud Celle. These stallions belonged to four different warmblood breeds, Quarter Horse, Arabian and English Thoroughbred.

2.3 Materials and methods

2.3.1 Stallions

In this study, we analyzed reports on fresh and frozen-thawed semen collected from 121 fertile stallions that have been regularly used for AI at the Lower Saxon National stud Celle, Germany. The data comprise 4,681 reports on fresh and frozen-thawed semen collected between 1998 and 2014. The stallions were 2 to 24 years old. Semen analyses of frozen-thawed semen were done in 2014-2016.

All stallions were kept in individual boxes bedded with straw, were exercised regularly and individually (riding, carriage driving), and had daily access for approximately one hour to a paddock without any direct mare contact. They were fed three times daily with hay, oats, barley, corn, and pellets supplemented with minerals and had ad libitum access to water at all times. The stallions were held according to national regulations and institutional animal care and use protocols.

2.3.2 Collection of semen

Semen was collected three times a week during the non-breeding (September to February) and breeding season (March to August), using a dummy and artificial vagina (both Hanover model). Semen was collected in a container covered with sterile gauze to minimize debris and the gel portion of the ejaculate. All semen collections were carried out from the right side of the stallion and in the presence of a stimulus mare. The stallions were always led by the same handler using a bit in the mouth. The mare was positioned in a stall perpendicular to the dummy with her head facing the stallion.

2.3.3 Cryopreservation of semen

For cryopreservation, extended samples were centrifuged at 600 x g for 10 min; after removing the supernatant the sperm concentration was determined using a NucleoCounter SP-100 (ChemoMetec A/S, Allerød, Denmark) sperm cell counter, and the cells were diluted at 400 x

10^6 cells mL^{-1} with INRA-82. An equal amount of INRA-82 containing twice the final concentration of the desired cryoprotectant and egg yolk was then added, resulting in 200 x 10^6 cells mL^{-1}, 2.5% of egg yolk, and 2.5% glycerol. The remainder was cooled at ~0.1°C min^{-1} to 5°C by placing the samples in a container with water at room temperature and leaving them in a cooling cabinet for 2.5 h. Straws of 0.5 mL were filled in the cooling cabinet, after which they were transferred to racks. The racks were transferred to a controlled-rate freezer (Minidigitcool, IMV-Technologies, L'Aigle, France) and cooled to -140°C at 60°C min^{-1} and subsequently stored in liquid nitrogen. The straws were thawed in a water bath at 37°C for 30 seconds prior to analysis.

2.3.4 Semen analysis: sperm concentration, progressive motility, membrane and chromatin integrity.

Volume of gel-free ejaculate (mL) was evaluated in a calibrated measuring cylinder and the sperm concentration (10^9/mL) measured using a SpermaCue photometer (Minitube, Tiefenbach, Germany). The total sperm count (total number of sperm (TNS), 10^9/mL) was calculated by multiplying gel-free volume and sperm concentration. The ejaculate was diluted with pre-warmed (37°C) skim milk-based extender (INRA82).

The percentage of progressively motile sperm was analyzed under a phase-contrast microscope with heated stage (37°C) at magnification x 200 (Olympus CH-II, Olympus Optical, Hamburg, Germany). For the motility evaluations, one drop of the sample was placed on a warm (37ºC) slide with coverslip. The progressive motility percentage was visually evaluated by one highly experienced technician in fresh semen after collection and in frozen-thawed semen during all years. Computer assisted sperm analysis (CASA) may provide a more objective analysis of sperm motility but for this long-time span CASA data were not available.

The sperm concentration of the raw samples was determined photometrically on a SpermaCue photometer (Minitube) according to the manufacturer's instructions. Aliquots of the raw ejaculate were placed in a micro-cuvette specific to the SpermaCue, placed in the device and analyzed. After centrifugation, the sperm concentration was measured with the NucleoCounter SP-100 according to the manufacturer's instructions.

In thawed samples, the non-viable sperm count (the damaged membrane integrity) was determined using a two-step procedure by measuring the total cell count and the non-viable cell count on the NucleoCounter SP-100 according to the manufacturer's instructions. After dilution of the sample with reagent S100 lysis of the viable cell membrane takes place, rendering all the cell nuclei susceptible to staining with propidium iodide (PI) and resulting in a total cell count. Non-viable cells diluted with PBS (phosphate buffer saline) are permeable without treatment, however, and are stained directly with PI, resulting in a non-viable sperm count in percent. The difference between the two measurements represents the viable cell count or percent viability.
The sperm chromatin structure assay (SCSA) was employed to evaluate chromatin integrity [22]. Sperm samples stored in liquid nitrogen were used. After thawing in a water bath at 37°C, the samples were diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.4) at approximately 2×10^6 sperm mL^{-1}. A volume of 400 µL acidic detergent solution (0.08 NHCl, 0.15 M NaCl, 0.1% Triton-X 100, pH 1.2) was added to 200 µL of this suspension and mixed for 30 s, after which 1.2 mL staining solution (0.15 M NaCl, 0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M EDTA, pH 6.0) containing 6 µg mL^{-1} acridine orange (Polysciences, Warrington, PA, USA) was added. After incubation on ice for 3 min, flow cytometric analysis was performed on 10 000 cells. The flow cytometer (FACScan, Becton-Dickinson, Heidelberg, Germany) was equipped with a 488 nm argon-ion laser with an output of 15 mW for excitation and a 530/30 nm band pass filter as well as a 650 nm long pass filter for detecting green and red fluorescence, respectively. Acridine orange shows green fluorescence upon intercalation with intact double-stranded DNA, whereas denatured single-stranded DNA shows red fluorescence. The DNA fragmentation index (DFI) was calculated from sperm fractions with single and double-stranded DNA.

2.3.5 Statistical analyses

The reports on fresh and frozen-thawed semen from 121 stallions of seven breeds, including Arabian, Thoroughbred, Quarter Horse and Hanoverian, Holstein, Dutch, Oldenburg warmblood were analyzed in this study (Table 1). We investigated on average per season and stallion 10.6 (spring/summer), 21.3 (autumn) and 23.1 (winter) semen samples. Of the 121 stallions, 26/121 were sampled in one season, 90/121 in two seasons and 5/121 in all three

seasons. Means, standard deviations, minima and maxima of fresh semen traits (before freezing) and post-thaw semen traits are given in Table 2 for all breeds and in Table 3 for Hanoverian stallions only. A linear mixed animal model was employed to analyze the fixed effects of the year and month of semen collection, age class and breed of the stallion and the random effect of the stallion within the breed. Targeted semen traits in the fresh semen were gel-free volume, sperm concentration, progressive motility, total number of sperm (TNS), total number of progressively motile sperm (TNMS), and in the frozen-thawed semen post-thaw progressive motility, DFI and percentage of non-viable sperm. The mixed linear model (model I) employed for the data was as follows:

$$Y_{ijklmn} = \mu + YEAR_i + MONTH_j + AGE_k + BREED_l + stallion(breed)_{lm} + e_{ijklmn}$$

Y_{ijklmn}	=	semen trait of the ijklmn-th ejaculate from the lm-th stallion
μ	=	model-dependent constant
$YEAR_i$	=	fixed effect of year classes (i=1-4, 1=1998-2001, 2=2002-2005, 3=2006-2010, 4=2011-2014)
$MONTH_j$	=	fixed effect of month in classes (j=1-3; 1=January to February, 2=March to August, 3=September to December)
AGE_k	=	fixed effect of age class (k=1-4; 1=2-6, 2=7-12, 3=13-18, 4=19--24 years)
$BREED_l$	=	fixed effect of the breed (l=1-7)
$stallion(breed)_{lm}$	=	random effect of the stallion (m=1-121) within breed
e_{ijklmn}	=	random residual effect

These analyses were performed using the MIXED procedure of SAS, version 9.4 (Statistical Analysis System Institute, Cary, NC, USA, 2018). Least-squares means (LSM) were calculated for fixed effects. The relative proportion of the total variance of the stallions within breed w_S ($w_S = \sigma^2_S/(\sigma^2_S + \sigma^2_e)$) was calculated using the variance components among stallions within breeds (σ^2_S) and within stallions (σ^2_e). P-values <0.05 were considered as significant. In addition, we tested the two-way-interactions month by breed and month by age class. The month by breed interaction was not significant for any of the semen quality traits and thus, not

considered in further analyses. An additional random interaction effect of stallion by breed and month was added to this extended model to evaluate whether variance components among stallions differ significantly between months (model II). Semen collection from March to August was within breeding season and from January to March as well as September to December outside the breeding season. The relative proportion of the interaction component among stallions and months within breed w_{SxM} ($w_{SxM}= \sigma^2_{SxM}/(\sigma^2_S + \sigma^2_{SxM} + \sigma^2_e)$) was calculated using the respective variance components. In addition, analyses of variance were performed only for Hanoverian stallions using model I and II under omission of the breed effect (model I and II) and the random interaction effect of stallion by breed (model II).

2.4 Results

The analyses of variance for all data with seven breeds revealed the fixed effects of month and year of semen collection as significant for semen traits evaluated in this study with exception of the month effect for progressive motile sperm post-thawing and of the year effect for progressive motility of fresh semen (Table 4). Age class was significant for sperm concentration, TNS, TNMS and progressive motile sperm post-thawing. The month by age class interaction was significant for all semen traits with exception of sperm concentration and DFI (Table 4). A significant effect of the breeds was seen for sperm concentration, TNS, TNMS and DFI.

LSM for all data with seven breeds were lowest for gel-free volume, sperm concentration, progressive motility and DFI in the breeding season, whereas LSM for TNS, TNMS, progressive motile sperm post-thawing and non-viable sperm were highest in the breeding season (Table 5).

Stallions in the age class between 2-6 years had the lowest LSM for all sperm traits (Table 6). Oldenburg stallions showed the highest LSM for sperm concentration and the lowest LSM for DFI (Table 7). LSM for TNS and TNMS were highest for Hanoverian stallions. Significant breed differences were found among Hanoverian and Arabian as well as Quarter Horse stallions for sperm concentration, TNS and TNMS. Thoroughbred stallions had significantly higher DFI than all warmblood stallions.

Variance components among stallions within breeds and among Hanoverian stallions were significant for all semen traits in model I (Table 8). The between-stallion variance within breeds

explained between 27 and 71% of the total variance. In Hanoverian stallions, relative inter-stallion variances were at 24-71%. The two-way interaction component stallion by month within breed (model II) was significant for all semen traits with relative proportions of variance at 1.6 to 6.9% (Table 9). Similar results were obtained for the stallion by month interaction component in Hanoverian stallions with relative proportions of the stallion by month interaction component at 1.2 to 7.4%.

2.5 Discussion

The present study showed significant seasonal variation for all semen quality traits but frozen-thawed motility. In addition, a significant stallion by month interaction was obvious. A significant interaction component between month and age class was observed for most of the semen traits analyzed. The largest proportion of variation was explained by stallions within breeds. The same results showed the analysis only for Hanoverian stallions.

In agreement with other studies [12-14,17,19,21,23-26], we demonstrated significant seasonal differences in gel-free volume, sperm concentration, progressive motility, TNS, TNMS, DFI and non-viability. Semen samples obtained in the breeding season differed significantly from semen in the winter and autumn. Highest LSM for TNS and TNMS were observed in spring/summer which is in agreement with data of Pickett et al. [23], Jasko et al. [24], Janett et al [19], but in contrast to Gamboa et al. [26] and Magistrini et al. [24]. LSM for gel-free volume decreased in breeding season compared to autumn and winter. Other studies reported increasing ejaculate volumes with spring and summer [19,24] or highest volumes for July and March [23,25]. Our study contained stallions from seven several breeds and thus specific breed effects should be cancelled out for LSM of month. Stallion management, frequency of semen collection, breed type, use of a specific horse breed and sample size may contribute to deviating results in various studies [2,13,19,28]. Increasing daylength and light intensity with spring and summer may explain elevated number of sperm in breeding season [29-32].

LSM for progressive motility in fresh semen were clearly lower in season than in autumn and winter. A similar result was shown for Franches–Montagnes and Swiss warmblood stallions [12,19], in a study with four stallions from ejaculates collected in December and May [33] and a further study with nine stallions from the Agricultural School of Coimbra in Portugal with

lowest percentage of progressive motile sperm for the period May to July [26]. Other studies reported contrasting data with no [27] or only small seasonal differences among seasons [23,25] or higher motility in summer [24]. Changes in ambient temperatures and seminal plasma volumes were discussed as possible reasons for seasonal differences in sperm motility [12,19,21,23,34]. In addition, seasonal changes in the composition of sperm plasma membrane may have an effect on sperm motility [21,35-37]. In the present study, progressive motility in fresh semen was significantly ($P<0.0001$) negatively correlated with DFI ($r=-0.20$) and non-viable sperm ($r=-0.13$) but seasonal changes of motility did not correlate with DFI. LSM of progressive motile sperm post-thawing were not significantly different among seasons in the present study, whereas previous data indicated higher [12,19,24] or lower [14] motility after freezing in ejaculates collected during winter months.

Significant age class effects were caused through the youngest group of stallions with an age of 2-6 years for sperm concentration, TNS, TNMS and progressive motile sperm post-thawing. Young stallions below 6 years of age had lower values in these semen traits. These data are in agreement with previous studies indicating increasing semen quality in stallions aged above 5 [37] or 6 years [13] or even with 10 years [7]. A decrease of semen quality from 6 to 18 years of age was not obvious in a previous study on a large sample of Hanoverian stallions and smaller samples of Thoroughbred, Holstein, Oldenburg and Dutch warmblood [13]. Post-thaw semen traits DFI and non-viable sperm did also not deteriorate in older stallions in the present study. This data indicated that stallions above the age of 13-18 years may still be used for collecting semen to be frozen. A bias due to retirement of older stallions with a marked decrease of semen quality is unlikely as a large number of stallions in the age class of 13-18 years was included in the present and previous study [13] and the large inter-stallion variance indicative for constant semen quality in stallions across different ages [1,4,15,17]. In contrast to these findings, Dowsett and Knott [2] recommend semen for freezing from stallions over 14 years of age only if semen quality is carefully examined. The month by age class interaction showed that stallions with an age of 2-6 years showed much lower semen quality in autumn and winter compared to spring/summer. Thus, a larger proportion of younger stallions may not be suited for semen collection outside the season.

The significant influences of breed on sperm concentration, TNS and TNMS were caused by high values of Hanoverian stallions and low values of Arabian and Quarter Horse stallions. For

DFI, Thoroughbred stallions showed highest LSM and Oldenburg and KWPN stallions lowest LSM. In comparison to previous data of Gottschalk et al. [12], breed effects of fresh semen quality traits are in good agreement. However, number of stallions from horse breeds other than Hanoverian are small and may not representative for these breeds. In contrast to Dowsett and Knott [2] who found the lowest percentages of dead sperm in Arabian stallions, LSM of non-viable sperm was highest in Arabian but not significantly different from the other horse breeds in our study. Breed effects for semen traits of light horse breeds within season are in line with those outside season as month by breed interaction effects were not detected in the present study.

The random stallion effect within breed as well as the inter-stallion variance in Hanoverians had the largest impact of variation in fresh and frozen-thawed semen traits. This data underlines the high inter-individual variability as shown in previous data [1,4,13,15-17]. The relative proportion of the variance due to the stallion by month interaction was with values at 2-7% much lower than the stallion variance with values at 30-71%. Very similar results gave the analyses only for Hanoverian stallions. The relatively small interaction component may be indicative that stallions with high semen quality during season also perform well outside breeding season.

In conclusion, this study demonstrates significant breed and stallion effects accounting a large and significant proportion of the variation in fresh and frozen-thawed semen traits. Young stallions below an age of 7 years seem not as well suited as older stallions as indicated by a significant month by age class interaction. Stallion by month interaction was small and thus, differences in semen traits among stallions within season are in the similar range and direction in autumn and winter months as in season.

2.6 Acknowledgements

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2.7 Conflicts of interest

The authors declare that there is no conflict of interest.

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Table 1

Number of stallions and reports on fresh and frozen-thawed semen by horse breed and number of stallions by age classes.

Breed	Abbreviation	Number of	
		Stallions (within age classes)*	Semen reports (within age classes)
Arabian	A	5 (1/2/1/1)	78 (14/19/32/13)
Thoroughbred	TB	3 (0/1/2/1)	86 (0/31/35/20)
Hanoverian Warmblood	HAN	96 (66/31/7/10)	4175 (2041/1281/442/411)
Holstein Warmblood	HOL	4 (1/2/1/0)	117 (33/72/12/0)
Dutch Warmblood	KWPN	3 (2/1/0/0)	75 (48/27/0/0)
Oldenburg Warmblood	OLD	5 (4/0/1/0)	92 (83/0/90)
Quarter Horse	QH	5 (1/3/1/0)	58 (6/39/13/0)
Total		121 (75/40/16/9)	4681 (2225/1469/543/444)

*Some Hanoverian stallions and a Thoroughbred stallion were repeatedly sampled in different age classes.

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

Table 2

Means ($\overline{x}$), standard deviation (SD), minimum (Min) and maximum (Max) of semen traits in 4,681 semen samples including all horse breeds.

Semen trait	$\overline{x}$	SD	Min	Max
Fresh semen reports				
Gel-free volume (mL)	35.4	17.2	2.5	150
Sperm concentration ($x10^6$/mL)	289.1	101.2	50	932
Progressive motility (%)	61.7	10.3	5	90
Total number of sperm ($x10^9$)	9.6	4.1	0.3	53.1
Total number of progressively motile sperm ($x10^9$)	6.0	2.8	0.1	19.4
Post-thaw semen traits				
Progressively motile sperm post-thawing (%)	33.1	10.0	1	70
DFI	10.8	5.8	0.5	47.5
Non-viable sperm (%)	52.7	10.8	13	92

Table 3

Means ($\overline{x}$), standard deviations (SD), minima (Min) and maxima (Max) of semen traits for Hanoverian Warmblood in 4,175 semen samples.

Semen trait/breed type	$\overline{x}$	SD	Min	Max
Fresh semen reports				
Gel-free volume (ml)	35.5	16.8	2.5	150
Sperm concentration ($x10^6$/ml)	293.5	99.7	55	932
Progressive motility (%)	61.9	10.2	5	90
Total number of sperm ($x10^9$)	9.8	4.2	0.9	53.1
Total number of progressively motile sperm ($x10^9$)	6.1	2.9	0.1	19.4
Post-thaw semen traits				
Progressively motile sperm post-thawing (%)	33.0	9.7	1	70
DFI	10.4	5.4	0.5	47.5
Non-viable sperm (%)	52.7	11.0	13	92

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

Table 4

Results of the analysis of variance for model I and month by age class interaction effect from model II showing error probabilities in analyzed semen traits.

Semen trait	Sources of variation				
	Month	Year	Age class	Breed	Month x Age class*
Fresh semen reports					
Gel-free volume (mL)	<0.0001	<0.0001	<0.0768	0.5922	0.0003
Sperm concentration ($x10^6$/mL)	<0.0001	<0.0001	<0.0001	0.0042	0.1701
Progressive motility (%)	0.0002	0.3822	0.2485	0.0902	<0.0001
Total number of sperm ($x10^9$)	0.0035	<0.0001	<0.0001	0.0131	<0.0001
Total number of progressively motile sperm ($x10^9$)	0.0054	<0.0001	<0.0001	0.0099	<0.0001
Post-thaw semen traits					
Progressively motile sperm post-thawing (%)	0.8382	<0.0001	0.0004	0.3932	<0.0001
DFI	<0.0001	<0.0001	0.1750	0.0241	0.5294
Non-viable sperm (%)	0.0073	<0.0001	0.9004	0.5350	0.0136

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

Table 5

Least squares means and their standard errors for semen traits by season of collection.

Semen trait (Stallions/samples)*	Winter (n=112/2583)	Spring/Summer (n=21/222)	Autumn (n=88/1876)
Fresh semen reports			
Gel-free volume (ml)	35.5 ± 2.5^{a}	29.4 ± 2.8^{b}	35.3 ± 2.5^{a}
Sperm concentration ($x10^6$/ml)	243.5 ± 15.2^{a}	239.1 ± 19.7^{a}	255.5 ± 15.1^{b}
Progressive motility (%)	61.6 ± 1.1^{a}	58.5 ± 1.4^{b}	60.7 ± 1.1^{c}
Total number of sperm ($x10^9$)	7.5 ± 0.5^{a}	$8.1 \pm 0.7^{a,b}$	7.9 ± 0.5^{b}
Total number of progressively motile sperm ($x10^9$)	4.5 ± 0.4^{a}	$4.9 \pm 0.5^{a,b}$	4.7 ± 0.4^{b}
Post-thaw semen traits			
Progressively motile sperm post-thawing (%)	33.9 ± 1.2^{a}	34.5 ± 1.5^{a}	33.9 ± 1.2^{a}
DFI	12.9 ± 1.0^{a}	11.9 ± 1.1^{b}	13.6 ± 1.0^{c}
Non-viable sperm (%)	54.1 ± 1.3^{a}	57.2 ± 1.6^{b}	$54.8 \pm 1.3^{a,c}$

*Some stallions were repeatedly sampled in different seasons.

Table 6

Least squares means and their standard errors of the means for semen traits by age classes (years) of stallions.

Semen trait (Stallions/samples)	2-6 (n=75/2225)	7-12 (n=40/1469)	13-18 (n=16/543)	19-24 (n=9/444)
Fresh semen reports				
Gel-free volume (ml)	30.2 ± 2.5[a]	32.4 ± 2.5[a]	33.5 ± 2.7[a]	32.5 ± 3.1[a]
Sperm concentration ($x10^6$/ml)	199.1 ± 15.7[a]	257.2 ± 15.6[b]	260.2 ± 16.7[b]	253.1 ± 19.4[b]
Progressive motility (%)	58.6 ± 1.2[a]	59.8 ± 1.1[a]	59.9 ± 1.3[a]	59.7 ± 1.6[a]
Total number of sperm ($x10^9$)	5.9 ± 0.5[a]	8.5 ± 0.5[b]	8.4 ± 0.6[b]	8.1 ± 0.7[b]
Total number of progressively motile sperm ($x10^9$)	3.5 ± 0.4[a]	5.2 ± 0.4[b]	5.0 ± 0.4[b]	4.7 ± 0.5[b]
Post-thaw semen traits				
Progressively motile sperm post-thawing	32.3 ± 1.2[a]	34.9 ± 1.2[b]	34.7 ± 1.4[b]	35.5 ± 1.7[b]
DFI	11.5 ± 1.0[a]	12.1 ± 1.1[a]	14.5 ± 1.4[a]	12.9 ± 2.1[a]
Non-viable sperm (%)	56.3 ± 1.5[a]	56.64± 1.5[a]	55.9 ± 1.9[a]	54.6 ± 2.4[a]

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

Table 8

Variance components among stallions within breed (σ^2_S) and Hanoverian stallions (σ^2_S) and their respective residual components (σ^2_e) with their relative proportions of total variance for semen traits from model I. Variance components only for Hanoverian stallions are given in brackets.

Semen trait	Variance components		Relative proportion on the total variance
	σ^2_S	σ^2_e	
Fresh semen reports			
Gel-free volume (mL)	169.00 (167.34)	149.06 (143.86)	0.53 (0.54)
Sperm concentration (x10^6/mL)	5577.91 (5205.09)	4725.11 (4816.89)	0.54 (0.52)
Progressive motility (%)	29.24 (25.62)	79.49 (81.01)	0.27 (0.24)
Total number of sperm (x10^9)	5.90 (6.49)	8.49 (8.78)	0.41 (0.42)
Total number of progressively motile sperm (x10^9)	3.07 (3.33)	4.00 (4.13)	0.43 (0.45)
Post-thaw semen traits			
Progressively motile sperm post-thawing (%)	35.87 (29.60)	73.16 (71.97)	0.33 (0.29)
DFI	23.88 (21.43)	9.55 (8.83)	0.71 (0.71)
Non-viable sperm (%)	29.58 (29.47)	76.84 (77.79)	0.28 (0.28)

Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses.

Table 9

Variance components among stallions within breed (σ^2_S), stallions by breed and month (σ^2_{SxM}) and residual components (σ^2_e) with their relative proportions of total variance for semen traits from model II. Variance components only among Hanoverian stallions are given in brackets.

Semen trait	Variance components			P-value for σ^2_{SxM}	Relative proportion of σ^2_{SxM} on the total variance
	σ^2_S	σ^2_{SxM}	σ^2_e		
Fresh semen reports					
Gel-free volume (mL)	162.05 (156.50)	7.42 (6.03)	146.22 (140.64)	0.0013 (0.0052)	0.024 (0.020)
Sperm concentration ($x10^6$/mL)	5401.30 (4966.18)	161.22 (206.75)	4657.13 (4735.57)	0.0075 (0.0109)	0.016 (0.021)
Progressive motility (%)	25.05 (22.48)	7.47 (7.97)	76.44 (77.69)	<0.0001 (<0.0001)	0.069 (0.074)
Total number of sperm ($x10^9$)	5.71 (6.03)	0.26 (0.24)	8.37 (8.57)	0.0070 (0.0106)	0.018 (0.027)
Total number of progressively motile sperm ($x10^9$)	2.93 (3.11)	0.20 (0.20)	3.91 (4.01)	0.0008 (<0.0001)	0.028 (0.027)
Post-thaw semen traits					
Progressively motile sperm post-thawing (%)	33.77 (28.26)	3.33 (2.19)	71.86 (69.87)	0.0021 (0.0105)	0.031 (0.022)
DFI	24.10 (21.84)	1.60 (2.08)	9.02 (8.19)	0.0053 (0.0046)	0.046 (0.065)
Non-viable sperm (%)	27.80 (24.48)	2.81 (1.21)	75.73 (76.93)	0.0571 (0.2319)	0.026 (0.012)

Table 7

Least squares means and their standard errors of the means for semen traits of seven horse breeds.

Semen trait (Stallions/samples)	A (n=5/78)	TB (n=3/86)	QH (n=5/58)	HAN (n=96/4175)	HOL (n=4/117)	KWPN (n=3/75)	OLD (n=5/92)
Fresh semen reports							
Gel-free volume (ml)	32.7 ± 6.1^{a}	44.1 ± 7.7^{a}	30.2 ± 6.2^{a}	33.2 ± 1.6^{a}	37.5 ± 6.7^{a}	30.5 ± 7.8^{a}	25.4 ± 6.2^{a}
Sperm concentration ($x10^{6}$/ml)	200.6 ± 35.9^{a}	$227.0 \pm 45.5^{a,b}$	192.2 ± 40.3^{a}	300.3 ± 10.2^{b}	$225.2 \pm 40.4^{a,b}$	$252.2 \pm 45.2^{a,b}$	324.6 ± 37.1^{b}
Progressive motility (%)	56.8 ± 2.7^{a}	60.2 ± 3.4^{a}	58.5 ± 2.8^{a}	61.7 ± 0.8^{a}	55.4 ± 2.9^{a}	64.2 ± 3.4^{a}	64.9 ± 2.8^{a}
Total number of sperm ($x10^{9}$)	6.5 ± 1.2^{a}	$8.7 \pm 1.5^{a,b}$	6.0 ± 1.4^{a}	9.9 ± 0.4^{b}	$8.1 \pm 1.4^{a,b}$	$7.7 \pm 1.5^{a,b}$	$8.3 \pm 1.2^{a,b}$
Total number of progressively motile sperm ($x10^{9}$)	3.5 ± 0.9^{a}	$5.3 \pm 1.1^{a,b}$	3.4 ± 1.0^{a}	6.1 ± 0.3^{b}	$4.3 \pm 1.0^{a,b}$	$4.8 \pm 1.1^{a,b}$	$5.4 \pm 0.9^{a,b}$
Post-thaw semen traits							
Progressively motile sperm post-thawing (%)	28.7 ± 3.0^{a}	33.4 ± 3.7^{a}	36.6 ± 3.0^{a}	34.9 ± 0.8^{a}	31.9 ± 3.2^{a}	37.7 ± 3.7^{a}	35.6 ± 3.0^{a}
DFI	16.8 ± 2.3^{a}	$19.3 \pm 3.0^{b,c,d}$	$13.6 \pm 2.4^{a,b,c,d}$	$10.8 \pm 0.8^{a,c,d}$	$11.6 \pm 2.6^{a,c,d}$	$9.2 \pm 3.0^{c,d}$	8.7 ± 2.3^{d}
Non-viable sperm (%)	60.9 ± 2.9^{a}	55.1 ± 3.7^{a}	54.5 ± 3.0^{a}	54.9 ± 1.1^{a}	54.3 ± 3.1^{a}	55.0 ± 3.7^{a}	52.9 ± 2.9^{a}

Abbreviations: A, Arabian; TB, Thoroughbred; QH, Quarter Horse, HAN, Hanoverian; HOL, Holstein; KWPN, Dutch Warmblood; OLD, Oldenburg Warmblo

Chapter 3

Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions

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Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions

3.1 ABSTRACT

Objectives of the present study were to estimate genetic parameters for frozen-thawed semen traits of 271 fertile German Warmblood stallions and genetic correlations with raw semen quality traits. Semen samples were collected from stallions utilized for semen collection and artificial insemination (AI) on the Lower Saxon National Stud Celle and the North Rhine-Westphalian National Stud Warendorf. Semen quality variables were analyzed in 63,972 raw (gel-free volume, concentration, progressive motility, number of sperm) and 3,681 frozen-thawed samples (motility, DNA fragmentation index (DFI), non-viable sperm). A multivariate linear animal model was used to estimate additive genetic and permanent environmental variances among stallions as well as estimated breeding values (EBVs) for all semen traits. Heritability estimates were greatest for DFI (h^2 = 0.45) and least for non-viable sperm counts (h^2 = 0.11). Additive genetic correlations between progressive sperm motility in raw semen and DFI (r_g = -0.79) as well as non-viable sperm (r_g = -0.45) were negative. The EBVs for frozen-thawed semen traits ranged from 49 to 181 with mean reliabilities of 0.28 to 0.43. The EBVs for progressively motile sperm post-thawing and DFI were the most highly correlated traits with EBVs for stallion fertility (r = 0.38 and r = -0.17). Stallions with relatively greater EBVs for progressive motility in raw semen may be most suitable when freezing semen for storage and subsequently thawing it for AI. Using EBVs for semen traits in selection of stallions to AI mares appears as an option for genetic improvement to enhance fertility after AI.

3.2 Introduction

Horse breeders have started to become increasingly aware of the importance of post-thawing stallion semen quality and are wanting to understand if stallions can be genetically selected for post-thawing sperm quality traits. The inter-individual and additive genetic variation of post-thaw semen traits are largely unknown and there is not an understanding of the genetic correlations

between raw and post-thawed semen traits. With raw semen variables, there is considerable variation among stallions.

Significant heritability estimates for raw semen traits were reported for a number of breeds. Estimates were moderate to high in Dutch maiden (Parlevliet et al., 1994), Shetland pony (Van Eldik et al., 2006), Friesian (Ducro et al., 2011), Hanoverian (Labitzke et al., 2014) and German warmblood (Gottschalk et al., 2016) stallions. The inter-stallion variability in values for semen variables varied from 37% to 85% in warmblood horses from Germany (Labitzke et al., 2014, Gottschalk et al., 2016) and between 37% to 69% in a French study including Thoroughbred, French trotter, Breton and Selle Francaise stallions (Rousset et al., 1986). The large estimates of inter-stallion variances in these previous reports are indicative that there has been little selection pressure for traits related to stallion sperm quality. For warmblood stallions to be used for breeding of a large number of mares, these stallions have to undergo strong performance competitions before being licensed as a breeding stallion. After licensing, warmblood stallions are selected based on the minimum requirements for semen quality standards and suitability of semen for cooled-storage and cryopreservation. Infertile warmblood stallions or stallions with a less than desirable foaling rate in the first breeding season will not be used for further breeding. An intense selection for stallion fertility is not a common practice in horse breeding due to the large economic value of stallions and their genetic potential as sport horses.

Improvement of semen quality through selective breeding requires knowledge of genetic parameters of semen quality traits and estimated breeding values (EBVs) with a sufficient accuracy to achieve genetic progress. In addition, the direction of selective breeding on semen quality traits with regard to stallion fertility is an important issue.

Stallions with relatively greater fertility may have a selective advantage because of the possibility of siring a larger number of progeny and potential future breeding animals in comparison to less fertile stallions. Previous reports on correlations between semen quality traits and averages of pregnancy rates per estrous cycle (PC), pregnant rates per first estrous cycle (FCP) and non-estrous-return-rates per stallion indicated positive outcomes and thus, indicate there is a positive phenotypic relationship between semen quality and foaling rate variables. Jasko et al. (1992) reported that there were significant correlations between the percentages of motile (r =

0.40), progressively motile (r = 0.46), morphologically normal (r = 0.36) sperm and computer-aided analysis of percentage of motile spermatozoa (r = 0.34) with PC. In a study including 88 stallions, correlations between values for progressive motility and stallion fertility were determined as PC and FCP and were significant with a value for r = 0.52 and r = 0.56, respectively (Love, 2011). Total sperm motility (r = 0.64) was the semen trait most highly correlated with FCP. In 3-year-old Dutch Warmblood stallions, there was a significant positive correlation between values for morphologically normal sperm and first estrous cycle non-return rates (Parlevliet and Colenbrander, 1999). Lesser DNA integrity as determined by DNA fragmentation indices was correlated with lesser PCs (r = -0.63) in Swedish warmblood stallions (Morrell et al., 2008). Insemination results for 196 donor mares in 496 estrous cycles indicated there were greater embryo recovery rates when sperm motility, viability, DNA quality, normal morphology, concentration, and total number were greater in an insemination dose (Love et al., 2015).

Positive relationships among EBVs for semen quality traits and fertility of stallions as indicated by EBVs for the paternal component of the pregnancy rate per estrous cycle (EBV-PAT) may be expected. Results of a study with 100 German Warmblood stallions indicated that for total number of progressively motile sperm in raw semen there was a significant positive correlation of 0.36 with EBV-PAT (Gottschalk et al., 2017). The EBVs of 100 to 110 for sperm concentration, progressive motility, total number of sperm and total number of progressively motile sperm were associated with the EBV-PAT of greater than 120. There are no studies analyzing associations of frozen-thawed semen quality traits that include assessments of EBV and PAT.

The objectives of the present study, therefore, were to estimate permanent environmental and genetic variances for frozen-thawed semen quality traits of fertile German Warmblood stallions routinely used for AI and analyze the genetic relationships with semen quality traits from raw semen. The EBVs and the reliabilities of frozen-thawed semen quality traits for genetic selection should allow for determination of whether the freezing capacity of stallion semen may be improved through use of breeding value information such as associations between EBVs and EBV-PAT values.

3.3 Materials and methods

3.3.1 Stallions

Semen traits were recorded in 241 fertile German Warmblood stallions routinely used in AI at the Lower Saxon National Stud Celle and the North Rhine-Westphalian National Stud Warendorf. This data set was used in a previous study by Gottschalk et al. (2016) to estimate genetic parameters and EBVs for raw semen traits (Data Set I). In the present study, this data set was further analyzed with values for post-thaw sperm variables being recorded for 121 fertile German Warmblood stallions (Dataset II) with 91 of 121 stallions having values recorded for raw semen in Dataset I. Data set I was comprised of 63,972 values for raw semen. In Dataset II, there were 3,681 values for frozen-thawed semen. Semen samples for which there were values in Dataset I were collected between 2001 and 2014 and for Dataset II between 1998 and 2014. All stallions included in the present study were approved for AI and had conception rates of at least 70% when there were greater than nine mares inseminated with semen from the specific stallion. All stallions included in the study were, therefore, considered to be fertile. A preselection of the stallions at the national studs was not conducted for the present study because as many stallions as possible were included in both datasets. Stallions were 3 to 30 years old and registered in German Warmblood horse breeding associations including Hanoverian, Holstein, Oldenburg, Rhinelander and Westphalian. Stallions registered with the different horse breeding organizations had pedigrees with common ancestors and there was an exchange of stallions between the different horse breeding organizations. All stallions were kept in individual box stalls bedded with straw without direct contact with mares and with 1 hour of access daily to a paddock. The stallions were supplied with water ad libitum and fed three times a day a diet of hay, oats, barley, corn, and pellets supplemented with minerals. Housing conditions of stallions at the State studs were consistent with the standards included in the institutional animal care and national welfare regulations.

3.3.2 Evaluation of semen variables

A detailed description of semen collection and examination of raw semen samples has been previously described (Gottschalk et al., 2016). Briefly, all semen samples were collected during the months of February to August using an artificial vagina (Hanover model). Semen was collected

once daily for 6 consecutive days every week during the months February to August using a "phantom" for the stallion to mount and an artificial vagina (Hanover model). Prior to evaluation of semen variables, semen was filtered with sterile conditions being utilized to remove the gel portion of the ejaculate. Afterwards, semen quality variables were evaluated. Semen quality variables included gel-free volume, sperm concentration, total number of sperm (TNS), progressively motile sperm and total number of progressively motile sperm (TNMS). Sperm concentration (10^6/mL) was measured using a SpermaCue photometer (Minitube, Tiefenbach, Germany). For calculation of the TNS ($x10^9$), the gel-free volume was multiplied by sperm concentration. After dilution with a pre-warmed (37°) skim milk-based extender (INRA82), progressive motility was determined using a phase-contrast microscope with a heater stage at 200x magnification (Olympus CH-II, Olympus Optical, Hamburg, Germany). There was subjective visual estimation of progressive motility by specialized and trained technicians at the respective national stud. A potential bias of single technicians was largely precluded because technicians examined many different stallions per breeding season and thus, different technicians evaluated samples of a stallion.

For cryopreservation, semen was collected three times a week from September to January using a "phantom" for stallions to mount and an artificial vagina (Hanover model) in the presence of a mare for sexual stimulation. To prepare the semen for cryopreservation, it was centrifuged for 10 minutes at 600 x *g*. After removal of the supernatant, the sperm concentration was assessed using a sperm cell counter (NucleoCounter SP-100, ChemoMetec A/S, Allerød, Denmark) according to the manufacturer's instructions. The semen was then diluted to a concentration of 400 x 10^6 cells/mL with INRA-82. The INRA-82, cryoprotectant and egg yolk were then added to the semen, resulting in 200 x 10^6 cells/mL, 2.5% of egg yolk, and 2.5% glycerol. The sample was placed in a vessel and the vessel was placed for 2.5 h in a flask containing water at room temperature, followed by cooling to 5 °C with ~0.1 °C/min in a cooling cabinet. Straws of 0.5 mL were automatically filled in the cooling cabinet and were subsequently transferred to a controlled rate freezer (Minidigitcool, IMV-Technologies, L'Aigle, France) where samples were cooled to -140 °C at 60 °C/min and subsequently stored in liquid nitrogen. The quality of the frozen-thawed semen was evaluated after thawing two straws in a water bath at 37 °C for 30 seconds after which

one straw was used for determining the percentage of non-viable cells and progressive motility and the other straw was used for determination of the DNA fragmentation index (DFI).

In frozen-thawed samples, sperm total and progressive motility were visually examined by a regularly trained and experienced technician using a phase-contrast microscope with a heated stage at 37 °C at 200x magnification (Olympus CH-II). The total cell count and the non-viable cell count were evaluated using a two-step procedure utilizing a NucleoCounter SP-100 according to the manufacturer's instructions. The counter functions on the principle of fluorescence microscopy and comprises a NucleoCounter instrument for analysis, a NucleoCassette for safe handling, lysis buffer (reagent S100) and stabilizing buffer (phosphate buffered saline, PBS). The NucleoCassette is a plastic cartridge pre-filled with fluorescent dye propidium iodide (PI) for staining cell nuclei. In the first phase, there is sperm processing for determining total cell count and the non-viable cell counts. In the first phase, the total cell count was determined by diluting an aliquot of the semen sample (50 µL) with 5 mL of reagent S100 which consequently results in lysis of the plasma membranes of viable cells which makes the nuclei susceptible for staining with PI. The semen sample was then loaded into the NucleoCassette and inserted into the NucleoCounter for analysis. In the second phase, another aliquot (50 µL) of the same semen sample was diluted with 5 mL PBS and loaded into the NucleoCassette and inserted into the NucleoCounter for analysis of the non-viable cells. The device is used to determine the non-viable cell count of samples due to the PI permeability of cell membranes of non-viable cells. The percentage of non-viable cells was determined as the ratio of the non-viable cell count to the total number of cells.

Values for sperm concentration measures as determined with use of the NucleoCounter were highly correlated (r = 0.85, P<0.001) with sperm concentration results obtained using the Bürker chamber. Values for viability of sperm as assessed using the NucleoCounter and flow cytometer were also correlated (r = 0.73; Morrell et al., 2010). The NucleoCounter SP-100 is accepted as a standard and is a convenient method with acceptable reproducibility (small coefficient of variation, CV) between experiments and operators to monitor total and viable cell counts (Shah et al., 2006; Morrell et al., 2010). In the laboratory where the research was conducted, there was a CV of 11.48% for within month and stallion repeated NucleoCounter SP-100 measurements. This result is consistent with those reported previously (Shah et al., 2006).

Use of the sperm chromatin structure assay (SCSA) allows for making an estimate of chromatin integrity in semen samples (Evenson et al., 2002). Initially, the semen sample was thawed for 30 seconds in a water bath at 37 °C and then diluted in TNE buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA, pH 7.4) at approximately 2×10^6 sperm/mL. There were 200 µL of this suspension and 400 µL acidic detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton-X 100, pH 1.2) used for mixing on a test tube shaker for 30 s, followed by addition of 1.2 mL staining solution (0.15 M NaCl, 0.037 M citric acid, 0.126 M Na2HPO4, 0.0011 M EDTA, pH 6.0) containing 6 µg/mL acridine orange (Polysciences, Warrington, PA, USA). After incubation for 3 min on ice, a flow cytometric analysis was performed on 10,000 cells using a flow cytometer (FACScan, Becton-Dickinson, Heidelberg, Germany) containing a 488 nm argon ion laser (15 mW) for excitation, a band pass 530/30 nm filter and a long pass 650 nm filter for identification of red and green fluorescence. Intact double-stranded DNA appeared fluorescent green, whereas denatured single-stranded DNA appeared fluorescent red. Sperm fractions with single and double-stranded DNA were used to calculate DFI.

Means, standard deviations, minimum and maximum values for semen variables from raw semen and frozen-thawed semen samples are presented in Table 1

3.3.3 Statistical analysis

A multivariate linear animal model with restricted maximum likelihood (REML) was used to estimate genetic parameters and EBVs for raw and frozen-thawed semen traits using data from Dataset I and II simultaneously. This model included the fixed effects of month and year of semen collection, type of horse breed registration, the age of stallions at the time of semen collection as covariates and a random permanent environmental effect among stallions as well as an additive genetic effect calculated based on the records of 271 stallions and their ancestors. At least 10 generations of known ancestors were included in the pedigree files. Variance and covariance parameters as well as the standard errors were estimated using the Variance Components Estimation (VCE 6) package, version 6.0.2 (Groeneveld et al., 2008). Parameterization of models was different for raw and frozen-thawed semen traits to allow different fixed effects and effect levels.

The model equation used for Dataset I was as follows:

$$Y_{ijklmnop} = \mu + Year_i + Month_j + b_1(Age)_k + b_2(Age)^2_k + b_3 \log(Age)_k + Stud_l + Reg_m + stallion_n + animal_o + e_{ijklmnop}$$

with $Y_{ijklmnop}$ = semen trait of the *ijklmnop*-th ejaculate including gel-free volume, sperm concentration, progressive motility, total number of sperm and total number of progressively motile sperm; μ = model constant; $Year_i$ = fixed effect of the year (i = 1-14); $Month_j$ = fixed effect of the month (j = 1-5; 1 = February - March, 2 = April, 3 = May, 4 = June, 5 = July - August); Age = age of the stallion in months at the time of semen collection; b_1, b_2, b_3 = linear, quadratic and logarithmic regression coefficients; $Stud_l$ = fixed effect of the national stud (l = 1-2; 1 = Celle, 2 = Warendorf); Reg_m = fixed effect of the horse breeding registration (l = 1-5; 1 = Hanoverian, 2 = Westphalian, 3= Holstein, 4 = Oldenburg, 5 = Rhinelander); $stallion_n$ = random permanent environmental effect of the stallion (n = 1-241); $animal_o$ = random additive genetic effect of the animal (o = 11,917) and $e_{ijklmnop}$ = random residual effect.

For frozen-thawed semen traits with use of the Dataset II model equation is as follows:

$$Y_{ijkmnop} = \mu + Year_i + Month_j + b_1(Age)_k + b_2(Age)^2_k + b_3 \log(Age)_k + Reg_m + stallion_n + animal_o + e_{ijkmnop}$$

with $Y_{ijkmnop}$ = frozen-thawed semen trait of the *ijkmnop*-th ejaculate including post-thawing motility, non-viable sperm and DFI; $Year_i$ = fixed effect of the year (i = 1-9); $Month_j$ = fixed effect of the month (j = 1-5; 1 = September to October, 2 = November, 3 = December, 4 = January, 5 = February); Age = age of the stallion in months at semen collection; b_1, b_2, b_3 = linear, quadratic and logarithmic regression coefficients; Reg_m = fixed effect of the horse breed registration (l = 1-3; 1 = Hanoverian, 2= Holstein, 3 = Oldenburg); $stallion_n$ = random permanent environmental effect of the stallion (n = 1-121); $animal_o$ = random additive genetic effect of the animal (o = 11,917) and $e_{ijkmnop}$ = random residual effect.

Heritabilities (h^2) for semen traits were estimated as $h^2 = \sigma^2_a/(\sigma^2_a + \sigma^2_p + \sigma^2_e)$, where σ^2_a = additive genetic variance for stallions; σ^2_p = permanent environmental variance among stallions; σ^2_e = residual variance. Genetic correlations among semen traits were determined as r_g = cov(a-1,a-2)/($\sigma_{a\text{-}1}$ x $\sigma_{a\text{-}2}$), where cov(a-1,a-2) = additive genetic covariance between semen trait 1 and 2, $\sigma_{a\text{-}1}$ and $\sigma_{a\text{-}2}$ = additive genetic standard deviations for semen trait 1 and 2. The EBVs were

estimated using a multivariate linear model utilizing PEST (Prediction and Estimation), version 4.2 (Groeneveld et al., 1990) and the (co-)variance component estimates from the REML analysis. The EBVs were standardized to a mean of 100 with a standard deviation of 20 using the respective stallions with semen records as reference. The EBVs of greater than 100 for semen traits indicate stallions transmitting a greater genetic component for the respective trait than the average of the recorded stallions. Reliabilities (r^2) of EBVs were calculated as follows: $r^2 = 1 - (PEV/\sigma^2_a)$ with PEV = predicted error variance and σ^2_a = additive genetic variance.

Stallion fertility was based on pregnancy rates per estrus at which there was AI using semen from Hanoverian stallions from the Lower Saxon National Stud Celle. The paternal component of the pregnancy rate per estrus was calculated from the additive genetic component of the stallion in the model for estimating breeding values (EBV for stallion fertility, EBV-PAT). The dataset and model used were previously described by Gottschalk et al. (2017). Briefly, recordings of pregnancy rates occurred during breeding seasons from 1997 to 2005 with 96,114 estrous cycles of 19,897 broodmares and 246 stallions used for AI. Heritability for the paternal component of the pregnancy rate per estrus was 1.1%. All EBVs for stallion fertility were standardized to a mean of 100 and a standard deviation of 20. The EBVs-PAT means of greater than 100 indicate conception rates that were greater than the population average. The mean reliabilities of the EBVs-PAT were 0.7.

Associations among EBVs for semen traits and EBV-PAT were determined for 106 stallions using correlation analysis with the procedure CORR of SAS, version 9.4 (Statistical Analysis System, SAS Institute, Cary, NC). Linear, quadratic and cubic regressions of EBVs for frozen-thawed semen traits were used for EBV-PAT to calculate the explained variance. With use of the general linear model analysis utilizing the GLM procedure of SAS, version 9.4, EBV-PAT were considered in three classes with Class 1 including stallions with a EBV-PAT of greater than 80 (n = 16 and on average 761 estrous cycles per stallion), Class 2 including stallions with a EBV-PAT of 80 to 120 (n = 67 and on average 603 estrous cycles per stallion) and Class 3 including stallions with a EBV-PAT of greater than 120 (n = 23 and on average 661 estrous cycles per stallion). Least-squares means (LSM) of EBVs for semen traits were tested for significance among the three classes of EBV-PAT.

3.4 Results

Heritabilities estimated for frozen-thawed semen traits of German Warmblood stallions were greatest for DFI (h^2 = 0.45) and lesser for non-viable sperm (h^2 = 0.11) and progressively motile sperm post-thawing (h^2 = 0.13; Table 2). Additive genetic correlations among raw semen traits and post-thaw semen motility were moderately positive for progressive motility (r_g = 0.39) and sperm concentration (r_g = 0.52) but negative for gel-free volume (r_g = -0.30). The DFI and progressive motility in raw semen were genetically negatively correlated (r_g = -0.79). For non-viable sperm, there were moderate negative additive genetic correlations for progressive motility (r_g = -0.45) and sperm concentration (r_g = -0.51) in raw semen. Additive genetic and residual correlations among progressively motile sperm post-thawing, DFI and non-viable sperm variables were close to zero.

The inter-stallion variance accounted for 28% to 71% of the total variance for frozen-thawed semen variables (Table 3). Estimated values for the permanent environmental variance were greatest for DFI (26%) and there were lesser values for progressively motile sperm post-thawing and non-viable sperm. The total variance among German Warmblood stallions was greatest for DFI (71%) and was 28% and 29% for non-viable sperm and progressively motile sperm post-thawing.

The EBVs for frozen-thawed semen traits ranged from 49 to 181 on a scale of 100 ± 20 for the 106 stallions with records (Table 4). Average reliabilities of EBVs were greatest for DFI (0.43) and was 0.28 for progressively motile sperm post-thawing and non-viable sperm for individual stallions with records.

The EBVs of progressively motile sperm post-thawing differed among classes of EBV-PAT (Table 5). The variance of EBV-PAT explained by the EBVs for progressively motile sperm post-thawing was 15.4%.

Correlations estimated among EBV-PAT and EBVs for semen traits were moderately positive and significant for progressively motile sperm post-thawing and DFI (Table 6). There was the greatest correlation for EBV-PAT with progressively motile sperm post-thawing (r = 0.38).

3.5 Discussion

In the present study, there was an estimation of the genetic parameters of post-thawed semen variables when data were collected during the non-breeding season and the genetic correlations with raw semen quality traits were calculated for German warmblood stallions. Due to repeated sampling of semen, permanent environmental and additive genetic effects of the stallions were estimable. To evaluate consequences of breeding with frozen semen, correlations among EBVs for semen traits and stallion fertility were reported for a subset of 106 stallions.

In the present study, there was a moderate to high heritability estimates for frozen-thawed semen traits as well as considerable inter-individual variance among stallions. These results in the present study are consistent with those from previous reports on heritabilities for raw semen quality traits.

In Shetland pony, Warmblood and Friesian stallions, heritability estimates ranged from a $h^2 = 0.13$ to $h^2 = 0.57$ (Van Eldik et al., 2006; Ducro et al., 2011; Labitzke et al., 2014; Gottschalk et al., 2016). Computer assisted sperm analysis (CASA) may provide a more objective analysis of sperm motility (Ball et al., 2003; Love, 2011) but due to the long-time span of the present study, CASA data were not available. The CASA data may have an effect on the outcome of a genetic analysis because differences in progressively motile sperm among stallions may not be accurate when using other procedures for this determination. Due to the large number of semen samples per stallion and considering there were only highly experienced technicians that conducted the procedures in the present study, a potential bias of genetic parameters is small.

Standard errors for heritabilities were slightly greater for frozen-thawed semen traits in comparison to raw semen traits in the present study but were still less than previously estimated for the Shetland Pony (Van Eldik et al., 2006), Friesian (Ducro et al., 2011) and Hanoverian (Labitzke et al., 2014) breeds. The large number of stallions in the present study with repeated records ensured the generally lesser standard errors as compared with most previous studies as well as allowing for the capacity to conduct a multivariate analysis of all data. The relatively lesser standard errors resulted in smaller confidence intervals and greater reliability of the estimates due to a smaller error variation and in addition, findings should be more representative for the population that was studied (Gottschalk et al., 2016). A possible bias due to preselection of stallions

for the present study can be precluded because all Hanoverian stallions were routinely located at the national stud in Celle were included in the present study for assessment of frozen-thawed semen variables. Excluding stallions with very poor semen quality that were less than the minimum requirements for AI may decrease the estimates of additive genetic and residual variances but not necessarily heritability estimates. Heritability estimates in the present study were in the same range as those for the Dutch Warmblood (Parlevliet et al., 1994), Shetland Pony (Van Eldik et al., 2006) and Friesian (Ducro et al., 2011) breeds. The data for these previous studies were from stallions that had a breeding soundness examination prior to studbook registration and these stallions were not selected for semen quality traits. In the present study, an average of 30 frozen-thawed semen samples was examined per stallion and this number is large enough to obtain repeatability values of greater than 80% (Barrier Battut et al., 2016). The additive genetic variances in the present study study are still large enough to allow for selective breeding of stallions for improving semen quality traits, particularly for cryopreservation through genetic selection of animals to produce future generations.

Heritability estimates and proportion of inter-stallion variance provided evidence about the important contribution of the stallion to post-thaw semen quality. The results of the present study are indicative of the effect of selective breeding to improve raw semen quality and freezing properties of semen in Warmblood stallions as previously reported for raw semen variables by Parlevliet et al. (1994), Van Eldik et al. (2006), Ducro et al. (2011), Labitzke et al. (2014) and Gottschalk et al. (2016). In addition, due to the significant genetic correlation of progressive motility in raw semen with post-thaw semen traits it is proposed that sperm progressive motility in raw semen is a possible indicator trait for stallions for which semen is stored using cryopreservation. Specifically, there is a genetic antagonism on progressive motility in raw semen with DFI after freezing in almost all stallions. Considering these results, it is proposed that greater emphasis be placed on progressive motility when making stallion selection decisions for breeding if semen is going to be cryopreserved. In contrast to progressive motility, there was virtually no genetic correlation with TNMS indicating this variable would not be useful for selecting stallions for improved sperm quality in semen samples stored using cryopreservation. Contrasting results were reported by Ducro et al. (2011) where it was suggested that consideration of the TNMS in

stallions selection decisions should occur due to the significant genetic correlations with sperm concentration, progressive motility, normal sperm morphology and frequency of abnormal acrosomes in raw semen samples.

Recording of semen traits on stallions routinely used for AI is important for breeding purposes. The more records per stallion available the more reliable the EBVs. Based on results in the present study, these factors also are important when considering frozen-thawed semen varibles. With frozen-thawed semen samples, there was the greatest correlations between progressively motile sperm and stallion fertility with these findings being consistent with previous results reported for raw semen (Barrier Battut et al., 2016; Gottschalk et al., 2017; Jasko et al., 1992; Love, 2011). These results are indicative of the importance of recording progressive motility in raw and post-thawed semen because correlated responses with freezing capacity of semen and stallion fertility may be expected.

When implementing breeding programs in horses, there should be consideration of EBVs for raw and frozen-thawed semen quality traits to allow for the option of breeders to improve semen quality and get a positively correlated selection response for stallion fertility. The EBVs for post-thaw semen traits appear to be worthwhile factors for consideration to enhance fertility when there is use of sperm that has been cryopreserved and thawed before using it for AI. Further study including evaluations of sperm variables that were assessed in the present study using the CASA seem to be worthwhile. In conclusion, continued selection for semen quality has a positive effect on the genetic potential for stallion fertility.

3.6 Conflict of interests

The authors declare that they have no competing interests.

3.7 Acknowledgements

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Table 1

Means ($\bar{x}$), standard deviation (SD), minimum (Min) and maximum (Max) for raw data of semen variables in raw and post-thawed semen from German Warmblood stallions

Semen variable	$\bar{x}$	SD	Min	Max
Raw semen variables (*n*=63,972)				
Gel-free volume (mL)	37.9	18.1	2	290
Sperm concentration ($x10^6$/mL)	213.1	88.9	1	695
Progressive motility (%)	60.8	9.8	1	95
Total number of sperm ($x10^9$)	7.3	3.0	0.6	36.0
Total number of progressively motile sperm ($x10^9$)	4.5	2.0	0.3	21.9
Post-thaw semen variables (*n*=3,681)				
Progressively motile sperm post-thawing (%)	32.97	9.75	1	70
DFI (%)	10.39	5.39	0.5	47.5
Non-viable sperm (%)	52.83	10.95	13	92

Raw semen traits: gel-free volume (mL); sperm concentration ($x10^6$/mL), progressive motility (%), TNS: total number of sperm ($x10^9$), TNMS: total number of progressively motile sperm ($x10^9$), post-thaw semen traits: progressively motile sperm post-thawing (%), DFI: DNA fragmentation index (%), non-viable sperm (%)

Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions

Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions

Table 2

Heritabilities (on the diagonal in bold), additive genetic (above the diagonal) and residual correlations (below the diagonal) with their standard errors for traits of raw and post-thaw semen estimated in a multivariate linear animal model for 271 German Warmblood stallions.

Semen variable	Gel-free volume	Sperm concentration	Progressive motility	TNS	TNMS	Progressively motile	DFI	Non-viable sperm
Gel-free volume	0.25 ± 0.02	-0.69 ± 0.06	-0.16 ± 0.08	0.54 ± 0.07	0.46 ± 0.07	-0.30 ± 0.07	-0.22 ± 0.08	0.36 ± 0.11
Sperm concentration	-0.37 ± 0.00	0.14 ± 0.03	0.11 ± 0.06	0.18 ± 0.07	0.24 ± 0.07	0.52 ± 0.14	0.31 ± 0.09	-0.51 ± 0.11
Progressive motility	-0.09 ± 0.00	0.02 ± 0.00	0.12 ± 0.01	-0.20 ± 0.08	0.16 ± 0.08	0.39 ± 0.08	-0.79 ± 0.05	-0.45 ± 0.13
TNS	0.55 ± 0.00	0.40 ± 0.00	-0.06 ± 0.00	0.14 ± 0.01	0.94 ± 0.01	0.03 ± 0.08	0.21 ± 0.05	-0.02 ± 0.06
TNMS	0.47 ± 0.00	0.37 ± 0.00	0.34 ± 0.00	0.90 ± 0.00	0.12 ± 0.01	0.16 ± 0.08	-0.05 ± 0.04	-0.19 ± 0.07
Progressively motile sperm post-thawing						0.13 ± 0.04	-0.29 ± 0.14	-0.01 ± 0.18
DFI						-0.21 ± 0.04	0.45 ± 0.04	0.30 ± 0.13
Non-viable sperm						-0.33 ± 0.03	0.18 ± 0.03	0.11 ± 0.04

For abbreviations, see Table 1

Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions

Table 3

Proportion of the permanent environmental (PERM), additive genetic (ADD) and total variance among 121 stallions (STALLION) for the phenotypic variance of frozen-thawed semen traits (σ^2) estimated in a linear multivariate animal model for German Warmblood stallions.

Semen variable	PERM	ADD	STALLION	Trait variance (σ^2)
Progressively motile sperm post-thawing (%)	0.16	0.13	0.29	98.86
DFI	0.26	0.45	0.71	31.02
Non-viable sperm	0.17	0.11	0.28	114.16

For abbreviations, see Table 1

Table 4

Minimum (Min), maximum (Max) and range of estimated breeding values (EBVs) and the reliability (r^2) for frozen-thawed semen traits of 121 German Warmblood stallions

Semen variable	Min	Max	Range	r^2_{mean}	r^2_{Min}	r^2_{Max}
Progressively motile sperm post-thawing (%)	51	156	105	0.28	0.01	0.59
DFI	49	181	132	0.43	0.07	0.73
Non-viable sperm	52	155	103	0.28	0.02	0.56

For abbreviations, see Table 1

Table 5

LSMs and their SEs (±SE) for correlations of estimated breeding values (EBVs) of frozen-thawed semen traits with EBVs for stallion fertility (EBV-PAT) classes in 106 German Warmblood stallions

EBV of semen trait	EBV-PAT		
	< 80	80-120	>120
Progressively motile sperm post-thawing	90.3 ± 2.4[a]	102.9 ± 1.0[b]	103.0 ± 2.0[b]
DFI	107.1 ± 2.8	104.8 ± 1.1	100.7 ± 2.4
Non-viable sperm	100.5 ± 2.8	100.1 ± 1.1	100.6 ± 2.4

For abbreviations, see Table 1; Different letters indicate differences $P < 0.01$.

Table 6

Correlation coefficients (*r*) among estimated breeding values (EBVs) for frozen-thawed semen traits and EBVs for stallion fertility (EBV-PAT) and variance explained (%) through frozen-thawed semen variables on EBV-PAT for 106 German Warmblood stallions

EBV of semen variable	EBV-PAT		
	r	*P*-value	Variance explained (%)
Progressively motile sperm post-thawing	0.38	<0.0001	15.4
DFI	-0.17	0.0556	3.0
Non-viable sperm	-0.05	0.5991	1.8

For abbreviations, see Table 1

Chapter 4

General discussion

General discussion

In recent years artificial insemination (AI) has become the most used breeding method in equine reproduction, although insemination of frozen-thawed semen often yields in low pregnancy rates (KUISMA et al. 2006). LOVE et al. (2015) inseminated 196 donor mares in 496 estrus cycles, emphasizing that embryo recovery rates rise when motility, viability, DNA quality, normal morphology concentration and total number of sperm increased in the insemination dose. AI reveals highest reproductive efficiency and genetic progress. Most varieties in the semen traits evaluated in equine semen were explained by specific stallions and environmental factors and diversity in semen quality in particular explains over 80% of the variation in fertility (JASKO et al. 1991; GOTTSCHALK et al. 2016b). Therefore, analyses have to be made in terms of influencing factors on semen quality in stallions used for AI. Hence, a great dataset of reports on fresh and frozen-thawed semen were analysed in the current study.

Significant interaction stallion by month and age by month was observed for most of the semen traits analysed. The seasonal differences observed in gel-free volume, sperm concentration, progressive motility, total number of sperm (TNS), total number of progressively motile sperm (TNMS) in fresh semen and DNA fragmentation index (DFI) and non-viable sperm in frozen-thawed semen, were consistent with previous studies (JANETT et al. 2003b; GOTTSCHALK et al. 2016a; KUHL et al. 2016; LABITZKE et al. 2014; AURICH 2016; PICKETT et al. 1976; MAGISTRINI et al. 1987; JASKO et al. 1991; GAMBOA et al. 2010; WRENCH et al. 2010). In nine stallions from the Agricultural School of Coimbra in Portugal (GAMBOA et al. 2010), the lowest percentage of progressive motile sperm was observed from May to July, and from June to August in Franches–Montagnes stallions (JANETT et al. 2003b), as well as in a study where ejaculates were collected in December and May (BLOTTNER et al. 2001). This is consistent with the least-squares mean (LSM) for progressive motility in fresh semen seen in our study, where the values were lower in season than in autumn and winter, but contrasts with KUHL et al. (2016) who found highest values (77%) in summer (Jun-Aug), or no seasonal differences whatever were seen (WRENCH et al. 2010). To date, it is not entirely clear, which factor is influential, but: changes in ambient temperature, seminal plasma volume, the composition of the sperm plasma membrane and also different testicular- and parenchymal weights and different seminiferous tubular volumes are discussed (JASKO et al. 1991; JANETT et al. 2003a, b; AURICH 2008, 2016; PICKETT et al. 1975, 1976; AURICH et al. 1996; PENA

et al. 2011). Further changes in the routine of the stallions, such as stall management, frequency of semen collection and shows at the weekends, or simply different types of horse breed registration may explain the differences in the various studies (DOWSETT and KNOTT 1996; GOTTSCHALK et al. 2016a; JANETT et al. 2003a; COLENBRANDER et al. 1992). In our study the data do not agree with previous studies, where higher (JANETT et al. 2003a, b; MAGISTRINI et al. 1987) or lower (KUHL et al. 2016) post-thawing motility was seen when collecting semen in autumn or winter, as the LSM for motility after thawing were not significantly different between the month of semen collection. In the current study LSM for sperm concentration were least in spring/summer which matches experiments made by JANETT et al. (2003a) performed on 10 warmblood stallions from the National Stud Farm in Avenches. Increasing LSM for TNS and TNMS were observed in spring/summer, which is consistent with the data reported by PICKETT et al. (1976), JASKO et al. (1991) and JANETT et al. (2003a) but in contrasts with GAMBOA et al. (2010) and MAGISTRINI et al. (1987), and may correlate with the increasing day length and light intensity (HARRIS et al. 1982; JOHNSON and THOMPSON 1983; HOFFMANN and LANDECK 1999; GERLACH and AURICH 2000). The LSM for gel-free volume differ across all studies. In our studies, LSM decreased in breeding season, in contrast to JANETT et al. (2003a) and MAGISTRINI et al. (1987), who observed increasing volumes in summer, and JASKO et al. (1991) who even found peak volumes in July/March. Our study contained stallions from seven breeds and thus specific breed effects should be cancelled out for seasonal LSM. Nevertheless, the specific effect of the month where semen collection took place on age should be noted and mentioned especially for breeders, as stallions aged 2-6 years showed much lower semen quality in autumn and winter compared to spring/summer. Consequently, a larger proportion of younger stallions may not be suited to semen collection outside the breeding season. Stallions under 6 years in any case had lower values in these semen traits, but the significant age class effects for sperm concentration, TNS, TNMS and post-thaw motility were also caused by the youngest group of stallions aged 2-6 years. PICKETT et al. (1989) already discussed, if the influence of season and age on testes size could be the reason for these findings. Our findings are consistent with other studies (AURICH et al. 2003; GOTTSCHALK et al. 2016a; AMANN and PICKETT 1987), in which increasing semen quality was documented for stallions aged over 5 years, but in contrast to KUHL et al. (2016) who mentioned that the highest percentage (75%) of acceptable ejaculates

were found in stallions aged from 2-4 years. A further study on 168 stallions representing 9 breeds, evaluated that semen quality traits had lowest values in fresh semen like gel-free volume, sperm concentration and TNS in stallions aged under 3 years, and greatest values for dead spermatozoa (DOWSETT and KNOTT 1996). Our findings are similar, as we estimated higher percentages of non-viable sperm in stallions aged from 2-6 years, but in contrast to low percentages for the DFI. This indicates, that younger stallions react more on environmental influences. Moreover, breeders should consider that post-thaw semen traits such as DFI and non-viable sperm remained stable in older stallions. Hence, stallions aged over 13-18 years can still be used for breeding purposes and even their semen can still be cryopreserved. In contrast to these findings, DOWSETT and KNOTT (1996) recommend that semen from stallions over the age of 14 year should only be utilized if semen quality is examined carefully and AURICH et al. (2003) stated that semen abnormalities raises when draught horse stallions are aged over 10 years and testosterone is less produced in stallions aged over 9 years. A breed effect on semen traits like the total number of sperm, gel-free volumes and sperm motility, can also be seen when taking ponies, Shetland ponies, miniature ponies, warmblood, light and draught horse breeds into account (DOWSETT and KNOTT 1996; DOWSETT and PATTIE 1982; PACCAMONTI et al. 1999; AURICH et al. 2003; TORRES-BOGGINO et al. 1995; STOUT and COLENBRANDER 2011; GOTTSCHALK et al. 2016a). In the current study the significant influences of breed on sperm concentration, TNS and TNMS were caused by greatest values for Hanoverian stallions and least values for Arabian and Quarter Horse stallions. Moreover, TORRES-BOGGINO et al. (1995) examined four draft stallions, where no ejaculate reached an acceptable percentage in post-thaw semen traits like spermatozoan motility. GOTTSCHALK et al. (2016a) mentioned similar trends in fresh semen traits.

Furthermore, EVENSON and JOST (2000) stated that no in vitro fertilization ended in pregnancy when mares were inseminated with an ejaculate containing > 27% sperm with damaged DNA. In our study, thoroughbred stallions had the highest LSM for DNA damaged semen, whereas Oldenburg and KWPN stallions had the lowest LSM. Further studies should be undertaken to find more reasons for the damaged DNA in the semen of thoroughbred stallions and therefore on the one hand the questionable suitability for cryopreservation and on the other hand reduced fertility as described by LOVE and KENNEY (1998). DOWSETT and KNOTT (1996) found the lowest percentages of dead sperm and high percentages for sperm

concentration in Arabian stallions. This is in contrast to the current study, where the LSM of non-viable sperm was greatest in Arabian stallions and sperm concentration showed a low LSM value, but not significantly different from the other horse breeds examined. In warmblood stallions JANETT et al. (2003a) found a membrane integrity of 57.6%, which is similar to our findings, where the percentages of non-viable sperm ranged from 52% to 55%.

Nevertheless, the most important impact on the variation in fresh and frozen-thawed semen resulted from the random stallion effect, which was already outlined in previous studies where high inter-individual variability was seen for example in warmblood horses (37%-85%) or in a French study, including Thoroughbred, French trotter, Breton and Selle Francaise stallions (37% to 69%) (ROUSSET et al. 1986; GOTTSCHALK et al. 2016a; PATTIE and DOWSETT 1982; BARRIER BATTUT et al. 2016; LABITZKE et al. 2014). PICKETT et al. stated already in 1970 that the TNS and the gel-free volume is dependent on the individual stallion. The relative proportion of the variance due to the stallion from seasonal interaction was much lower (2%-7%) than the stallion variance (27%-71%). This small interaction value may be indicative of the fact that stallions with an ordinarily high semen quality during the season also perform well outside of the breeding season, which is consistent with AURICH (2016) and MAGISTRINI et al. (1987). The suitability of the individual stallion, and being a „good“ freezer is more interesting than collecting the semen in or outside of the breeding season. A significant breed and stallion effect on the variation in fresh and frozen-thawed semen traits and a significant month by age class interaction for young stallions below an age of 7, became obvious.

To estimate heritabilities and additive-genetic correlations of raw and post-thawing semen variables, this study additionally tried to find genetic parameters of frozen semen quality traits in breeding stallions. Hence, repeated records of raw and frozen-thawed semen of a large number of breeding stallions were taken. Moreover, a multivariate estimation of additive-genetic and permanent environmental variances among stallions as well as estimated breeding values for all semen traits were used. All Hanoverian stallions involved in this study were routinely employed at the national stud in Celle. Excluding stallions having very poor semen qualities may lower the estimates of additive genetic and residual variances but not automatically heritabilities. PARLEVLIET et al. (1994) underlined the correlation of semen quality and quantity in semi-siblings and recommended that the data showed a tendency to

heritability of semen quality. Heritability estimates in previous studies ranged for gel-free volumes from h^2=0.16 to h^2=0.57 and for progressive motility from h^2=0.20 to h^2=0.46 in Shetland pony, warmblood and Friesian stallions (VAN ELDIK et al. 2006; DUCRO et al. 2011; LABITZKE et al. 2014). This matches our findings for gel-free volume (h^2=0.25), but not for progressive motility in raw semen (h^2=0.12). High heritability was seen for the DFI (h^2=0.45) which makes it reasonable to find semen traits in raw semen, that are genetically correlated. Consequently, the significant genetic correlation of progressive motility in raw semen with post-thawing semen traits could be used as an indicator for stallions for which semen is stored using cryopreservation. We propose that an emphasis should be placed on the impact of breeding measures to improve semen quality (VAN ELDIK et al. 2006; DUCRO et al. 2011; LABITZKE et al. 2014; GOTTSCHALK et al. 2016b), especially when selection decisions is made on the base of semen which is going to be cryopreserved. In contrast to DUCRO et al. (2011), who found high genetic correlations for TNMS with sperm concentration, progressive motility and normal sperm morphology, our study found almost no genetic correlation. Hence, we would not propose using this particular semen variable.

To inform breeders on the semen quality of stallions, EBVs should be published for all semen traits, also post-thawing. Therefore, it is important to record as much semen traits as possible of stallions, as these EBVs become even more reliable, the more records per stallion are available. In this study the relationship among EBVs for post-thawing semen traits and EBV-PAT in a sample of 106 German warmblood stallions were reported. LSM of EBV for progressive motility post-thawing showed highest values for stallions with EBV-PAT greater than 120. As also seen in previous studies regarding motility and fertility in raw semen, where a significant positive correlation of 0.31 for the progressive motility in raw semen and EBV-PAT was seen (BARRIER BATTUT et al. 2016; GOTTSCHALK et al. 2017; JASKO et al. 1992; LOVE 2011), the current study revealed the greatest correlation in frozen-thawed progressive motility and stallion fertility. Hence, stallions with good EBVs for progressive motility in raw semen may be most suited when freezing semen for storage and later thawing it for AI. Breeding programs should consider raw and frozen-thawed semen quality traits, giving breeders the chance to improve semen quality and in turn, stallion fertility.

In conclusion, our study found that breed and stallion effects cause raw and frozen-thawed semen traits to vary significantly. For breeding purposes, younger stallions do not appear to be as well suited as older stallions, as indicated by a significant month by age class interaction. Further, it becomes clear that DNA intactness in the semen is attributable more to the stallion, while the membrane integrity is influenced more by the environment. Lastly, EBVs for post-thaw semen traits appear to be a helpful tool with respect to semen quality and therefore freezing abilities, stallion fertility and selection on semen quality has the potential to raise genetic progress in stallion fertility.

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Chapter 5

Summary

Summary

Theresa Greiser (2019)

Analysis of breed effects and genetic parameters of semen quality traits for frozen-thawed semen in stallions

Despite improvements in recent years in the techniques of freezing semen for reproduction purposes, a considerable amount of stallions' semen is still not suitable for providing frozen-thawed semen that fulfils the minimum quality requirements. Semen quality traits vary considerably between horse breeds, month and age of the stallions under similar environmental conditions. Therefore, the objectives of our study were to analyse the systematic effects on the quality traits of semen (fresh and frozen-thawed) collected in the breeding and non-breeding season.

5.1 Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses

The objectives of the present study were to analyze systematic effects on semen quality traits from fresh and frozen-thawed semen collected in and outside season. A total of 4,681 reports on semen traits of 121 stallions representing Arabian, Thoroughbred, Quarter Horse and four warmblood breeds used for artificial insemination at the Lower Saxon National stud Celle were edited for analysis of gel-free volume, sperm concentration, total number of sperm and total number of motile sperm in fresh semen and in frozen-thawed semen progressive motility, DNA fragmentation index and non-viable sperm. Month, year, age, breed and stallions' effects were analyzed with a linear mixed model procedure. Breed differences were significant for sperm concentration, total number of sperm, total motile sperm number and DNA fragmentation index. Hanoverian stallions showed significant higher least squares means for sperm concentration, total number of sperm and total motile sperm number in fresh semen, whereas Thoroughbred had significant higher least squares means for DNA fragmentation index. Stallions with an age of 2-6 years had significant lower least squares means in sperm concentration, total number of sperm and total motile sperm number and progressive motile

sperm post-thawing than the other age groups. Month was significant for all semen traits but progressive motile sperm post-thawing. Month by age class interaction showed a significant influence. Inter-stallion variance accounted for 27-71% and the two-way stallion by month interaction for 2-7% of the total variance of semen quality traits. The largest proportion of inter-individual variance among stallions was explained by the DNA fragmentation index.

5.2 Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions

Objectives of the present study were to estimate genetic parameters for frozen-thawed semen traits of 271 fertile German Warmblood stallions and genetic correlations with raw semen quality traits. Semen samples were collected from stallions utilized for semen collection and artificial insemination (AI) on the Lower Saxon National Stud Celle and the North Rhine-Westphalian National Stud Warendorf. Semen quality variables were analyzed in 63,972 raw (gel-free volume, concentration, progressive motility, number of sperm) and 3,681 frozen-thawed samples (motility, DNA fragmentation index (DFI), non-viable sperm). A multivariate linear animal model was used to estimate additive genetic and permanent environmental variances among stallions as well as estimated breeding values (EBVs) for all semen traits. Heritability estimates were greatest for DFI (h^2 = 0.45) and least for non-viable sperm counts (h^2 = 0.11). Additive genetic correlations between progressive sperm motility in raw semen and DFI (r_g = -0.79) as well as non-viable sperm (r_g = -0.45) were negative. The EBVs for frozen-thawed semen traits ranged from 49 to 181 with mean reliabilities of 0.28 to 0.43. The EBVs for progressively motile sperm post-thawing and DFI were the most highly correlated traits with EBVs for stallion fertility (r = 0.38 and r = -0.17). Stallions with relatively greater EBVs for progressive motility in raw semen may be most suitable when freezing semen for storage and subsequently thawing it for AI. Using EBVs for semen traits in selection of stallions to AI mares appears as an option for genetic improvement to enhance fertility after AI.

Chapter 6

Zusammenfassung

Zusammenfassung

Theresa Greiser (2019)

Analyse der rassespezifischen Effekte und genetischen Parameter von Qualitätsmerkmalen in Tiefgefriersamen bei Deckhengsten

Obwohl sich die Techniken der Herstellung von Tiefgefriersamen in den letzten Jahren stetig weiterentwickelt haben, erfüllt nach wie vor eine große Anzahl von Pferdeejakulaten nicht die qualitativen Mindestanforderungen von Tiefgefriersamen. Unter ähnlichen Umweltbedingungen divergiert die Qualität des Frisch- und Tiefgefriersamens sehr stark zwischen den einzelnen Rassen, dem Monat der Samengewinnung und dem Alter des Hengstes. Ziel dieser Studie war es zu ermitteln, in welchem Umfang Rasseeffekte und genetische Parameter die Qualität von Tiefgefriersamen beeinflussen und mögliche Ursachen für die Varianz von Tiefgefrierspermamerkmalen zu finden. Eine Einschätzung über den genetischen Einfluss auf die Qualität des Tiefgefriersamens lieferten die additiv-genetische Varianz, die permanente Umweltvarianz, die Heritabilitäten und die genetische Korrelation von Frisch- und Tiefgefriersamenparametern. Der Einsatz von geschätzten Zuchtwerten ermöglicht die Selektion von Deckhengsten auf Samen, der eine gute Einfriertauglichkeit aufweist.

6.1 Analyse der rasse- und hengstspezifischen Effekte auf den Tiefgefriersamen von Warmblut-, Vollblut-, und Quarter Horse Pferden

In der vorliegenden Studie wurden die systematischen Effekte auf die Parameter der Spermienqualität von Frisch- und Tiefgefriersamen untersucht, welche sowohl innerhalb als auch außerhalb der Decksaison abgenommen wurden. Insgesamt wurden von 121 Hengsten 4.681 Samenprotokolle ausgewertet. In die Studie gingen Pferde der Rassen arabisches und englisches Vollblut, Quarter Horse sowie vier Warmblutrassen mit ein, wobei alle Hengste ausschließlich bei der künstlichen Besamung des Niedersächsischen Landgestüts Celle eingesetzt wurden. Folgende Samenparameter wurden untersucht: Volumen, Spermiendichte, Gesamtspermienzahl (GSZ) und Gesamtanzahl vorwärts-beweglicher Spermien im Frischsamen sowie die Vorwärtsmotilität nach dem Auftauen, der DNA-Fragmentationsindex (DFI) und die Anzahl membrandefekter Spermien im Tiefgefriersamen, innerhalb und

außerhalb der Decksaison. Die systematischen Effekte des Monats und des Jahres der Samengewinnung, der Altersklasse des Hengstes, des Zuchtgebiets sowie der zufällige Effekt des Hengstes wurden mittels gemischter Modelle auf Signifikanz geprüft. Rasseunterschiede waren signifikant für die Samenparameter Spermienkonzentration, GSZ, Gesamtmotilität und DFI. Vor allem Vollblüter zeigten signifikant höhere least-square Mittelwerte für den DFI-Wert, wobei Hannoveraner Hengste höhere least-square Mittelwerte für die Spermienkonzentration, die GSZ und die Gesamtmotilität im Frischsamen aufwiesen. Die Gruppe der 2-6- jährigen Hengste hatte signifikant niedrigere least-square Mittelwerte als die anderen Altersgruppen, für die Spermienkonzentration, GSZ und die Gesamtmotilität sowie die progressive Auftaumotilität. Mit Ausnahme der Auftaumotilität zeigten alle Samenqualitätsmerkmale saisonale Schwankungen. Der Interaktionseffekt Monat der Samengewinnung und das Alter des Hengstes hatte einen signifikanten Einfluss. Im Alter von 2-6 Jahren zeigten die Hengste im Frühling und Winter eine schlechtere Samenqualität als im Sommer und Herbst. Dies lässt darauf schließen, dass diese Altersgruppe weniger gut für eine Samenabnahme außerhalb der Saison geeignet ist. Die Hengste im Alter von 2-6 Jahren hatten außerdem einen hohen Prozentsatz nicht-lebender Spermien, wiesen jedoch die besten DFI-Werte auf. Die Werte dieser beiden Qualitätsmerkmale blieben auch im weiteren Verlauf des Lebens der Hengste stabil. Der zufällige Hengsteffekt hatte einen Anteil von 27-71% an der Gesamtvarianz, wobei der größte Hengsteffekt auf den DFI-Wert zurückzuführen war. Die Ergebnisse zeigen, dass überwiegend der Hengst für eine intakte DNA in den Spermien verantwortlich ist, die Membranintegrität jedoch eher Umwelteinflüssen unterliegt. Generell lässt sich sagen, dass vor allem der Hengst die Spermienqualität bestimmt und dass signifikante Interaktionen zwischen dem Hengst und der Umwelt, Schwankungen in den ausgewerteten Spermamerkmalen bewirken.

6.2 Genetische Analysen und geschätzte Zuchtwerte von Frisch- und Tiefgefriersamen in deutschen Warmbluthengsten

Essentiell für eine erfolgreiche Pferdezucht und gezielte Selektion, sind eine gute Samenqualität, eine gute Auftauqualität und eine gute Befruchtungsfähigkeit der Spermien. Management, Umweltfaktoren, genetische Einflüsse und der individuelle Hengsteffekt führen zu einer signifikanten Varianz in der Qualität der Tiefgefriersamen. Deshalb war ein weiteres

Ziel unserer Arbeit, genetische Parameter für die Merkmale des Tiefgefriersamens (oder der Tiefgefrierportion) von fruchtbaren Warmbluthengsten zu erheben und genetische Korrelationen zu Frischsamenparametern zu finden. Insgesamt wurden 63.972 Frischsamenprotokolle und 3.681 Tiefgefrierprotokolle von 271 Hengsten aus dem Niedersächsischen Landgestüt Celle und dem Nordrhein-Westfälischen Landgestüt Warendorf ausgewertet. Ein multivariantes lineares Tiermodell wurde eingesetzt, um additiv-genetische Effekte und permanente Umweltvarianzen zwischen Hengsten und EBVs der Spermamerkmale zu schätzen. Die Spannweite der Relativzuchtwerte reichte von 49 bis 181 und deren mittlere Zuverlässigkeit von 0,28 bis 0,49. Die Heritabilitätsschätzung ergab hohe Werte für den DFI (h^2=0,45) und niedrige Schätzwerte für die Anzahl membrandefekter Spermien pro Ejakulat (h^2 = 0,11). Sowohl zwischen der progressiven Motilität des Frischsamens und dem DFI-Wert (r_g = -0,79), als auch den membrandefekten Spermien (r_g = -0,45) bestand eine negative additiv-genetische Korrelation. Die Spermienkonzentration im Frischsamen zeigte eine hohe additiv-genetische Korrelation, sowohl mit der progressiven Auftaumotilität, als auch mit dem DFI.

Schlussendlich wurde der Zusammenhang zwischen den Zuchtwerten (EBVs) für Tiefgefriersamen und den Zuchtwerten für die paternale Komponente der Trächtigkeitsrate pro Rossezyklus (EBV-PAT) bei 106 Hengsten untersucht. Zuchtwerte werden genauer, je mehr Samenprotokolle pro Hengst zur Verfügung stehen und ausgewertet werden können. Am höchsten korrelierten die Zuchtwerte für die progressive Vorwärtsbeweglichkeit im Tiefgefriersamen und für den DFI-Wert mit EBV-PAT (r = 0,38 und r = -0,17), wobei der Zuchtwert für die membrandefekten Spermien nur wenig korrelierte (r= -0,05). Zusammenfassend lässt sich sagen, dass Hengste mit einem hohen Zuchtwert für die progressive Motilität im Frischsamen eine gute Voraussetzung für die Herstellung von Tiefgefriersamen aufweisen. Die Verwendung von Zuchtwerten stellt eine Option der Selektion auf Hengste mit guter Samenqualität nach dem Einfrierprozess dar.

Chapter 7

List of publications

List of publications

Theresa Greiser, Harald Sieme, Gunilla Martinsson, Ottmar Distl (2019): Breed and stallion effects on frozen-thawed semen in Warmblood, Light and Quarter horses. Theriogenology Sep 19;142:8-14 (doi: 10.1016/j.theriogenology.2019.09.033)

Theresa Greiser, Harald Sieme, Gunilla Martinsson, Ottmar Distl (2019): Genetic parameters and estimated breeding values for traits of raw and frozen-thawed semen in German Warmblood stallions. Journal of Animal Reproduction Science Nov;210:106195 (doi: 10.1016/j.anireprosci.2019.106194. Epub 2019 Sep 23)

Chapter 8

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