Associations between dairy cow inter-service interval and probability of conception

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Abstract
Recent research has indicated that the interval between inseminations in modern dairy cattle is often longer than the commonly accepted cycle length of 18-24 days. This study analysed 257,396 inseminations in 75,745 cows from 312 herds in England and Wales. The interval between subsequent inseminations in the same cow in the same lactation (inter-service interval, ISI) were calculated and inseminations categorised as successful or unsuccessful depending on whether there was a corresponding calving event. Conception risk was calculated for each individual ISI between 16 and 28 days. A random effects logistic regression model was fitted to the data with pregnancy as the outcome variable and ISI (in days) included in the model as a categorical variable. The modal ISI was 22 days and the peak conception risk was 44% for ISIs of 21 days rising from 27% at 16 days. The logistic regression model revealed significant associations of conception risk with ISI as well as 305 day milk yield, insemination number, parity and days in milk. Predicted conception risk was lower for ISIs of 16, 17 and 18 days and higher for ISIs of 20, 21 and 22 days compared to 25 day ISIs. A mixture model was specified to identify clusters in insemination frequency and conception risk for ISIs between 3 and 50 days. A "high conception risk, high insemination frequency" cluster was identified between 19 and 26 days which indicated that this time period was the true latent distribution for ISI with optimal reproductive outcome. These findings suggest that the period of increased numbers of inseminations around 22 days identified in existing work coincides with the period of increased probability of conception and therefore likely represents true return estrus events.

Keywords
Estrous cycle; inter-service interval; conception; pregnancy; dairy cow; mixture model

1. Introduction
Good reproductive performance in dairy herds is essential for efficient milk production. At cow level, good reproductive performance involves two main steps: 'submitting' cows for insemination in a timely manner, followed by conception and maintenance of pregnancy. Successfully detecting and inseminating cows in estrus is important as it is one of the most commonly used strategies to submit cows for artificial insemination, particularly in the UK. A good understanding of the physiology of the cow's estrous cycle has potential to improve both aspects. Better insight into expected interval between estrus events can help with accurate heat detection monitoring, and has potential to inform improved heat detection strategies on farm. Exploring associations between inter-service interval (ISI) and subsequent fertility may provide insights which help to improve conception risk (the probability of an insemination resulting in a pregnancy).

The average length of the estrous cycle of the cow is commonly quoted as 21 days, with a normal range of 18 to 24 days [1, 2]. A number of small-scale studies have identified mean estrous cycle lengths in excess of 21 days [3-5]. A much larger study evaluating the time between successive inseminations in the same cow in the same lactation, the inter-service interval (ISI), suggests that ISIs in excess of the normal range are frequent, with 22 days being the modal interval and that the traditional normal range of 18 to 24 days may poorly reflect the observed distribution of intervals in the population [6], a similar pattern has been identified in progesterone profiles [7]. Some of these longer ISIs could be the result of late embryonic death [8-10], this may impact on their chance of conception at the next insemination. It is not clear whether cows inseminated at these apparently abnormal intervals are as likely to conceive as those inseminated within the traditional expected range.

Estrous cycles in *Bos taurus* cattle typically consist of two or three follicular waves, with three wave cycles tending to result in an inter-ovulatory interval (IOI) longer than that of two wave cycles [11]. Some authors have hypothesised that due to the relatively increased time taken for development of the pre-ovulatory follicle in two wave cycles that these may be less fertile than three wave cycles. However there is no clear consensus with some studies finding reduced conception risk in two wave cycles [12] and others finding no difference [4, 13]. There are several potential mechanisms by which ISI could plausibly impact on conception risk as well as late embryonic death. Whilst follicular wave number, IOI and ISI may not necessarily correlate substantially, there are potential mechanisms for conception risk to vary with ISI. To the authors' knowledge, no studies exist evaluating the relationship between ISI and fertility on a large number of cows.

The aim of this study was to investigate the variation in conception risk by ISI in a large number of dairy cows in order to further our understanding of the possible effects of ISI on fertility, and to explore the expected estrous cycle length of a previously inseminated modern dairy cow. The authors hypothesise that conception risk will vary with ISI.

2. Materials and methods

2.1 Data collection, organisation and descriptive analysis

Management data was collected from farms that were clients of one of twenty veterinary surgeons in England and Wales as part of larger project [14, 15]. Records for 468 dairy farms considered to have good quality data were collated and converted to a standard format. An assessment of data quality was carried out to identify herds with accurate recording of calving and insemination. Measures used to screen for data quality included the proportion of calving events with corresponding inseminations and the proportion of inseminations leading to a pregnancy (see Hudson, Bradley [15] for more detail). This left data for 257,396 inseminations in 75,745 cows from 312 herds. The data were structured with a single insemination as a line of data, with the insemination, cow, parity and herd identity recorded. For each insemination, the date of insemination as well as the variables shown in Table 1 were recorded or calculated. The interval between subsequent inseminations in the same lactation from the same cow was calculated (inter-service interval, ISI); first inseminations were excluded. Inseminations were categorised as successfully resulting in a pregnancy when the cow was recorded as having calved 266 to 296 days after insemination, based on the expected range of gestation length for the common dairy breeds [16, 17]. Where two inseminations occurred within this range, the closest to 283 days gestation was categorised as successful. Plots were produced to examine the distribution of ISIs between 3 and 50 days to visualise the increased frequency of insemination occurring at the expected time of around 21-22 days. The distribution of the proportion of inseminations resulting in a pregnancy (conception risk) at each ISI between 3 and 50 days was also plotted using GraphPad Prism (Version 7.02, California, USA); with confidence intervals for risk at each ISI calculated using the modified Wilson interval [18]. From this distribution, a range of 16 to 28 days was selected for further exploration as it appeared to encompass the range of increased conception risk and insemination frequency at which most first returns would be expected, predominantly reflecting accurately detected estrus events. This final dataset, used to fit the regression model, contained 60,094 ISIs from 33,122 cows in 312 herds. Initial data restructuring and analysis was carried out in Microsoft Excel 2010 (Microsoft Corporation, Redmond, Washington).

2.2 Statistical modelling

A logistic multivariable regression model with the binary event of insemination success as the outcome variable was fitted to the data to account for the potential effect of other measurable confounding variables on conception risk. A multilevel model was used with a three level structure, with insemination as the bottom level and cow and herd-level random effects used to account for clustering at each level. The model was created by stepwise forward selection, with each variable being offered to the model and retained if the magnitude of its estimated coefficient was at least double the standard error of the estimate (equivalent to $p<0.05$). ISI was forced in to the model as a categorical variable, with each discrete one-day interval represented as a category. An ISI of 25 days was selected as the reference ISI as it represented a conception risk is the middle of the range. Where one or more categories of a variable were significant, all the categories for that variable were retained in the model. A list of all variables offered to the model is given in Table 1. Polynomial functions were tested for all continuous variables up to power three. Biologically plausible first order interaction effects were also tested, including ISI with milk yield, days in milk, parity and service number. All rejected variables were re-offered to the final model, and retained if they met the criteria described above.

The model took the conventional form

$$Pregnancy_{ijk} \sim Bernoulli\left(mean = \pi_{ijk}\right) \tag{1}$$

$$\ln\left(\frac{\pi_{ijk}}{1-\pi_{ijk}}\right) = \beta_0 + \boldsymbol{\beta x}_{ijk} + v_{0k} + u_{0jk} \tag{2}$$

$$v_{0k} \sim N(0, \sigma_{v0}^2) \tag{3}$$

$$u_{0jk} \sim N(0, \sigma_{u0}^2) \tag{4}$$

where Pregnancy_{ijk} is whether the i^{th} insemination in the j^{th} cow in the k^{th} herd resulted in a pregnancy; π_{ijk} is the fitted probability of Pregancy_{ijk}; β_0 is the regression intercept, $\boldsymbol{\beta}$ is the vector of coefficients for the vector of predictor variables $\mathbf{x}$; v_{0k} and u_{0jk} are the random effects to represent herd and cow level variation respectively.

The model was fitted using MLwiN version 2.35 [19]. Initial parameter estimates were calculated using iterative generalised least squares (IGLS) and final parameter estimates generated using a Bayesian approach, Markov Chain Monte Carlo (MCMC) with Gibbs sampling [20, 21]. A burn-in length of 5,000 iterations was used followed by a monitoring chain of 50,000 iterations. MCMC chains for the parameter estimates were visually checked to ensure adequate convergence. Model fit was checked by comparing observed and predicted number of pregnancies for each decile of risk, and ensuring that the observed number was within the 95% coverage interval of the predicted number for each decile.

To aid in interpretation, the model was used to predict the probability of achieving pregnancy for an insemination following an ISI of each length, whilst all other explanatory variables were fixed at their population mean values. These predictions were illustrated graphically by plotting predicted probability for each ISI as a bar chart, along with the corresponding 95% credible intervals.

An unsupervised latent class analysis was conducted to identify and define clusters of similar ISIs based on the daily frequency of inseminations and probability of conception. The analysis was carried out in R version 3.3.1 [22] using the *mclust* package [23, 24]. A finite Gaussian mixture model was fit to the insemination frequency and conception risk data using an expectation-maximisation algorithm, the optimum number of classifications was selected based on maximising the Bayesian Information Criterion (BIC) [23, 24]. The probability of each ISI falling within each classification was calculated and graphical plots used to illustrate the results. ISIs were assigned to the cluster that they had the highest probability of belonging to, the uncertainty was also illustrated.

3. Results

Figure 1 shows the distribution of ISIs between 3 and 50 days: a clear increase in the frequency of insemination occurring around 22 days is evident. Figure 1 also shows the observed conception risk across the same range. A peak in conception risk is apparent around multiples of the expected estrous cycle (around three and six weeks). The conception risk is low at 16 days (27%) and increases from 18 days up to a peak of 44% at 21 days, there is then a more gradual decline to a plateau of around 35% from 27 days ISI.

The final parameter estimates for the logistic regression model (with the outcome of a pregnancy (yes or no) resulting from an insemination) are shown in Table 2. The distribution of predicted mean conception risk by ISI is shown in Figure 2. Predicted conception risk was significantly lower for ISIs of 16, 17 and 18 days (mean predicted conception risk of 30, 27 and 32% respectively), whereas predicted mean conception risk for ISI of days 20, 21 and 22 were significantly higher than the reference interval of 25 days. There was no significant difference in predicted mean conception risk between ISIs of 21 or 22 days (47 and 46% respectively).

There were significant associations of milk yield, parity, days in milk, insemination number, month and year of insemination with conception risk. As milk yield increased conception risk tended to decrease, there was a trend for decreasing conception risk with increasing parity, with a significant decline in conception risk for parity five or greater. There was a quadratic association between the natural logarithm of days in milk and conception risk: conception risk tended to increase up to around 160 days in milk and then decline in later lactation. Third or later inseminations were less likely to result in pregnancy than second inseminations.

Three latent classes (clusters) were identified in the data, broadly corresponding to days with a lower chance of conception with lower number of inseminations (LL), a higher chance of conception and lower number of inseminations (HL), a higher chance of conception and a higher number of inseminations (HH). Figure 3 illustrates the estimated probability for each ISI (day) being a member of each latent class. Inseminations carried out between 19 and 26 days were most likely to fall in the HH classification. Figure 4 illustrates the clustering of each ISI by conception rates and number of inseminations on each day.

4. Discussion

These findings are useful in reconsidering estimates of normal or expected ISI. The lowest conception risk identified in the analyses were for inseminations given at ISIs of less than 18 days (Figure 1). This supports the common interpretation that these inseminations frequently represent inaccurately detected estrus events, with either the first or the second insemination of the interval having occurred when the cow was not truly in estrus [25, 26]. However, it is interesting to note that ISIs of 18 days (traditionally considered to be within the normal cycle range) had outcomes similar to the "short" intervals of 16-17 days and significantly lower than ISIs of 19 to 26 days. This finding is supported by the findings of the mixture model, where by taking in to account the number of inseminations and the average conception rate a, cluster of ISI with high insemination frequency and high conception rate was identified between 19 and 26 days. This would seem to support the idea that 18 day ISIs more often represent inaccurate heat detection than a normal cycle of this duration. It remains plausible that 18 day cycles predominantly represent true pairs of estrus events, but are associated with reduced fertility. The fact that 18 day ISIs are similar to shorter intervals, both in terms of frequency and outcome, would tend to support the theory that these are much more commonly the result a "false positive" detection of estrus. In this case, 18 to 24 days would not be an appropriate expected range by which to define a normal ISI in the modern dairy cow and 19-26 days may be more appropriate. This may have implications for the interpretation of some standard methods for assessing distribution of ISIs as a measure of heat detection performance, as well as for the generation of expected heat dates in on-farm management systems.

The conception risk appears to peak at multiples of the expected cycle length (i.e around 22 and 44 days, Figure 1); it is likely these intervals predominantly represent pairs of true estrus events. However, the conception risk remains markedly higher for intervals of 28-37 days (i.e. those between the approximate expected lengths of one and two cycles) than for intervals shorter than 18 days. This is likely to be due to different possible explanations for ISIs within this range. This is also reflected in the uncertainty of classifying these ISIs in the mixture model. In some cases, these intervals are likely to be the result of cows being served outside of true estrus events. In this case, a missed estrus and a wrongly identified estrus would need to occur in succession. As these could occur in either order, the second insemination of the interval may or may not be given when the cow is not in estrus, and a lower conception risk would therefore be expected. These intervals may also occur following late embryonic death [8-10], or as a result of pharmacological cycle manipulation following negative pregnancy diagnosis [27]. In either case, the second insemination is likely to relate to a true ovulation event, but would in some cases perhaps be expected to have a somewhat reduced risk of conception (depending on the cause of LED, or the type of cycle manipulation employed). Whilst the use of fixed time AI was not captured in the data, the authors would expect the use of these techniques (particularly within 30 days of a previous insemination) to be minimal in UK dairy herds at the time of data capture, this is supported by the clear increase in frequency of ISIs at the expected unmanipulated cycle lengths of three and six weeks. Any impact of cycle manipulation would also fall outside of the 16-28 day window of interest. Finally, it is also possible that these may represent normal physiological cycles of longer length than traditionally expected. The current results suggest that 26 day intervals are associated with similar outcomes to the 25 day reference category (Table 2), as are those at 23 and 24 days. Intervals of 27 or 28 days were associated with a significantly decreased odds of pregnancy, which is likely to reflect the combined impact of the possible explanations described above.

This study used ISI as a proxy for IOI; this was considered useful as it enabled a much larger number of herds to be studied than has previously been the case in this field. However, since this study excludes the first insemination of each lactation (where there is no ISI), it is likely that late embryonic death (as described above), will mean that the distribution of ISIs shown in Figure 1 is not likely to reflect accurately the distribution of IOIs in the same population. Without a marker for the presence of the embryo, or a group of "control" cattle that are not inseminated, it is impossible to know to what extent the results are affected by embryonic death and how much is due to estrous cycle length. However, recent work evaluating progesterone profiles of 1,418 estrous cycles in 1009 lactations has shown a markedly similar pattern in estrous cycle length [7] independent of whether cows were inseminated. This is also consistent with studies on smaller numbers of cattle where estrous cycles were found to be longer than expected [3]. This suggests that the increased frequency of cows being inseminated at intervals slightly greater than 24 days and the decreased frequency of inseminations carried out at intervals of 18 days from a previous serve is consistent with the cow return estrous cycle being longer than the traditionally accepted 18-24 days. This is important clinically, but also in research, where some studies may incorrectly categorise estrous cycles greater than 24 days as abnormal [7].

Individual studies commonly find significant differences between the durations of two- and three-wave cycles, the mean IOI in each group varies substantially between studies, with some reporting the mean duration of a three-wave cycle at around 22 days [13] whilst others found this to be greater than 24 days [12], this variation in reported inter-ovulatory interval is shown in supplementary material. Some authors have hypothesised that fertility will be reduced in cows undergoing two-wave (and therefore shorter) cycles, as a result of longer follicular maturation time [11]. The potential for substantial overlap between the distributions of two- and three-wave cycles makes it impossible to accurately separate cycles into follicular wave categories by ISI. This is especially difficult where data comes from a large number of herds and is confounded by factors such as embryonic death discussed previously. In this study, slightly shorter cycles (20-21 days) tended to be slightly more fertile rather than less (compared to 23 days); although speculative this would seem not to provide support for the two- versus three-wave hypothesis as an important factor in determining conception risk across a large, multi-herd dataset.

Other findings in this study are consistent with existing work, showing a negative association of conception risk with increasing parity and increasing number of inseminations as well as a non-linear association between days in milk and conception risk [28]. There was a statistically significant but small negative association between increasing milk production and conception risk. For categorical variables effect size can be seen in Table 2 using the absolute predicted risk, for example month effects were very small as the absolute risk is very similar across months. Care needs to be taken when interpreting these associations and there are many confounding factors which cannot be measured or were not available to include in the model [29]. There also appeared to be a seasonal effect, with conception risks declining in the summer. This is consistent with the likely effects of heat stress or nutritional changes during the summer [30].

The data collected in this study represent a convenience sample of those farms where data quality was sufficient for the analysis. It is possible that this would select for farms with better management which may influence the results. Whilst care needs to be taken before generalising these findings to all farms it seems unlikely that farm management would influence the results to a great degree, and the hierarchical multilevel structure of the regression model would account for any between farm variations in "baseline" ISI.

5. Conclusions

Inseminations carried out at intervals of 19-26 days following previous insemination were significantly more likely to result in a pregnancy than those carried out at shorter or longer intervals, within the 16 to 28 day range. This work also provides support for the hypothesis that the expected range for inter-service interval in the modern dairy cow should be considered longer than the traditional 18-24 days, with the alternative range of 19-26 appearing to be most supported by this study.

Table 1. Variables tested for inclusion in a logistic regression model with the outcome of conception risk

Variable	Type
Pregnancy	Outcome variable, binary
ISI, days	Categorical (16 days, 17 days…28 days)
Month of insemination ending ISI	Categorical
Year of insemination ending ISI	Categorical
Parity	Categorical (1,…,5+)
305 day lactation milk yield	Continuous
Days in milk at insemination ending ISI	Continuous
Number of inseminations (in this lactations)	Categorical (2,3+)
Cow ID	Random effect
Herd ID	Random effect

Table 2 Parameter estimates for a Bayesian multilevel logistic regression model with conception risk as an outcome variable and the inter-service interval (ISI) preceding the insemination forced in to the model as a categorical variable. *Absolute predicted risk has been calculated for all categorical variables by fixing all other model variables at their population means, and predicting absolute risk (with a 95% credible interval for the prediction) for each of the categories within the variable. Absolute predicted risks are not presented for continuous variables.

Variable	**Coefficient estimate**	**Standard error**	**Odds ratio (95% credible interval)**	**Absolute predicted risk* (95% credible interval)**
Pregnancy	Outcome			
Fixed Part				
Intercept	0.155	0.058		
ISI_16	-0.531	0.095	0.58(0.48-0.70)	0.3 (0.27-0.34)
ISI_17	-0.722	0.087	0.48(0.40-0.57)	0.27 (0.24-0.3)
ISI_18	-0.446	0.068	0.64(0.56-0.73)	0.32 (0.3-0.35)
ISI_19	-0.082	0.05	0.92(0.83-1.01)	0.4 (0.38-0.43)
ISI_20	0.098	0.04	1.10(1.01-1.19)	0.45 (0.43-0.46)
ISI_21	0.167	0.037	1.18(1.09-1.27)	0.46 (0.45-0.48)
ISI_22	0.152	0.037	1.16(1.08-1.25)	0.46 (0.45-0.47)
ISI_23	0.075	0.037	1.07(1.00-1.15)	0.44 (0.43-0.45)
ISI_24	0.054	0.04	1.05(0.97-1.14)	0.44 (0.42-0.45)
ISI_25	Reference			0.42 (0.41-0.44)
ISI_26	-0.034	0.047	0.96(0.88-1.05)	0.41 (0.4-0.43)
ISI_27	-0.205	0.053	0.81(0.73-0.90)	0.37 (0.36-0.4)
ISI_28	-0.144	0.058	0.86(0.77-0.97)	0.39 (0.37-0.41)
305 day milk yield ('000s of litres)	-0.022	0.005	0.97(0.96-0.98)	Continuous
Parity 1	Reference			0.45 (0.44-0.46)
Parity 2	-0.028	0.025	0.97(0.92-1.02)	0.45 (0.43-0.46)
Parity 3	-0.052	0.027	0.94(0.90-1.00)	0.44 (0.43-0.45)
Parity 4	-0.077	0.031	0.92(0.87-0.98)	0.43 (0.42-0.45)
Parity 5+	-0.276	0.026	0.75(0.72-0.79)	0.39 (0.38-0.4)
January	Reference			0.45 (0.44-0.47)
February	-0.038	0.035	0.96(0.89-1.03)	0.44 (0.43-0.46)
March	-0.068	0.036	0.93(0.87-1.00)	0.43 (0.42-0.45)
April	-0.12	0.039	0.88(0.82-0.95)	0.42 (0.4-0.44)
May	-0.053	0.041	0.94(0.87-1.02)	0.44 (0.42-0.46)
June	-0.126	0.042	0.88(0.81-0.95)	0.42 (0.4-0.44)
July	-0.202	0.045	0.81(0.74-0.89)	0.4 (0.38-0.42)
August	-0.188	0.047	0.82(0.75-0.90)	0.41 (0.39-0.43)
September	-0.27	0.046	0.76(0.69-0.83)	0.39 (0.37-0.41)
October	-0.079	0.041	0.92(0.85-1.00)	0.43 (0.41-0.45)
November	-0.094	0.037	0.91(0.84-0.97)	0.43 (0.41-0.44)

December	-0.037	0.033	0.96(0.90-1.02)	0.44 (0.43-0.46)
2002	Reference			0.49 (0.47-0.51)
2003	-0.063	0.04	0.93(0.86-1.01)	0.47 (0.46-0.49)
2004	-0.201	0.039	0.81(0.75-0.88)	0.44 (0.43-0.45)
2005	-0.265	0.039	0.76(0.71-0.82)	0.42 (0.41-0.44)
2006	-0.319	0.039	0.72(0.67-0.78)	0.41 (0.4-0.42)
2007	-0.327	0.038	0.72(0.66-0.77)	0.41 (0.4-0.42)
2008	-0.372	0.047	0.68(0.62-0.75)	0.4 (0.38-0.41)
Insemination number 2	Reference			0.44 (0.43-0.46)
Insemination number >2	-0.136	0.022	0.87(0.83-0.91)	0.41 (0.4-0.42)
Log_eDiM	0.17	0.035	1.18(1.10-1.26)	Continuous
$(Log_eDiM)^2$	-0.345	0.071	0.70(0.61-0.81)	Continuous
Random Part				
Herd level variance	0.06	0.008		
Cow level variance	0.001	0		

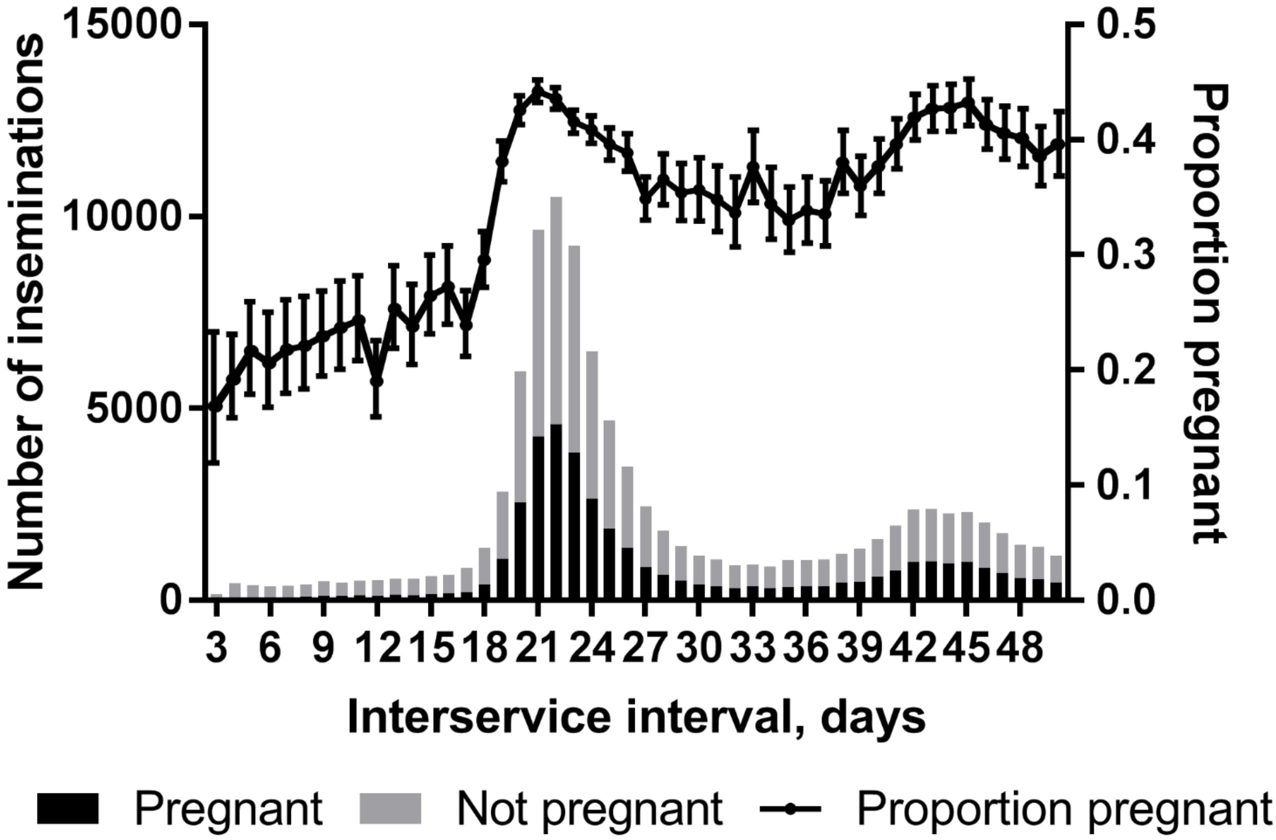


Figure 1 The distribution of interservice intervals (ISIs) from a large dataset of UK dairy cows, showing the number of inseminations (left axis) both resulting in a pregnancy (black bars), the number not resulting in a pregnancy (grey bars) and the change in mean conception risk across the range of inter-service intervals (line) with 95% confidence intervals (error bars)(right axis).

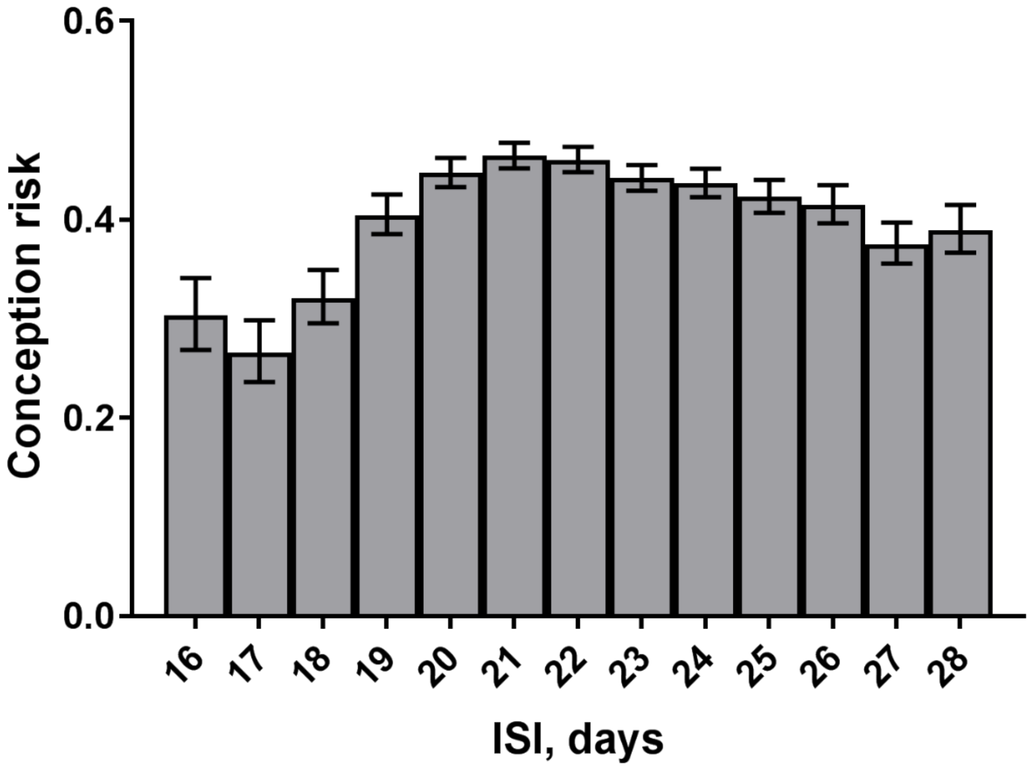


Figure 2 Predicted mean conception risk from multilevel model for different inter-service intervals, error bars show the 95% Bayesian credible interval. Bar height shows the average predicted conception risk at inseminations of different inter-service intervals (ISI) with all other explanatory variables fixed at the mean value

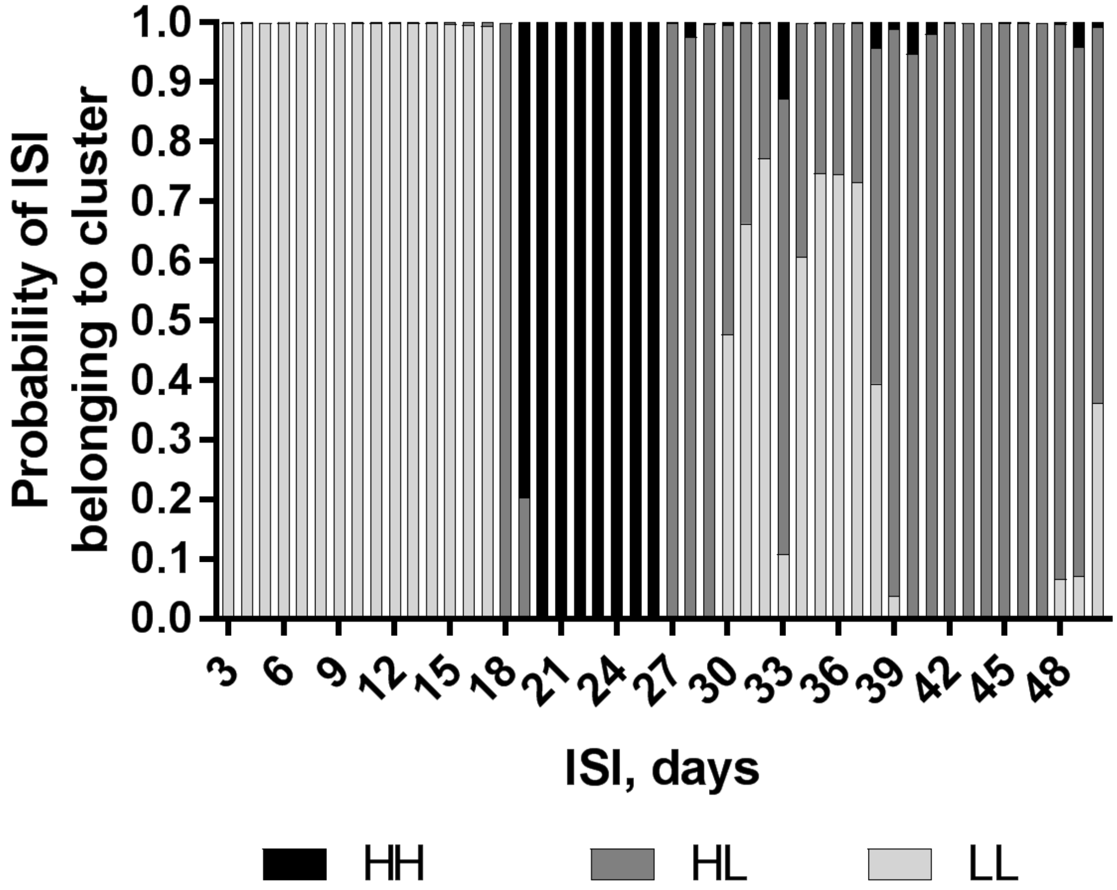


Figure 3 Probability of each ISI falling within the finite Gaussian model classifications: low conception rate, low number of inseminations (LL); Low number of inseminations, higher conception rate (LH); high number of inseminations and higher conception rate (HH)

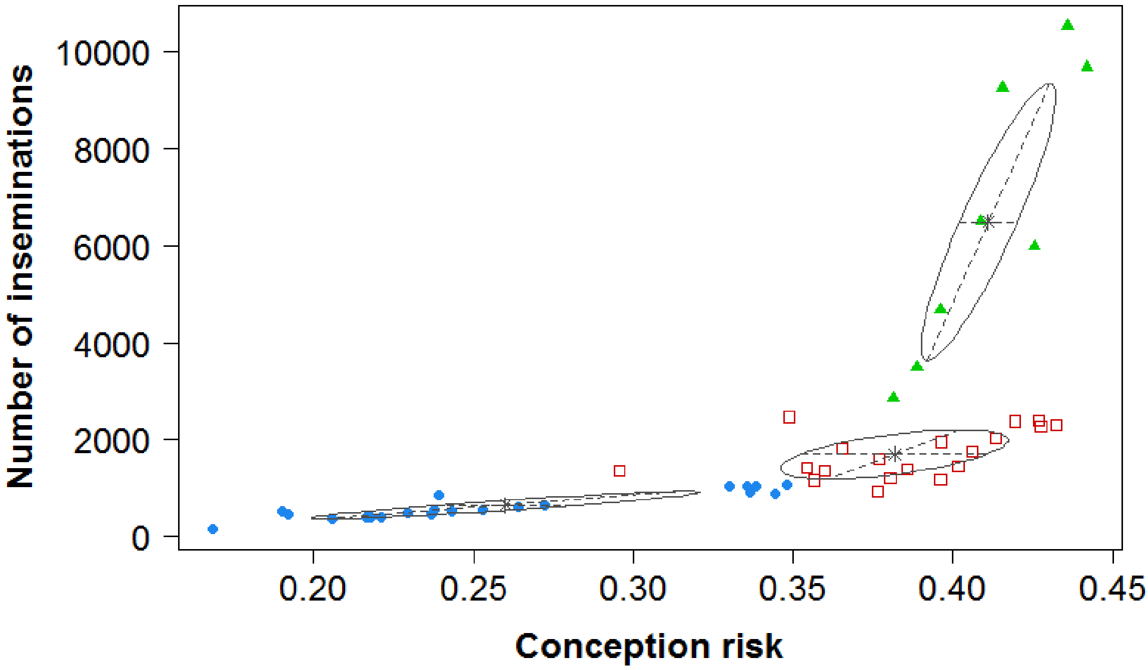
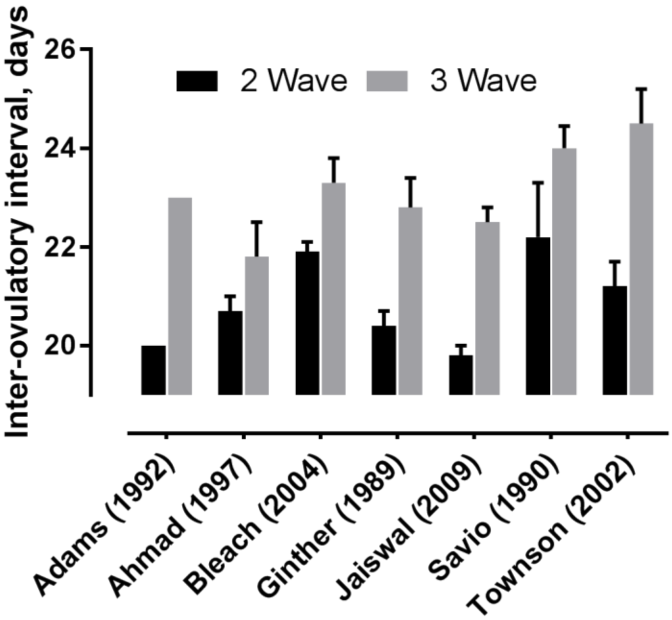


Figure 4 Finite Gaussian mixture model classification of ISIs by average conception risk and number of inseminations at each ISI, the identified clusters are shown by the different symbols (blue dots, LL; red squares, HL with; green triangles, HH) the mean of each cluster identified with the star

Supplementary material



Summary of existing studies evaluating inter-ovulatory intervals in two and three follicular wave estrous cycles, bars show the mean IOI for two and three wave cycles, where present, error bars show stated standard error [4, 12, 13, 31-34]

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