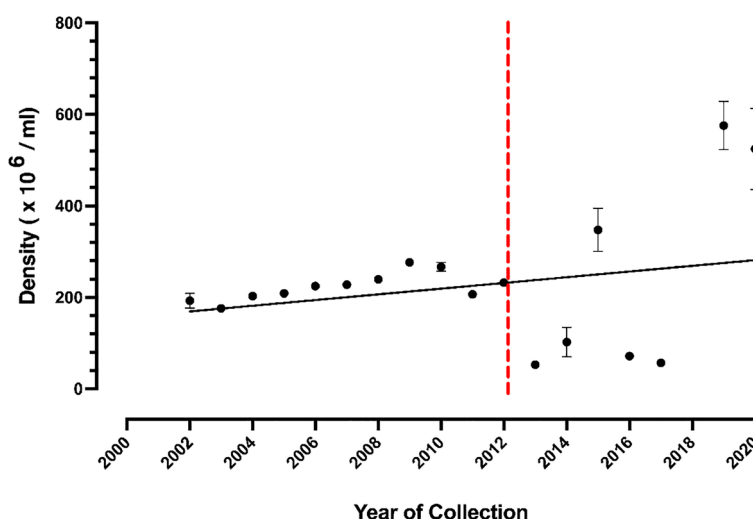## Supplementary Data: Sperm Concentration

We provide sperm concentration as supplementary data for readers' interest only. The method for assessing sperm concentration changed during the period of the study from a SpermaCue (2001–2012) to a NucleoCounter SP-100 (2013–2020). We did not feel it appropriate to display these findings within the report due to a change in assessment method. The NucleoCounter is a fluorescence-based automated cell counter instrument, providing accurate, precise, and sensitive readings on semen concentration [1]. The SpermaCue is a photometric device used to determine sperm concentration. In bulls, research has found no significant difference in sperm concentration measurements between photometric devices and other instruments [2]; however, research in stallions suggests that concentration readings by a SpermaCue are less accurate than those read by a NucleoCounter [3]. This inconsistency does not provide confidence in presenting the results, especially as n numbers, which can be viewed in **Supplementary Table 1**, were much lower when the method was changed in 2012.

The dataset for concentration, analyzed via the SpermaCue, indicated an increase of 94.77×10^6/mL between 2002 and 2012. From 2013, concentration was analyzed via a NucleoCounter, although this parameter was not as routinely assessed resulting in lower sample numbers. Variability in concentration post-2013 was observed. Data should be considered preliminary; however, significant differences in this parameter were reported for years of collection (REML: $p < 0.001$).

Supplementary Table 1: Overview of the number of stallions and subsequent ejaculates for semen concentration, per year.

	Concentration				
Year of collection	2001	2002	2003	2004	2005
Stallion/sample number	*	2/14	27/189	42/403	56/403
Year of collection	2006	2007	2008	2009	2010
Stallion/sample number	82/665	89/662	93/755	74/527	54/381
Year of collection	2011	2012	2013	2014	2015
Stallion/sample number	81/827	35/296	1/4	3/8	5/24
Year of collection	2016	2017	2018	2019	2020
Stallion/sample number	2/5	1/11	*	2/7	2/4

* Denotes where data was not available.

Supplementary Figure 1: Time trends in mean (± SEM) sperm concentration between 2001 and 2020. The red line at 2012 represents the shift in the analysis method (SpermaCue to NucleoCounter) and the point at which data should be treated as preliminary due to lower sample sizes. The black line denotes the regression slope (simple linear) of predicted. Each point represents the mean predicted value for that year. Error bars = ± 1 SEM. Graphs produced on GraphPad Prism version 9.0, GraphPad Software, California, CA, USA. The equation $y = mx + c$ determined overall trends; $y = 10.53X - 20862$.

References

[1] Brito LFC, Althouse GC, Aurich C, Chenoweth PJ, Eilts BE, Love CC, et al. Andrology Laboratory Review: Evaluation of sperm concentration. Theriogenology 2016;85:1507–27. https://doi.org/10.1016/j.theriogenology.2016.01.002.

[2] Atiq N, Ullah N, Andrabi SMH, Akhter S. Comparison of photometer with improved neubauer hemocytometer and makler counting chamber for sperm concentration measurement in cattle. Pakistan Veterinary Journal 2011;31:83–4.

[3] Comerford KL. Validation of a comercially available fluorescence-based instrument to evaluate stallion spermatozoal concentration and comparison to photometric systems. Doctoral Dissertation. Texas A&M University, 2009.