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

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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 3681 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.

1. 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%

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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 100–110 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.

2. Materials and methods

2.1. 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 3681 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 h 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.

2.2. 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 min at 600 × *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 × 10^6 cells/mL with INRA-82. The INRA-82, cryoprotectant and egg yolk were then added to the semen, resulting in 200 × 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 s 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 200× 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 s 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 Na_2HPO_4, 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.

Table 1
Means ($\bar{x}$), standard deviation (SD), minimum (Min) and maximum (Max) for raw data for 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-thawed 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 (%).

2.3. 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, 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 $Y_{ijklmnop}$ = the 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 type of 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 $h^2 = \sigma_a^2/(\sigma_a^2 + \sigma_p^2 + \sigma_e^2)$, where σ_a^2 = additive genetic variance for stallions; σ_p^2 = permanent environmental variance among stallions; and σ_e^2 = 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: $r^2 = 1 - (PEV/\sigma_a^2)$ with PEV = predicted error variance and σ_a^2 = 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 80–120 (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. 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%–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).

4. 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 were 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

Table 2
Heritabilities (on the diagonal in bold), additive genetic (above the diagonal) and residual correlations (below the diagonal) with the standard errors for traits of raw and post-thawed semen estimated using a multivariate linear animal model for 271 German Warmblood stallions.

Semen variable	Gel-free volume	Sperm concentration	Progressive motility	TNS	TNMS	Progressively motile sperm post-thawing (% post-thawing)	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.

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) for 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 of 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.

(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 correlation 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 studies 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.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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