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RESEARCH PAPER

Evaluation of Predictive Ability of Bayesian Regularized Neural Network Using Cholesky Factorization of Genetic Relationship Matrices for Additive and Non-additive Genetic Effects

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Abstract

This study aimed to explore the effects of additive and non-additive genetic effects on the prediction of complex traits using Bayesian regularized artificial neural network (BRANN). The data sets were simulated for two hypothetical pedigrees with five different fractions of total genetic variance accounted by additive, additive x additive, and additive x additive x additive genetic effects. A feed forward artificial neural network (ANN) with Bayesian regularization (BR) was used to assess the performance of different nonlinear ANNs and compare their predictive ability with those from linear models under different genetic architectures of phenotypic traits. Effective number of parameters and sum of squares error (SSE) in test data sets were used to evaluate the performance of ANNs. Distribution of weights and correlation between observed and predicted values in the test data set were used to evaluate the predictive ability. There were clear and significant improvements in terms of the predictive ability of linear (equivalent Bayesian ridge regression) and nonlinear models when the proportion of additive genetic variance in total genetic variance (σ_a^2/σ_G^2) increased. On the other hand, nonlinear models outperformed the linear models across different genetic architectures. The weights for the linear models were larger and more variable than for the nonlinear network, and presented leptokurtic distributions, indicating strong shrinkage towards 0. In conclusion, our results showed that: a) inclusion of non-additive effects did not improve the prediction ability compared to purely additive models, b) The predictive ability of BRANN architectures with nonlinear activation function were substantially larger than the linear models for the scenarios considered.

Introduction

Additive genetic variance generally accounts for most or all of total genetic variance in complex traits (Van Tessel 2000; Nagy *et al.* 2013). Nonetheless, there is a long-standing controversy and paradox about the importance of non-additive genetic effects in breeding programs. For example, some authors argue that the prediction of genetic response may be biased upwards if non-additive genetic variances are not included in genetic models (Cheverud, & Routman 1995; Carlborg & Haley 2004; Hallander & Waldmann 2007). In addition, many authors have reported that accounting for non-additive effects in the genetic effects might improve the estimation of additive effects, resulting in less biased prediction (e.g., Wittenburg *et al*. 2011; Colleen *et al.*2013; Powell *et al.* 2013).

Interactions between genes are known to be common and are a key concept for understanding adaptation and evolution of species as well as long-term response to selection in breeding programs (Alvarez-Castro & Carlborg 2007; Ingileif & Yuster 2008). An alternative to better understand the genetic architecture of complex traits is to include intralocus (dominance) and inter-locus (epistasis) interaction of alleles when fitting a model to a trait (Jamrozik *et al.* 2005; Oakey *et al.* 2006; Valentina *et al.* 2007; Wittenburg *et al*. 2011). However, modelling additive and non-additive effects simultaneously poses challenges in terms of computational demand, data

analysis and interpretation of results. Several statistical and computational methods have been devised for studying the association between complex traits and high dimensional data sets. Classical single-marker regressions and, more recently, Bayesian linear regression models of various types (Gianola *et al*. 2011; Meuwissen *et al*. 2009; de los Campos *et al*. 2009; de los Campos *et al*. 2010) are focused on additive inheritance and ignore interactions and non-linearity. In fact, a study by Wittenburg *et al*. (2011) confirmed that parametric methods have difficulties in identifying and estimating some non-genetic effects. Alternatively, artificial neural networks (ANNs) provide a powerful technique for learning about complex traits by predicting the future state of an outcome variable based on training data. ANNs can capture non-linear relationships between predictors (e.g. SNP markers) and responses (e.g. phenotypes) and learn about functional forms in an adaptive manner (Okut *et at*. 2011; Gianola *et al*. 2011). The ability of computing complex nonlinear relationships between response and input variables, including any kind of interactions between input variables, with many training algorithms available makes ANN extremely interesting for analysis of complex traits (Lampinen &Vehtari 2001; Okut 2016; Okut 2021).

One of the most serious problems that can occur when training an ANN is overfitting. Since the ultimate goal of ANN is attaining generalization such overfitting problems should be detected and addressed carefully. Bayesian Regularized Artificial Neural Network (BRANN) is more resistant to overcome the problem of overfitting problem than conventional ANN, resulting in improved generalization performance (Gianola *et al*. 2011; Okut *et al*. 2013). The idea of Bayesian regularization (BR) is to make the network response smoother, through modification of the objective function by adding a penalty term consisting of the mean square of all network coefficients (Gencay & Qi 2001). BR minimizes a combination of squared errors and weights, and then determines an appropriate combination so as to produce a network that generalizes well (Marwala 2007; Ripley 2007; MacKay 2008). As such, BR can be viewed as a nonlinear analog of ridge regression. The Bayesian approach to neural network modeling consists of arriving at the posterior probability distribution of weights by updating a prior probability distribution by means of a training data set (Okut *et al*. 2013).

The objectives of this paper were to use the ANNs to explore the impact of non-additive genetic effects (additive x additive, and additive x additive x additive) on the prediction of complex traits when additive and non-additive effects were jointly considered when fitting a model to a trait. For such, BRANN architectures differing in terms of number of neurons and activation functions were used and compared in terms of their prediction ability. Details on data simulation and modeling are presented in the next section. The scenarios used to generate data by including only additive or a certain portion of non-additive genetic effects is explained in this section. On the second section, we summarize the results obtained from the different BRANNs. A final section presents a discussion and concluding remarks.

Materials and Methods

Data sets

The data considered in this paper were simulated for 5 non-overlapping generations. Two hypothetical pedigrees, referred to as population 1 (Pop1) and population 2 (Pop2), were generated. The number of animals in the base populations were 100 (50 nuclear families) and 300 (150 nuclear families), respectively. Animals used in the base populations were assumed to be unrelated and not inbred. Four additional generations were created with 100 and 300 animals in each generation for each population. Each family in the base population and subsequent generations had only 2 offspring. In both populations throughout the five generations (base and four additional generations), animals were randomly mated within their generations. At the end of the simulation the total number of animals in Pop1 was 500 (274 female and 226 male) and in Pop2 was 1500 (767 female and 733 male). A relationship matrix (**A=CC',** here **C** is lower triangular Cholesky factor decomposition) from the animals of the 5 generations was then constructed for both populations and the elements of the lower triangular Cholesky factor decomposition of these relationship matrices (a_{kl}) were considered as input variables ($\boldsymbol{p}_i = a_{kl}$) in BRANN architectures.

The phenotypic records ($\boldsymbol{t}_i$) were generated for all animals in the pedigree structure and were a function of genetic and random environmental effects from a normal distribution. For each generation, 50 and 150 nuclear families consisting of the two sibs values were obtained by adding a normally distributed environmental effect with 0 mean and unit variance. Target variables (t_i) which represent animals' phenotypes were generated according to the following equation:

$$\begin{aligned}\mathbf{t} &= \mathbf{a}_1 + \mathbf{a}_2 + \mathbf{a}_3 + \mathbf{e} \\ &= [\mathbf{A}^{1/2} \times \mathbf{u}_1 \times \sigma_a] + [(\mathbf{A}\#\mathbf{A})^{1/2} \times \mathbf{u}_2 \times \sigma_{aa}] \\ &\quad + [(\mathbf{A}\#\mathbf{A}\#\mathbf{A})^{1/2} \times \mathbf{u}_3 \times \sigma_{aaa}] + [\mathbf{u}_4 \times \sigma_e]\end{aligned}$$

where $\boldsymbol{a}_1$, $\boldsymbol{a}_2$ and $\boldsymbol{a}_3$ are vectors of additive, additive x additive, and additive x additive x additive genetic effects, and $\boldsymbol{e}$ is a vector of random residual effects. In the equation above, the matrix **A** represents the numerator relationship matrix, is the Hadamard product, and $\boldsymbol{u}_1$, $\boldsymbol{u}_2$, $\boldsymbol{u}_3$ and $\boldsymbol{u}_4$ are random vectors from multivariate standard normal distribution, i.e. $\mathbf{u}_j \sim N(\mathbf{0}, \mathbf{I})$, j=1… 4, where **0** is a column vector of zeros

and **I** is an identity matrix. With these settings we have that:
$\mathbf{a}_1 \sim N(\mathbf{0}, \mathbf{A}\sigma_a^2)$,
$\mathbf{a}_2 \sim N(\mathbf{0}, \mathbf{A}\#\mathbf{A}\sigma_{aa}^2)$,
$\mathbf{a}_3 \sim N(\mathbf{0}, \mathbf{A}\#\mathbf{A}\#\mathbf{A}\sigma_{aaa}^2)$ and
$\mathbf{e} \sim N(\mathbf{0}, \mathbf{I}\sigma_e^2)$

and that the total phenotypic variance is given by:
$\mathrm{Var}(\mathbf{t}) = \mathbf{A}\sigma_a^2 + \mathbf{A}\#\mathbf{A}\sigma_{aa}^2 + \mathbf{A}\#\mathbf{A}\#\mathbf{A}\sigma_{aaa}^2 + \mathbf{I}\sigma_e^2$.

In the simulations, the total variance was assumed to be $\mathrm{V}(t) = \sigma_T^2 = 2$, and the variance of

genetic and non-genetic effects was assumed to be $\sigma_G^2 = 1 \text{and } \sigma_e^2 = 1$, respectively. Here, σ_G^2 is the total genetic variance and σ_e^2 is the residual (random environmental) variance. Five different scenarios were considered as σ_a^2 / σ_G^2 =0, 0.1, 0.5, 0.9, and 1 (namely, five different fractions of variance accounted by additive genetic effect) for simulating phenotypic values. Heritability (h^2) was kept to be 0.5 for the all scenarios. The fractions of both non-additive effects (additive x additive, $\sigma_{aa}^2 / \sigma_G^2$, and additive x additive x additive, $\sigma_{aaa}^2 / \sigma_G^2$) were assumed to be equal in each model. For example, when the fraction of variance accounted by additive genetic effects was σ_a^2 / σ_G^2 = 0.5 then the fractions of non-additive genetic effects were assumed $\sigma_{aa}^2 / \sigma_G^2$ =0.25 and $\sigma_{aaa}^2 / \sigma_G^2$ =0.25 (Table 1).

Feed-forward neural networks

A fully connected, two-layer feed-forward BRANN with backpropagation was used in this study and is illustrated in Figure 1. In Figure 1, $\mathbf{p}_i$ is an input vector of elements of the relationship matrix (a_{kl}) at the left-most layer. The elements of relationship matrix (Cholesky factorization) are connected to the neurons in a single hidden (middle) layer via weights (w_{jk}) with a bias (intercept) specific to each neuron. For example, if there are *S* neurons in the architecture (Figure 1 depicts four neurons), the biases in the hidden layer are $b_1^{(1)}, b_2^{(1)} \ldots\ldots b_S^{(1)}$. A hyperbolic tangent and a linear activation function were applied to the hidden and output layers. The input into neuron *j*, prior to activation, is $b_j + \sum_{k=1}^{R} w_{jk} p_k$. This weighted input is transformed ("*activated*") using hyperbolic tangent activation function *f(.)* (Figure 1) as $f_j\left(b_j + \sum_{k=1}^{R} w_{jk} p_k\right)$. This activated emission is then sent to the output layer and collected as $\sum_{j=1}^{S} w_j' f_j\left(b_j + \sum_{k=1}^{R} w_{jk} p_k\right) + b$, where w_j (*j* = 1,2…,*S*) are weights specific to each neuron and *b* is another bias parameter. Finally, this is activated again

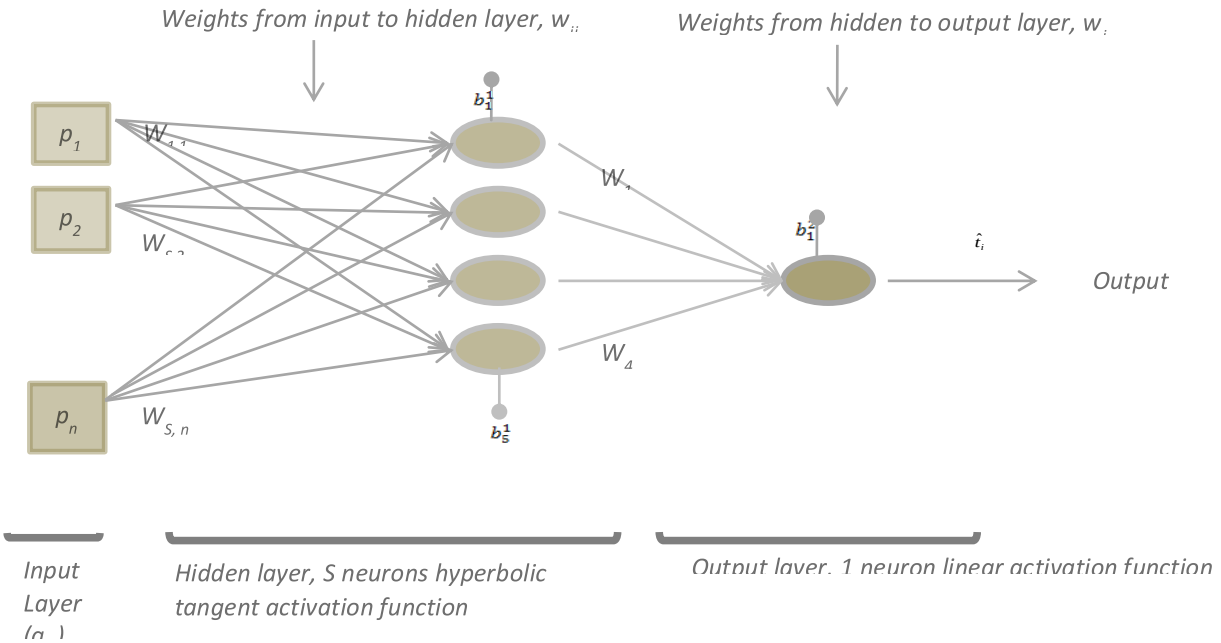

Figure 1. Artificial neural network design used in this study. The elements (a_{kl}) of relationship matrix (**A**) were used as inputs (p_i). Each input, pi is connected to up to S neurons via coefficients w_{ij} (*j* denotes neuron, *i* denotes input). Each hidden and output neuron has a bias $b_j^{(k)}$ (*j* denotes neuron, *k* denotes layer).

Table 1. The fraction of additive and non-additive genetic variances for the 5 different models considered.

Models	σ_a^2/σ_G^2	σ_{aa}^2/σ_G^2	$\sigma_{aaa}^2/\sigma_G^2$	σ_e^2
1	0	0.5	0.5	1
2	0.1	0.45	0.45	1
3	0.5	0.25	0.25	1
4	0.9	0.05	0.05	1
5	1	0	0	1

σ_G^2 =Total genetic variance, σ_a^2 = additive genetic variance, σ_{aa}^2 =additive*additive genetic variance, σ_{aaa}^2 = additive*additive*additive genetic variance. σ_e^2 = residual variance

with linear function *g(.)* as $g\left[\sum_{j=1}^{S} w_j' f_j(.)+b\right]$. Thus, the output is assumed to be a linear combination of output from hidden neurons and a Gaussian error term N(0, σ^2). This becomes the predicted value of t_i in the training set. Both input and target variables were normalized prior to network training and BRANN training was implemented according to the Levenberg-Marquardt optimization (Foresee & Hagan 1997). After training, the output (i.e., the predicted value of phenotypes) is calculated as:

$$\hat{t}_i = g\left\{\sum_{j=1}^{S} w_j' f_j\left(\sum_{k=1}^{R} w_{jk} p_{ik} + b_j\right)+b\right\}; \quad i=1,2...,n \quad (1)$$

Bayesian regularization

ANN conventional training aims to reduce the sum of squared error, $F=E_D$. Pre-Bayesian "training" of neural networks involved finding the network parameters, w, to minimize the error term equivalent in the probabilistic interpretation to maximum likelihood. As with other highly parameterized or ill-posed problems, this led to overfitting (Titerington 2004). In Bayesian ANNs (e.g., BRANNs), the objective function *F* has an additional quadratic penalty term that penalizes large weights to achieve a smoother mapping. Gradient-based optimization is then used to minimize the following function, which is equal to a penalized natural log-likelihood:

$$F=\beta E_D(D|w,M)+\alpha E_W(w|M), \quad (2)$$

In equation (2), the $E_D(D|\mathbf{w},M)=\sum_{i=1}^{n}(\hat{t}_i - t_i)^2$ and $E_W(w|M)=\sum_{i=1}^{m} w_i^2$ are the sum of squares of error and network weights, *m* is the number of weights, and α and *β* are positive regularization parameters which need to be estimated. *M* denotes a specific network architecture which consists of a specification of the number of layers, the number of neurons in each layer, and the type of activation functions used. The second part of equation (2) is called weight decay, which penalizes large weights to achieve a *smoother* mapping. Therefore, the Bayesian approach involves a probability distribution of network weights, so the predictions from the network can also be casted in a probabilistic framework (Sorich *et al*. 2003). Conditional on the data, given *α, β,* and *M,* the posterior distribution of *w* is:

$$P(\mathbf{w}|D,\alpha,\beta,M)=\frac{P(D|\mathbf{w},\beta,M)P(\mathbf{w}|\alpha,M)}{P(D|\alpha,\beta,M)}, \quad (3)$$

where *D* is the training data set. In (3) the $P(\mathbf{w}|\alpha,M)=\left(\frac{\alpha}{2\pi}\right)^{m/2}\exp\left\{-\frac{\alpha}{2}\mathbf{w}'\mathbf{w}\right\}$ is the prior distribution of weights and $P(D|w,\beta,M)$ is the likelihood function, which is the probability of the data given *w*, while $P(D|\alpha,\beta,M)\int P(D|w,\beta,M)P(w|\alpha,M)dw$ is a normalization factor, which does not depend on *w* (Kumar *et al.* 2004). In this Bayesian framework, the optimal weights should maximize the posterior probability P(*w | D, α,* P, *M)*. Maximizing the posterior probability of *w* is equivalent to minimizing the regularized objective function $F=\beta E_D+\alpha E_W$ (Foresee and Hagan 1997). While minimization of *F* is identical to finding the (locally) *maximum a posteriori* estimates w^{MP}, minimization of E_D by back-propagation is identical to finding the maximum likelihood estimates w^{ML} if n>m, where m is the number of the parameters (MacKay 2008). Consider the joint posterior density

$$P(\alpha,\beta|D,M)=\frac{P(D|\alpha,\beta,M)P(\alpha,\beta|M)}{P(D|M)} \quad (4)$$

If the prior density *P(α, β|M)* is uniform, maximization of *P(α β | D, M)* with respect to *α* is equivalent to the maximization of the likelihood *P(D | α, β, M*) in equation (4). This likelihood is *evidence for α* and *β*, which is the normalization factor for equation (3). According to MacKay (1992) we have;

$$P(D|\alpha,\beta,M)=\frac{P(D|\mathbf{w},\beta,M)P(\mathbf{w}|\alpha,M)}{P(\mathbf{w}|D,\alpha,\beta,M)}=\frac{Z_F(\alpha,\beta)}{(\pi/\beta)^{n/2}(\pi/\alpha)^{m/2}}, \quad (5)$$

where *n* and *m* are the number of observation and parameters, respectively. The objective function, $F=\beta E_D(D|w,M)+\alpha E_W(w|M)$ has the shape of a quadratic in a small area surrounding the minimum point of the posterior density w^{MAP}, where the gradient is zero. A Laplacian approximation to $Z_F(\alpha,\beta)$ in equation (5) yields;

$$Z_F(\alpha,\beta) \propto \left|\mathbf{H}^{\mathbf{MAP}}\right|^{-\frac{1}{2}} \exp(-F(w^{MAP})) \quad (6)$$

where $\mathbf{H}^{\mathbf{MAP}}$ is the Hessian matrix of the objective function evaluated at w^{MAP}.

Bayesian optimization of the regularization parameters requires computation of the Hessian matrix of the objective function F evaluated at the optimum point w^{MAP} (Xu *et al*. 2006). However, directly computing the Hessian matrix is not always necessary. As proposed by MacKay (1992), the Gauss-Newton approximation to the Hessian matrix can be used if the Levenberg-Marquardt (LM) optimization algorithm is employed to locate the minimum of F (Shaneh & Butler 2006). The LM algorithm is a robust method for approximation function and LM modification to Gauss-Newton is

$(J'J + \mu I)\ \delta = J'e$, (7)

and the Hessian matrix can be approximated as:

$$\mathbf{H} = \mathbf{J}'\mathbf{J} \quad (8)$$

where J is the Jacobian matrix that contains first derivatives of the network errors with respect to network parameters (the weights and biases), δ is the parameter update vector and μ is the Levenberg`s damping factor. The gradient is computed as g=J'e. The μ is adjustable at each iteration and guides to the optimization process. If reductions of the cost function F are rapid, then the parameter μ is divided by a constant (c) to bring the algorithm closer to the Gauss–Newton. On the other hand, if an iteration gives insufficient reduction in F, then μ is multiplied by the same constant giving a step closer to the gradient descent direction. Therefore, the Marquardt-Levenberg algorithm can be considered a trust-region modification to Gauss-Newton designed to serve as an intermediate optimization algorithm between the Gauss-Newton method and the Gradient-Descent algorithm (Battiti 1992).

If the expression $\gamma = m - 2\alpha^{MAP} tr(H^{MAP})^{-1}$ refers to the effective number of parameters in the neural network, where m is the total number of parameters ($0 \le \gamma \le m$), then it can be shown (MacKay 1992; Xu *et al*. 2006) that:

$$\alpha^{MAP} = \frac{\gamma}{2E_w(w^{MAP})} \quad and \quad \beta^{MAP} = \frac{n-\gamma}{2E_D(w^{MAP})} \quad (9)$$

Analyses

MATLAB (2009) was used for fitting the BRANN. The neural networks considered had two layers (hidden and output) and were fully connected feed-forward networks as shown in Figure 1. To avoid overtraining, improve predictive ability, and eliminate spurious effects caused by the starting values, the BRANNs were trained independently 12 times. Results were recorded as the average of the 12 independent runs. The number of epochs used was 1000. Training was stopped if: 1) the maximum number of epochs was reached; 2) performance had met a suitable level; 3) the gradient was below a suitable target; and 4) the Levemberg-Marquardt μ parameter exceeded a suitable maximum (training stopped when it became larger than 10^{10}). Each of these targets and goals were set at the default values set by the MATLAB implementation.

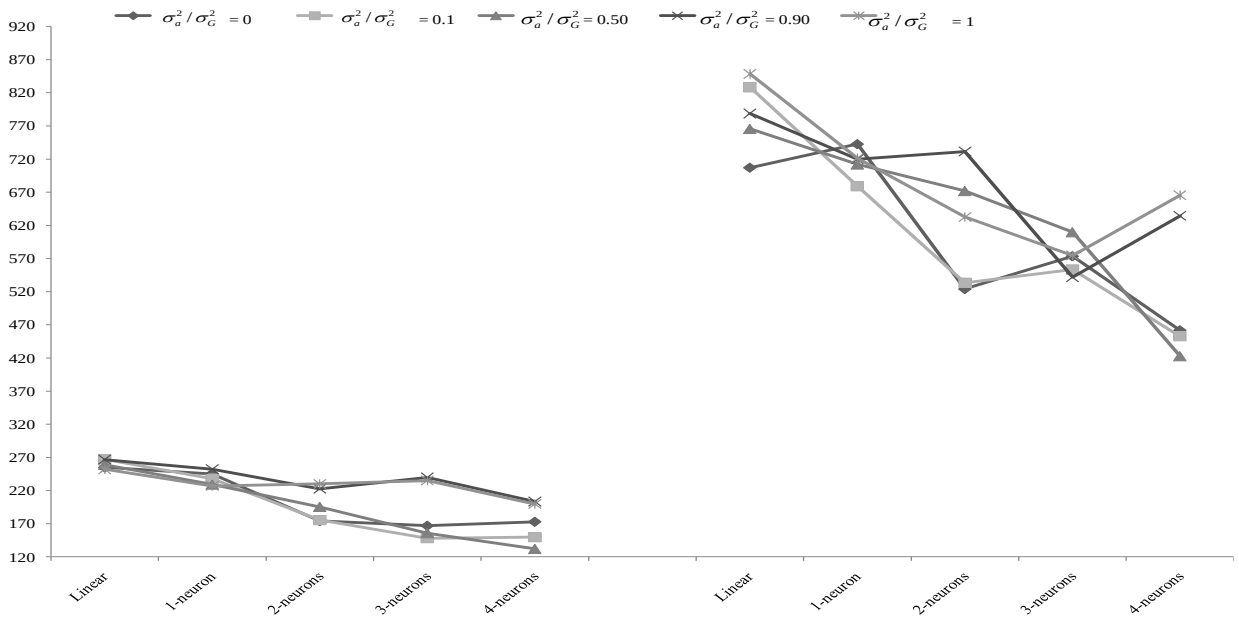

Figure 2. Effective number of parameters (γ) in populations I (at the left side) and population II (at the right side).

Table 2. Effective number parameters and their standard deviations after 12 running for different network architectures for the both populations (results are average of 12 independent running).

	Population I					*Population II*				
	Effective number of parameters γ					*Effective number of parameters γ*				
	Linear	1-neuron	2-neurons	3-neurons	4-neurons	Linear	1-neuron	2-neurons	3-neurons	4-neurons
$\sigma_a^2/\sigma_G^2=0$	255.0±40	245.0±30	174.0±59	167.0±59	173.0±44	707±83	742.5±63	524.0±163	573.4±165	461.8±179
$\sigma_a^2/\sigma_G^2=.1$	267.0±30	237.8±36	175.6±36	148.0±44	149.7±40	828.3±50	679.1±60	533.5±62	553.4±168	452.7±156
$\sigma_a^2/\sigma_G^2=.5$	259.4±31	229.3±30	195.4±43	155.8±41	132.0±42	765.5±168	711.9±66	671.8±172	609.7±118	422.7±96
$\sigma_a^2/\sigma_G^2=.9$	266.6±34	252.2±50	222.6±33	239.7±35	203.4±44	788.6±108	719.8±80	731.4±64	541.8±140	634.4±124
$\sigma_a^2/\sigma_G^2=1$	252.2±27	226.9±27	230.0±49	235.0±38	199.7±51	848.4±66	721.2±77	632.6±140	574.5±122.9	665.5±95

σ_a^2 =Additive genetic variance, σ_G^2 =Total genetic variance ($\sigma_G^2=\sigma_a^2+\sigma_{aa}^2+\sigma_{aaa}^2$)

Results

Degree of complexity and performance of BRANN

The performance of BRANNs was evaluated in terms of effective number of parameters (*γ*) and residuals sum of squares in the test data set (SSE_{test}). The estimated effective number of parameters associated with each network evaluated is summarized in Table 2. For both populations, a total of 600 BRANN architectures were examined (50 ANN architectures and 12 replications for each). Our focus was on comparing performances from different linear and nonlinear ANN architectures under practical conditions while varying the proportion of total genetic variance contributed by additive genetic effects.

Except for σ_a^2/σ_G^2 =0 in Pop2, the highest effective number of parameters (*γ*) was obtained from linear ANN architectures (linear activation function in hidden and output layers), indicating explicitly penalizing the complex models by nonlinear BRANN. For example, *γ* was equal to 255.0±30 and 173.0±44 in BRANNs for linear and nonlinear, respectively, with 4 neurons when the genetic model was assumed to be purely non-additive (σ_a^2/σ_G^2 =0). Here, γ reduced drastically (32%) even though the nominal number of parameters (*m*) increased from 501 to 2509 for the same BRANN architectures (Table 2 and Figure 2). In most cases (except for σ_a^2/σ_G^2 = 0 and σ_a^2/σ_G^2 = 0.1 in Pop2) the smallest effective number of parameters attained was obtained either with 3 or 4 neurons in nonlinear (hyperbolic tangent activation function in hidden layer) BRANN architectures, suggesting the penalization ability of ANNs via Bayesian regularization in complex nonlinear models to attain better performance.

The residuals sum of squares (SSE) in test data sets showed the same pattern in both populations across BRANNs: SSE substantially increased as the proportion of genetic variance accounted for by additive genetic effect (σ_a^2/σ_G^2) increased. The SSE of linear models for Pop1 and Pop2 ranged from 164.3 to 668.6 and from 525.1 to 1941.6, respectively. The minimum and maximum values of SSE in both populations were obtained for σ_a^2/σ_G^2 ≥ 0.1 and σ_a^2/σ_G^2 = 1. Except for σ_a^2/σ_G^2 =0.5 in Pop2 with two neurons, the SSE of nonlinear models were smaller than those of linear models when the ratio of additive genetic variance increased (σ_a^2/σ_G^2 >0). For example, the smallest SSE values in Pop1 and Pop2 for σ_a^2/σ_G^2 =0.9 were observed with 4 neuron nonlinear models which were 14 and 16% less than the linear models in the both populations. In general, the smallest SSE as well as *γ* values in both populations for σ_a^2/σ_G^2 ≥0.5 was attained with 3 or 4 neuron architectures, indicating the capability of BRANN to improve performance of complex models via shrinking and reducing the SSE (Table 3).

Predictive ability

The predictive ability (generalization) of the networks was assessed by means of the correlation between predicted and phenotypic observed values in the testing set and the distributions of weights. Such predictive correlations are given in Table 4 and Figure 3.

There were clear and significant improvements in terms of the predictive ability for both linear (equivalent Bayesian ridge regression) and nonlinear models when the proportion of additive genetic variance increased. In

Table 3. Residual sum of squares for BRANNs in testing data set for five models and two populations.

	σ_a^2/σ_G^2 =0	σ_a^2/σ_G^2 =0.1	σ_a^2/σ_G^2 =0.5	σ_a^2/σ_G^2 =0.9	σ_a^2/σ_G^2 =1
			Population I		
Linear	164.3	165.2	271.1	651.8	668.6
Nonlin-1neuron	174.1	150.9	257.7	543.6	620.5
Nonlin-2neurons	165.7	149.4	247..2	515.0	611.0
Nonlin-3neurons	162.6	151.4	246.6	571.5	593.1
Nonlin-4neurons	152.6	157.1	243.1	492.9	576.0
			Population II		
Linear	525.1	530.8	782.8	1716.2	1941.6
Nonlin-1neuron	542.8	486.8	758.5	1608.6	1771
Nonlin-2neurons	495.6	502.9	791.9	1546.4	1740.8
Nonlin-3neurons	492.2	486.3	733.4	1461.7	1771
Nonlin-4neurons	471.9	495.4	730.1	1446.7	1770

general, increasing in predictive ability was quite distinct for the nonlinear BRANN, but more markedly so for genetic models with σ_a^2/σ_G^2 >0.5.

The predictive abilities were smallest when σ_a^2/σ_G^2 <0.5, intermediately for σ_a^2/σ_G^2 =0.5 and highest when σ_a^2/σ_G^2 >0.5 for all BRANNs, suggesting that the high ratio of additive genetic variance to total variance might be a good approximation for analyzing the quantitative traits. For example, improvements σ_a^2/σ_G^2 = 1 vs σ_a^2/σ_G^2 = 0 in Pop2, were 57, 67, 54, 53 and 68 % for linear and nonlinear BRANN architectures of 1, 2, 3 and 4 neurons, respectively. There was no consistent upward trend on predictive correlations from linear to nonlinear BRANN when σ_a^2/σ_G^2 ≤0.1. Further, the same findings were observed for σ_a^2/σ_G^2≤0.1 when the number of neurons in the hidden layer gradually increased in the both populations (Table 4). For example, the predictive correlations for the linear model for the Pop1 and Pop2 when σ_a^2/σ_G^2= 0 were 0.406 and 0.358, respectively. The highest correlations for both population for σ_a^2/σ_G^2 = 0 with nonlinear BRANN were 0.441 (in 3-neuron BRANN architecture) and 0.407 (in 2-neuron BRANN architecture). Similar patterns were found for σ_a^2/σ_G^2 =0.1 for both populations. In contrast, the

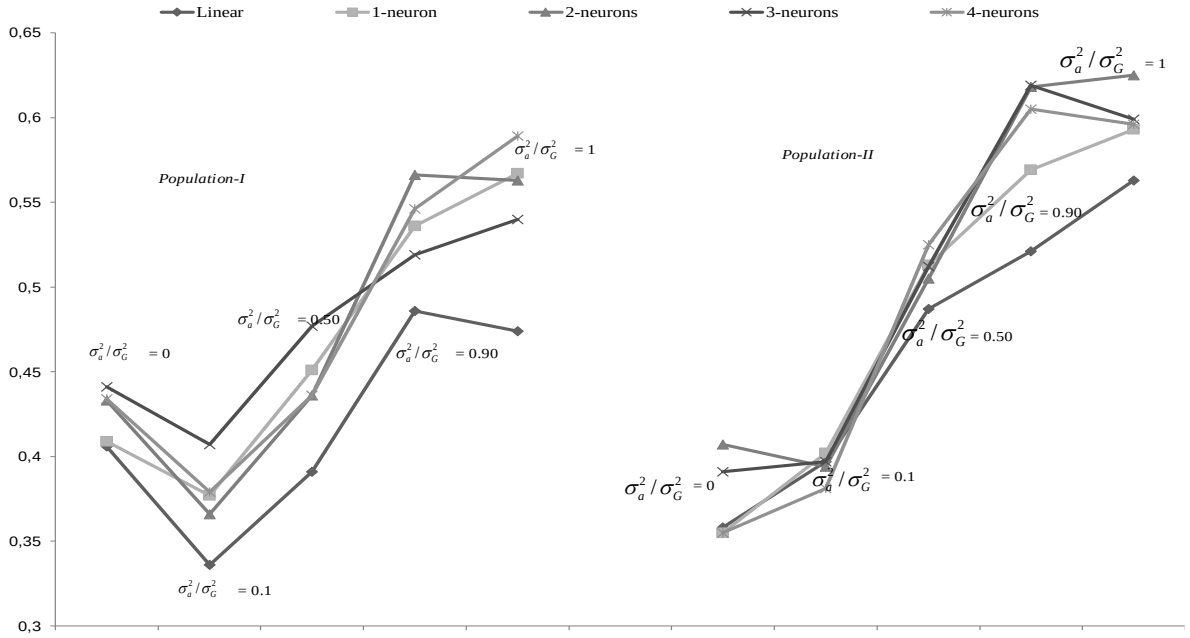

Figure 3. Predictive correlations in testing data set (r_{test}) for different ANN architectures for both populations' data sets for network architectures used in both populations.

Table 4. Predictive correlations of BRANN in testing data set for five models and two populations.

	σ_a^2/σ_G^2 =0	σ_a^2/σ_G^2 =0.1	σ_a^2/σ_G^2 =0.5	σ_a^2/σ_G^2 =0.9	σ_a^2/σ_G^2 =1
Population 1					
Linear	0.406	0.336	0.391	0.486	0.474
Nonlin-1neuron	0.409	0.377	0.451	0.536	0.567
Nonlin-2neurons	0.433	0.366	0.436	0.566	0.563
Nonlin-3neurons	0.441	0.407	0.477	0.519	0.54
Nonlin-4neurons	0.434	0.379	0.436	0.546	0.589
Population 2					
Linear	0.358	0.397	0.487	0.521	0.563
Nlin-1neuron	0.355	0.402	0.513	0.569	0.593
Nlin-2neurons	0.407	0.394	0.505	0.618	0.625
Nlin-3neurons	0.391	0.397	0.512	0.619	0.599
Nlin-4neurons	0.355	0.381	0.525	0.605	0.596

predictive ability of nonlinear BRANN architectures increased as the proportion of additive genetic variance increased beyond 0.5 (σ_a^2/σ_G^2 ≥0.5).

The distribution of weights after training a network also provides some indication of predictive ability; smaller values suggest better generalization while larger weights indicate a more local representation. The weight decay penalty term in the objective function in BRANN causes the weights to converge to smaller absolute values. Figure 4 depicts the distribution of weights in Pop2 for the linear and nonlinear networks with 4-neuron architectures for the σ_a^2/σ_G^2 =0, σ_a^2/σ_G^2 =0.5 and σ_a^2/σ_G^2 =1 scenarios. The average sum of squares of weights when σ_a^2/σ_G^2 =0 were 7.05 and 2.8 for linear and nonlinear specifications, respectively; however, the 7.05 for the linear model was the sum of squares of about 1500 weights whereas the 2.8 for the nonlinear model with 4 neurons was the sum of squares of about 6000 weights (4 x 1500). Similar results were obtained for Pop1 (not depicted here), indicating how strong the shrinkage is (towards 0) when utilizing BRANN. The weights in ANN are the measure the importance of the inputs. The contribution value of an individual input is simply the product of the absolute value of the weights going from a specific input unit to a specific output, summed over the *S* hidden units. Figure 4 shows that the contribution of an input variable to the target variable becomes larger as the proportion of additive genetic variance is increased. Therefore, the majority of weighs lie between ±0.05 and ±0.1 for σ_a^2/σ_G^2 =0 and σ_a^2/σ_G^2 =1 with four neurons, indicating the relative importance of input on the output increases as proportion of additive genetic variance increase.

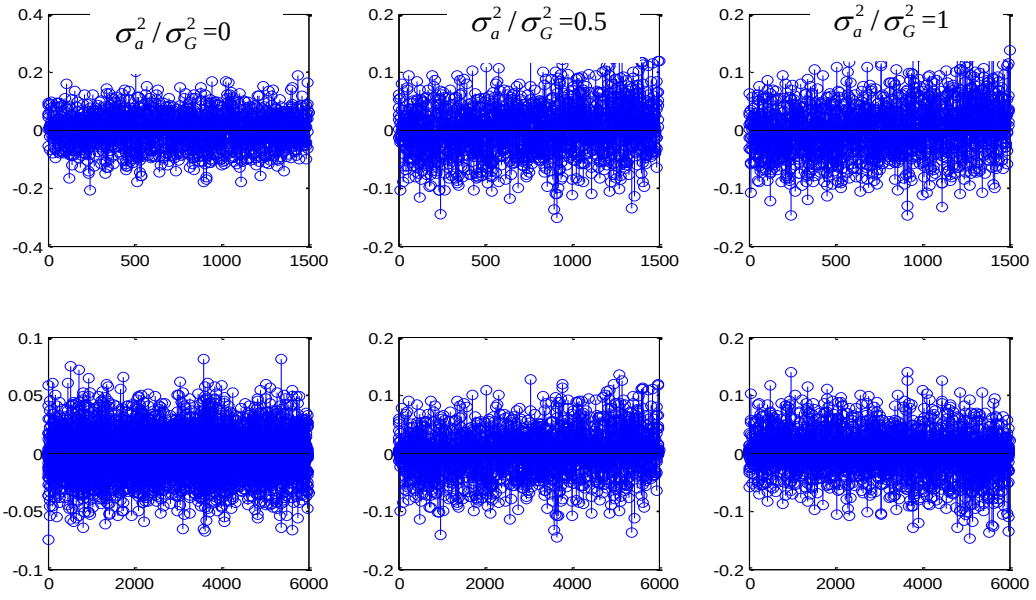

Figure 4. Distributions of weights in Pop2 for σ_a^2/σ_G^2 =0, σ_a^2/σ_G^2 =0.5 and σ_a^2/σ_G^2 =1 for linear (upper row) and nonlinear BRANN with 4 neurons in hidden layer (bottom row).

Discussion

In contrast to the early generation of the quantitative genetics, the non-additive components have a clear mechanistic knowledge in the framework of scientific advancement of the network model and the theories of systems quantitative genetics (Zhu *et al*. 2009). Many research articles (Bagheri & Wagner 2004; Bradshaw *et al*. 2005; Weinreich *et al*. 2005; Le Rouzic *et al*. 2008) have documented that there exists a widening consensus about the evolutionary operability of non-additive genetic components. With this study, the BRANN architectures differing in terms of number of neurons and activation functions were used to make predictions when additive and non-additive effects were jointly involved in fitting a model to a trait. It was shown that the inclusion of non-additive effects did not improve the predictive ability compared to purely additive models (Table 4). Only the σ_a^2 / σ_G^2 =0.9 scenario generated similar prediction abilities with all BRANN architectures compared to purely additive models. In contrast, the predictive ability of non-linear models decreased drastically when the proportion of non-additive effects ($\sigma_{aa}^2 / \sigma_G^2 \text{and} \sigma_{aa}^2 a / \sigma_G^2$) were assumed to be 0.5 or more. These results are in agreement with Allison *et al*. (2009; Calus, 2010) as they reported that partitioning into a complex model for non-additive effects is unnecessary, as these often represent a relatively small proportion of the total genetic variance. Results from our findings and other studies (Allison *et al*. 2009; Hill *et al*. 2008; Wittenburg *et al*. 2011) suggest that even when non-additive genetic effects are present, the total genetic variation may be explicable to a large degree by additive genetic variance (Pirchner 1983).

Results documented here are based on the additive genetic relationship information. Even though the data set used here is from a simulation study, it does provide a practical illustration of the methods presented. Given advances in molecular technology it is now easy to have genome-wide scans with more than one million SNPs for developing additive and non-additive relationship matrices with information from molecular markers. Genomic information can provide a more accurate representation of the relationships between individuals rather than using relationships based on pedigree information only. Gianola *et al*. (2011) reported that the use of genomic relationships led to a more reliable prediction of phenotypes than the use of pedigree information. The relative increase in strength of association, as measured by the correlation, is much larger in those predicted from genomic information than those from pedigrees. The same conclusion has been portrayed by Habier *et al*. (2007).

Traditionally, statistical theory and algorithms have been well developed for linear relationship models. However, quite often the relationship between variables requires nonlinear methods to allow for successful prediction of properties of interest (Hoffman *et al*. 2008). However, linear and nonlinear parametric statistical approaches have limited flexibility for modeling the high-order, non-linear interactions that may be important in complex traits (Gianola *et al*. 2006; Moore 2010). On the other hand, neural networks have the potential to capture non-linear relationships and may be useful in the study of quantitative traits under complex gene action, given suitable inputs. A nonlinear transformation (the hyperbolic tangent sigmoidal used herein) in $\hat{t}_i = g\left\{\sum_{j=1}^{s} w_j^{'} f_j\left(\sum_{k=1}^{R} w_{jk} p_{ik} + b_j\right) + b\right\}$; modifies the connection strength between additive relationship (Cholesky factor decomposition) and phenotype in an adaptive manner underlying the potential for an improvement in predictive ability (Okut *et al*. 2013). Our results revealed that the sigmoidal-type activation function used in the hidden layer in BRANN models outperformed the linear models. The predictive ability of BRANN architectures with nonlinear activation function were substantially larger than the linear models for the scenarios considered, except for σ_a^2 / σ_G^2 =0.1 in the Pop1. In a recent study, it was shown that non-linear neural networks outperformed a benchmark linear model when predicting phenotypes, especially in inbred wheat lines where cryptic gene actions and interactions are expected (Gianola *et al*. 2011).

The Levenberg-Marquardt algorithm was adopted to optimize weights and biases because previous evaluations with networks used a smaller number of weights to indicate that it was a suitable method (Demuth *et al*. 2009). However, the Levenberg-Marquardt is sensitive to initial values of weights as well as outliers in the data. These may lead to a overfitting problem in ANN. In the training process, overfitting often occurred, leading to loss of generalization of the predictive model. Bayesian regularization proposed by MacKay (1992) was used to avoid over-fitting and improve generalization. Adding Bayesian regularization to the Levenberg-Marquardt enables it to overcome the problem invoked in interpolating noisy data and overfitting. Since evidence provide an objective Bayesian criterion for stopping training, they are difficult to overtrain (Winkler & Burden 2004). The problem of overfitting and overtraining are also dealt with by this method so that the production of a definitive and reproducible model is attained.

Because highly parameterized models are penalized in the Bayesian approach, we were able to explore complex BRANN architectures. The complexity of a network is related to both the number of weights and the size of the weights. A model selection criterion related to complex BRANN is concerned with the number of weights. The more weights there are, relative to the number of training cases, the more overfitting amplifies noise in the target variables. For the networks trained with Bayesian regularization, we examined how

the effective number of parameters *γ* varied with architecture. As shown in Table 2, even though the fold number of parameters varied from about 501 to 2009 in Pop1 and from 1501 to 6009 in Pop2, the effective number of parameters varied only from 199.7 to 252 and from 665 to 848, for σ_a^2 / σ_G^2 =1 on average, indicating the effect of regularization was effective. The weight decay penalty term in objective function, $F = \beta E_D + \alpha E_W$ in BRANN causes the weights to converge to smaller absolute values than what they otherwise would. Thus, the effective number of parameters used in the models is less than the number of weights, as some weights do not contribute to the models. In the linear model this form of weight is equivalent to the ridge regression.

Conclusions

The Bayesian learning with a nonlinear model outperformed the linear model when the entire or considerable part of the total genetic variance was due to additive genetic effects. This was evident in our study when 50% or more of the total genetic variance was explained by additive genetic effects. BRANN architecture with a nonlinear activation function and linear models had similar predictive ability when the entire (σ_a^2 / σ_G^2 =1) or a considerable part (σ_a^2 / σ_G^2 = 0.9) of the total genetic variance was due to additive genetic effects. ANNs are a promising tool to handle complex data situations. Bayesian regularization ANN allowed estimation of all connection strengths even when *n<<p*, and the effective number of parameters was much smaller than the corresponding nominal number. The optimal values of posterior distribution of the connection strengths in BRANN can be automatically obtained through Bayesian regularized methods. This facilitates the selection of regularization parameters, possesses good robustness and excellent fitting. These advantages might even be more pronounced if further research can be done concerning the interpretation of their parameters.

In summary a feed forward ANN with BR was used to assess the performance and predictive ability of different nonlinear ANNs and linear models for complex traits with genetic architectures. It was shown that the inclusion of non-additive effects did not improve the predictive ability compared to purely additive models.

Author Contributions

HO conceived and performed computations, coordinate the study and drafted the manuscript; DG conceived, coordinate the study and provided critical insights and revised the manuscript; GJMR conceived, carried out the study, advised for computations, provided critical insights and revised the manuscript, KAW helped conceive and revised the manuscript.

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LIVESTOCK STUDIES

RESEARCH PAPER

Effects of Different Methods and Relationship Matrices on Reliabilities of Genomic Selection in Dairy Cattle

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Genomic relationship matrix
ssGBLUP
Reliability

Abstract
Since genomic prediction is widely used in dairy cattle, we aimed to evaluate the performance of pedigree based (ABLUP), SNP based (GBLUP) and single-step GBLUP (ss-GBLUP) methods with different sets of information in terms of reliability of genomic prediction. Four different methods were evaluated: (Method 1) ABLUP with all available phenotypes and pedigree; (Method 2) GBLUP with SNP genotypes and phenotypes of genotyped cows; (Method 3) single-step GBLUP with SNP genotypes, phenotypes of genotyped cows and all pedigree and (Method 4) single-step GBLUP with SNP genotypes, all phenotypes of both genotyped and nongenotyped cows and all pedigree. SNP based methods also used different genomic relationship matrices (GRMs) formed by different approaches: ***vanRaden***, ***Astle***, ***Yang*** and ***Endelman***. The simulated dataset replicates a common dairy cattle population.
A significant increase in reliability of prediction was observed in ss-GBLUP with all phenotypes and pedigree beside genotyped cows. This increase was apparent for both first lactation milk yield (LMY) and milk fat percentage (Fat%). Combining all available information with ss-GBLUP gave about 1.6 and 1.2 times higher reliabilities for LMY and Fat%, respectively, compared to those obtained from the other three methods.

Introduction

Livestock breeding, with the increase of today's nutritional problems, has brought with it the need for rapid progress in production processes. The genetic gain of the population subject to selection must be achieved in a shorter time than with conventional methods such as progeny testing (PT) in dairy cattle. Genomic selection (GS) may shorten the generation interval (GI), an important factor in terms of genetic gain for a given time and GI in cattle is equivalent to an average of 5.5 years in classical progeny testing program. However, this period can be reduced by almost half with GS methods (Schaeffer 2006).

Genomic selection has been used since the beginning of the 21st century, but it can be observed that the number of genotyped animals has become more and more economical in the last decade in the countries that have access to the genotyping process (Wiggans et al. 2017). Although this is a preferred point, computational times seem to be a more restrictive problem in terms of efficiency than the times in the earlier methods. In other words, the technological increase in the available information has also created the need for advanced technology in calculations (computations/computational hardwares)(Tsurata et al. 2021).

One of the problems encountered in GS in some countries is that when the number of SNP is less than the number of genotyped animals, the genomic relationship matrices (GRM) turn into a singular (non-invertable) structure and they have to be combined with the A matrix and included in the analysis (Misztal et al. 2020). Genomic selection has come to a point where it can be preferred despite all the difficulties in the field of animal breeding, since it has the convenience of making a decision by taking blood even from the fetus or embrios.

Several methods have been developed for the formation of GRMs, but GRMs have been obtained mainly by using SNP information. In the context of the Human Genome Project (IHGSC 2001). Due to the fact that these SNPs are scattered throughout the genome, GS has been used in breeding value estimation as a useful method in today's conditions, as it is consistent with the

basic theories of quantitative genetics (Meuwissen et al. 2001).

This study aimed to investigate the effects of four methods with GRMs formed by different approaches on the reliabilities of genomic estimated breeding values (GEBV).

Materials and Methods

Data sets

The data considered in this study were simulated for 3 discrete generations. The structure of data is given in Table 1 and has been generated according to the optimized scenario for the first lactation of Holstein dairy cattle by the steps below and the simulation study was performed in R environment (R Core Team 2022). The assumptions were:

- First lactation milk yield and fat percentage distributed as:

$$MVN \sim \left(\begin{bmatrix} 11000 \\ 3.4 \end{bmatrix}, \begin{bmatrix} 7954975 & -37.7904 \\ -37.7904 & 0.0067 \end{bmatrix}\right)$$

- Heritabilities of the traits are 0.17 and 0.30, respectively,

Table 1. The structure of simulated data

	Pedigree Information	SNP-effect	Phenotype	#Cow	#Sire*
Gen 0[1]	Yes	No	No	2200	200
Gen 1	Yes	No	Yes	2200	200
Gen 2	Yes	Yes	Yes	2000	-

*No records, just have pedigree

[1]Gen 0-2: generation 0 to 2

- Genetic correlation between traits is -0.80,
- 3 generations data were simulated:
  - Base population: 2200 dams without phenotypes and without SNP-genotypes
  - First generation: 2200 cows with phenotypes, without SNP-genotypes, and they were the progeny of the dams in the base population,
  - Second generation: 2000 cows with phenotypes and with SNP-genotypes, they were the progeny of the dams(cows) in the first generation,
- Each dam has one and female progeny (cow),
- Sires have average of 10 progeny and no phenotypic record,
- 54K SNP were simulated and each SNP has only additive effect,
- SNPs were mapped with reference to the bovine genome,

Method

In this study, the reliabilities of genomic predictions using different methods with different GRMs (**Henderson**, **vanRaden**, **Astle**, **Yang**, **Endelman**) developed by Henderson (1976), vanRaden (2008), Astle and Balding (2009), Yang et al. (2010) and Endelman and Jannink (2012) were compared. GRMs are based on pedigrees and/or SNP markers and each matrix calculation has the property of being idenditical by descent or state with additive relations. The Henderson relationship matrix (A) was used in classical ABLUP and single-step-genomic-BLUP (ss-GBLUP) analyses, while the other matrices were used in genomic-BLUP (GBLUP) and ss-GBLUP analyses.

Henderson relationship matrix (A) performs calculations based on the probability of gametic identity. It takes into account that each animal inherits a set of chromosomes from its ancestors and performs calculations for sets of chromosomes from common ancestor(s). The other GRMs, on the other hand, encode the markers as “0” for major homozygotes, “1” for heterozygotes and “2” for minor homozygotes as the basic framework, even though they are specifically separated. This is obtained by subtracting the mean from the coded matrix and dividing the variance of the SNPs by the total variance of the SNPs according to the equal/different number condition.

The vanRaden-GRM has been used most widely in genomic selection studies. This matrix subtracts the marker matrix from the expected values. The expected values computed by each calculated SNP frequencies from the sample or assuming the base population are known SNP frequencies, and divides it by 2pq, considering that the variance of the SNPs is equal and multiplied by the 2pq coefficient (vanRaden 2008):

$$GRM = \frac{ZZ'}{2\sum_i^m p_i(1-p_i)}$$

The Z matrix is the centered marker matrix and is the substraction of the marker matrix from a matrix of expected values of the SNPs. In this GRM, the diagonal elements correspond to 1+f. This is indeed inclined and consistent with the theory of inbreeding, measure of homozygosity in an individual. Additionally, the mean of the diagonal elements corresponding to 1+f is calculated to be 1. The correlation of inbreeding coefficients obtained with this matrix and inbreeding coefficients obtained by the classical method was calculated as 0.63 (vanRaden 2008)

The Endelmen-GRM has almost the same structure as the vanRaden-GRM. The only difference is that instead of taking the expected value the matrix, it assumes that the expected values of the SNPs are 0.5. The operations used in a GRM of this structure are no different from those in the vanRaden-GRM. These GRMs appear as the most appropriate predictors when the mean squared error is considered under one condition, which is when the number of markers is greater than the number of genotyped animals (Endelman and Jannink 2012).

Astle-GRM approach takes the kinship coefficients into account in the calculations of the GRM and handles the loci one by one. Although this matrix layout is not considered very suitable in animal breeding, it is used in plant breeding or human genetics studies. One more iteration to make unbiased of p at the expected value 2p and the p's can be recalculated. Negative values in the diagonal elements of the G indicate that less alleles are shared than the expected in line with the given p's (Astle and Balding 2009):

$$GRM_{i,j} = \frac{1}{L}\sum_{l}^{L}\frac{(x_l - 2p_l 1)(x_l - 2p_l 1)^T}{4p_l(1-p_l)}$$

Yang-GRM follows an approach that is very similar to the Astle method. The difference between them is that for an unbiased estimate of the inbreeding coefficient in the diagonal elements, the variance of SNPs is considered to be different, so that each SNP variance is affected by p (Yang et al. 2010). They divided the matrix into two elements, diagonal and off-diagonal, the expected value of the off-diagonal and diagonal elements will be zero and 1, respectively:

$$\frac{1}{N}\sum\nolimits_i G_{ijk} = \begin{cases} \frac{1}{N}\sum_i \frac{(x_{ij} - 2p_i)(x_{ik} - 2p_i)}{2p_i(1-p_i)}, j \neq k \\ \frac{1}{N}\sum_i \frac{{x_{ij}}^2(1+2p_i)x_{ij} + 2{p_i}^2}{2p_i(1-p_i)}, j = k \end{cases}$$

In our study, the simulated data were subjected to multi-trait-BLUP analyzes.

The mixed model equation used is as follows (Mrode, 2014):

Here it is assumed that u's are u~MVN(0, g), and e's are e~MVN(0, R). R and g matrices are covariance matrices and the elements of the matrices are calculated with REML algorithms. It is also assumed that there is no correlation among genetic effects and the environmental effects:

$$cov\begin{bmatrix} g_1 \\ g_2 \\ e_1 \\ e_2 \end{bmatrix} = \begin{bmatrix} g_{11}G & g_{12}G & 0 & 0 \\ g_{21}G & g_{22}G & 0 & 0 \\ 0 & 0 & r_{11} & r_{12} \\ 0 & 0 & r_{21} & r_{22} \end{bmatrix}$$

The analyzes were carried out using the rrBLUP (Endelman 2011) and Sommer (Covarrubias-Pazaran 2016) packages. Genomic parameters were estimated with four different methods:

Method 1: ABLUP: used full pedigree and all available records in the data,

Method 2: GBLUP: used only SNP-genotypes and the phenotypes of genotyped cows,

Method 3: ss-GBLUP: used full pedigree, SNP-genotypes and phenotypes of genotyped cows,

Method 4: ss-GBLUP: used full pedigree, SNP-genotypes and all available phenotypes.

Heritability and genetic correlation were estimated and the reliabilities of breeding value estimates were compared. The reliability formula is given below and the accuracies are the square root of the reliability of the predictions:

$$REL_i = 1 - \frac{PEV}{Vg}$$

where,

REL_i = reliability of estimated breeding value of ith cow,

PEV = prediction error variance,

V_g = additive genomic variance.

$$\begin{bmatrix} \hat{\mu}_1 \\ \hat{\mu}_2 \\ \hat{u}_1 \\ \hat{u}_2 \end{bmatrix} = \begin{bmatrix} X_1^T R^{11} X_1 & X_1^T R^{12} X_2 & X_1^T R^{11} Z_1 & X_1^T R^{12} Z_2 \\ X_2^T R^{21} X_1 & X_2^T R^{22} X_1 & X_2^T R^{21} Z_1 & X_2^T R^{22} Z_2 \\ Z_1^T R^{11} X_1 & Z_1^T R^{12} X_2 & Z_1^T R^{11} Z_1 + G^{-1} g^{11} & Z_1^T R^{12} Z_1 + G^{-1} g^{12} \\ Z_2^T R^{21} X_1 & Z_2^T R^{22} X_1 & Z_2^T R^{21} Z_1 + G^{-1} g^{21} & Z_2^T R^{22} Z_2 + G^{-1} g^{22} \end{bmatrix}^{-1} \begin{bmatrix} X_1^T R^{11} y_1 + X_1^T R^{12} y_2 \\ X_2^T R^{21} y_1 + X_2^T R^{22} y_2 \\ Z_1^T R^{11} y_1 + Z_1^T R^{12} y_2 \\ Z_1^T R^{21} y_1 + Z_1^T R^{22} y_2 \end{bmatrix}$$

Results

Descriptive statistics of first lactation milk yields (LMY) and milk fat percentage (Fat%) of 4200 dairy cattles from 2200 and 2000 cows in generation 1 (Gen1) and generation 2 (Gen2), respectively, are shown in Table 2.

In dairy cattle, half-sib family structure is very common because a sire has the ability to mate with more than one cow in a given time. The probability of these siblings being geneticaly related to each other is 25% if it is considered with the Henderson-A. Even though this possibility changes with crossing over, the general expectation is in this direction.

Table 2. The descriptive statistics of simulated traits

Trait	N	Mean	Median	Min	Max	SD	CV%
Fat%	4200	3.40	3.40	3.12	3.69	0.082	2.40
LMY	4200	10980.95	10950.77	1595.36	20457.09	2700.168	24.50

LMY: lactation milk yield; Fat%: milk fat percentage; N: number of observations used; Min: minimum value; Max: maximum value; SD: standard deviation; CV%: coefficient of variation

Since SNP-based GRMs are based on observations rather than probability, they also take crossingover into account and it allows one to make more valid predictions. Comparisons of coefficient in different GRMs of a half-sib family with a random individual within the family are given in Table 3. Genomic relationship among halfsibs varies around the classical expectation of 0.25.

The heritability and genomic correlations are given in Table 4. All methods slightly underestimated the heritabilities, however underestimation was more drastic for genomic correlation. It can be attributable to the number of observation simulated in this study.

Table 3. Genomic relationships based on different GRMs for the animal F10_3 with its some halfsibs

i^{th} animal	j^{th} halfsib	Henderson	Endelman	vanRaden	Astle	Yang
F10_3	F181_3	0.25	0.238	0.238	0.232	0.232
F10_3	F489_3	0.25	0.247	0.247	0.239	0.238
F10_3	F608_3	0.25	0.199	0.199	0.198	0.198
F10_3	F729_3	0.25	0.231	0.231	0.221	0.220
F10_3	F930_3	0.25	0.270	0.270	0.265	0.264
F10_3	F1370_3	0.25	0.169	0.169	0.164	0.163

Table 4. Heritabilities and genomic correlations

Method		LMY	Fat%	r^G
ABLUP	Henderson	0.152	0.272	-0.617
GBLUP[1] or ss-GBLUP	vanRaden	0.155	0.282	-0.533
	Astle	0.151	0.279	-0.518
	Yang	0.153	0.275	-0.523
	Endelman	0.155	0.282	-0.533

LMY: lactation milk yield; Fat%: milk fat percentage; h^2: heritability; r^G: eklemeli genomic correlation; ABLUP: used pedigree and phenotypes (from generation 0 to 2); GBLUP: used SNP-genotypes and phenotypes (in generaion 2 only)
[1]GBLUP and ss-GBLUP produced the same results

The reliabilities obtained from four methods with different GRMs for the first lactation milk yield (LMY) and milk fat percentage (Fat%) are given in Table 5 and Table 6, respectively. For LMY, reliability estimates were close but lower using Method 2 and 3 (varied from 0.126 to 0.315) than that of obtained from classical approach (Henderson ABLUP) (0.281). However, Method 4 gave more higher reliabity estimtes (varied from 0.340 to 0.447) coparing to the other three methods. For Fat%, reliability estimates were close but higher using Method 2 and 3 (varied from 0.271 to 0.414) than that of obtained from classical approach (Henderson ABLUP) (0.362). However, Method 4 provided much higher reliability (between 0.406 and 0.496) compared to the other three methods.

Phenotypes of nongenotyped cows in the first generation improved the reliabilities of the estimations for genotyped cows in the second generation dramaticaly in the ss-GBLUP analyses (Method 4). Presence of the phenotype and pedigree information of the dams of genotyped cows were contributed significantly to the reliability of the predictions for the both trait evaluated. Moreover, ss-GBLUP with full pedigree and all available phenotypes produced higher reliabily estimates for Fat% with smaller standard deviation than for LMY with larger standard deviation. Combining all available information with ss-GBLUP gave about 1.6 and 1.2 times higher reliabilities for LMY and Fat%, respectively, compared to those obtained from ABLUP (Method 1), GBLUP (Method 2) or ss-GBLUP (Method 3) using only phenotypes of genotyped cows.

Table 5. Descriptive statistics of reliability estimates of genotyped cows from different methods for the first laktation milk yield (LMY)

	Mean	Min	Max	S.D.								
Henderson	0.281	0.206	0.302	0.009								
	Method 2				Method 3				Method 4			
	Mean	Min	Max	S.D.	Mean	Min	Max	S.D.	Mean	Min	Max	S.D.
vanRaden	0.272	0.145	0.315	0.013	0.272	0.145	0.315	0.013	0.417	0.348	0.447	0.009
Astle	0.262	0.126	0.309	0.016	0.262	0.126	0.309	0.016	0.412	0.340	0.449	0.010
Yang	0.264	0.139	0.302	0.012	0.264	0.139	0.302	0.012	0.412	0.346	0.439	0.008
Endelman	0.272	0.145	0.315	0.013	0.272	0.145	0.315	0.013	0.417	0.348	0.447	0.009

Table 6. Descriptive statistics of reliability estimates of genotyped cows from different methods for the milk fat percentage (Fat%)

	Mean	Min	Max	S.D.								
Henderson	0.362	0.305	0.383	0.009								
	Method 2				Method 3				Method 4			
	Mean	Min	Max	S.D.	Mean	Min	Max	S.D.	Mean	Min	Max	S.D.
vanRaden	0.373	0.284	0.414	0.012	0.373	0.284	0.414	0.012	0.464	0.413	0.495	0.009
Astle	0.367	0.271	0.410	0.014	0.367	0.271	0.410	0.014	0.461	0.406	0.496	0.010
Yang	0.363	0.273	0.399	0.012	0.363	0.273	0.399	0.012	0.461	0.411	0.487	0.008
Endelman	0.373	0.284	0.414	0.012	0.373	0.284	0.414	0.012	0.464	0.413	0.495	0.009

Using different GRMs, formed in four different approaches, did not make any significant effect on the reliabilities of genomic prediction. All four approaches (vanRaden, Astle, Yang, Endelman) yielded almost the same results.

Discussion

The genomic prediction reliability of the methods using GRM and only the phenotype of the genotyped cows was slightly higher for Fat% than that of the conventional ABLUP approach, but slightly lower than that for LMY. This could be due to the fact that Fat% was simulated with a low standard deviation and a high heritability, while LMY had a high standard deviation and a low heritability. Moreover, using only phenotypes of genotyped animals (GBLUP/Method 2) or all pedigree (ss-GBLUP/Method 3) did not make an noteworthy change in the results. However, a significant degree of superiority was found among the methods. Including phenotypes of nongenotyped animals into the analyses (ss-GBLUP/Method 4) dramatically improved the reliability estimates of genomic prediction. This results are consistant with previous studies (Christensen and Lund 2010; Gray et al. 2012).

Another important finding of this study is that the reliability of genomic prediction is almost the same regardless of which GRM (vanRaden, Astle, Yang, or Endelman) is used.

Conclusions

The ss-GBLUP method, which considers the entire pedigree, genomic information and phenotypes of genotyped and non-genotyped cows, provides higher realiability of genomic prediction compared to traditional BLUP (ABLUP) and the other methods that use only genotyped individuals.

Author contributions

All authors contributed equally to this study.

Conflicts of interest

The authors declares that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

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LIVESTOCK STUDIES

REVİEW ARTICLE

Develeopment of Sensory Organs and Its Effects on Growth in Chicks

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Abstract

Recently, alterations in poultry farming have been much more than other branches of agriculture. This case also achieves a great deal of success in terms of efficiency. Increasing productivity in broiler production is one of the most important issue and researches are increasing day by day. Both during incubation and after hatching, applications are aimed at increasing productivity, growth and development. In this study, the development of sensory organs in chicks and the effects of the applications through sensory organs are emphasized. Especially in recent years, studies on odor, colour and light have been increasing. In the chick sex posed to some applications during incubation, the effects of these applications after hatching have been observed in most studies. To increase these effects positively, more intensive research should be conducted and positive developments should be recorded in broiler production. Broiler development and growth occurs with feed consumption. The earlier the chicks are fed and the more feed is eaten during growth, the more production occurs at slaughter age. For this reason, the importance of the senses in terms of their effect on the feeding behavior is discussed in this study.

Introduction

The increase in the world population, climate changes due to global warming and pandemic diseases affecting many regions have brought the importance of plant and animal production, which is necessary for a healthy and balanced nutrition of people, to the agenda. Due to the global changes that have occurred for many years, researchers have worked on sustainable agriculture and livestock policies (Ordu and Zengin, 2020). Accordingly, developments in poultry farming are also quite high. Livestock is one of the sub-branches of the agricultural sector and provides raw materials to different industries. Animal husbandry is crucial for societies because it provides basic nutrients (Er and Özçelik, 2016). Main animal products (eggs, milk and meat) constitute the main protein sources that people should take for a balanced and healthy life (Ordu and Zengin, 2020). Poultry was domesticated about 8000 years ago (Webster, 2002). This is a great development for humanity and has had a significant impact on meeting people's protein requirements. Since its first domestication, chicken has been bred for meat and eggs, and recently much effort has been done to increase chicken production and yield. There are many studies on increasing feed consumption. Today, poultry farming, particularly egg and meat poultry, has become the most intensive animal production area with significant capacity increase (Nkukwana, 2018; Sarıca *et al.*, 2018; Sheldon, 2000; Waarst *et al.*, 2015). Although almost all of the chicken meat and eggs are obtained from the intensive production in our country, there is a demand for poultry products obtained from traditional production for various reasons (Sarıca *et al.*, 2020).

Chicken is reared in intensive conditions, either as flock in the hen house or in groups in cages. There is an increasing interest in the social behavior of these animals to encourage more effective flock management and to get more efficiency from them (Göger, 1994). Additionally, chicken density in the poultry is one of the important factors affecting the yield. To generate more income in broiler enterprises both in Türkiye and other countries, the density of poultry is frequently exceeded and problems may occur (Teke *et al.*, 2019).

Early studies on chick behavior focused on identifying which behaviors were instinctive and which were acquired behavior. In one study, chicks showed instinctively grooming and scratching on the ground between day one and day three after hatching (Göger and Yenice, 2018). Studies have shown that chicks instinctively fear stinging insects but try catching flies (Krause *et al.*, 2006). In another study (Thomas *et al.*, 1998); one day old chicks were exposed to the smell of feathers, faeces, sawdust and feed and it was investigated whether these odors effected the chicks. It has been observed that chicks exposed to fecal odor exhibit a more headshaking behavior than the others (Thomas *et al.*, 1998). Sneddon *et al.* (1998) studied on the scent of strawberry, applied to the shells of the hatched eggs, and the behavior of the chicks after hatching. They observed that the chicks spent more time on the strawberry scented litter than the normal litter in the hen, and this was significant. In a study conducted, at the 20th day of the embryo, chicks exposed to odors containing amyl acetate and dichloroethane in various concentrations were observed to wake up by shaking their heads and making a clap-like sound with their beaks (Tolhurst and Vince, 1976). In chicks, the senses other than the sense of taste are highly developed and directly affect the behavior of chicks such as hatching, finding, and consuming feed. In many studies, it has been determined that the chick inside the egg hears the sound of the chicken and the chick easily finds its own mother after hatching (Edgar *et al.*, 2015). However, some sounds are known to trigger hatching (Vince 1968b, a; Vince *et al.*, 1984; White, 1984; Rumpf and Tzschentke, 2010; Sleigh and Casey, 2014; Tong *et al.*, 2015). The ability to distinguish colors in chicken is highly developed. Their development is supported by periodically using lights of different wavelengths (Classen, 2003). Chicks can easily select the bait by touching the bait with their beaks or they try to recognize a foreign object with their beaks (Kutlu, 2015; Göger *et al.*, 2018). Although it is thought that the sense of smell is not developed in chicks, it has been observed that this sense has developed considerably in recent studies and applications have been made to increase feed consumption, particularly in broilers.

The determining factors in the selection of feed in chicken; taste and smell of feed, visual cues, learning ability, nutritional needs and social interaction (Forbes, 1995). Research on visual discrimination has revealed that chicken can distinguish objects through their shapes (Göger and Yenice, 2018). Portella *et al.* (1988) reported that broilers prefer larger particles with increasing age. It is also reported that the location of the feed in the cage is a visual factor for feed separation (Kutlu, 1993). It has been reported that chickens generally prefer brightly colored feeds, especially pink and red, and that color is a strong cue for chickens in learning about preference and hatred (Forbes and Covasa, 1995; Çadırcı, 2014). It has been reported that the taste and smell of the feed have an important role on the selection of the feed, but has a secondary importance compared to the visual reagents during the feed selection as a result of the few taste receptors in the tongue and palate of the birds (Kutlu, 1993).

Sensory Organs in Chicks

All senses are used to recognize feed and a loss of one sense results in loss of the ability to select and digest. While mammals depend primarily on taste in recognizing feed, birds use appearance and quickly learn to combine the metabolic consequences of feed eaten with the sensory characteristics of a feed (Forbes and Covasa, 1995). For this reason, the sense of sight is one of the most important sense organs for feed consumption in poultry. After the eggs are placed in appropriate incubation conditions, a third layer, the mesoderm, develops in the space between the endoderm and ectoderm layers in a short time. These three layers form the basic structure of different organs and tissues of the chick during embryo development (Elibol, 2009). Elibol (2009) reports that the eye socket and ear canal begin to form at the 24th hour of the embryo, the nose and wings are formed on the 3rd day, the eyes are colored, and the beak takes its normal shape on the 6th day of incubation. The sense of hearing emerges at the 12th day of the embryo (Cohen and Fermin, 1978; Hirokawa, 1978; Rebillard and Pujol, 1983; Whitehead and Morest,1985a, b).

Sense of Taste

In chicken, taste sensitivity is different for specific taste stimuli (Liu *et al.*, 2018). Taste is important in guiding nutritive choices and motivating food intake and the sensory organs for taste are the taste buds, that transduce gustatory stimuli into neural signals (Liu *et al.*, 2018). Nevertheless, the study conducted by Tuncel *et al.*, (1995) state that feed taste is not an important factor that can affect the feed consumption of chickens, they can change their feed preferences by effecting sense of taste. Although it was stated in the previous studies on taste that the development of taste sense in birds very low, studies carried out in recent years also reveal that chickens have a better taste system and the sense of taste develops more than previously estimates. Mammals have a more developed taste system than poultry. Because of birds have less taste receptors than mammals there is a

broad consensus that birds have a lower taste than mammals (Liu *et al.*, 2018). According to Berkhoudt (1985) and Shi and Zhang (2005), the number of taste receptors are 5-12 in daily chicks, 24 in chickens, 6974 in humans and 17000 in rabbits. It is supported by some studies that the number of taste receptors varies between 240-360 depending on the chicken breed (Ganchrow and Ganchrow, 1985; Kudo *et al.*, 2010). Chickens can distinguish bitter, sweet, salty and sour tastes from each other to a certain extent (Kare and Mason 1986; McKeegan, 2004; Forbes 2010). Gentle (1972) reported that chickens respond even to low-concentration stimuli of hydrochloric acid and acetic acid. Accordingly, recent findings in chicken taste buds and taste sensation indicating that the chicken taste organ is better developed than previously thought and can serve as an ideal system for multidisciplinary studies including organogenesis, regenerative medicine, feeding and nutritional choices (Liu *et al.*, 2018). Taste sensitivity for specific taste qualities may be altered under certain conditions (Liu *et al.*, 2018).

Sense of Hearing

The sense of hearing in chicks is well developed. The inner ear is a complex three-dimensional structure that contains the auditory and vestibular sensory organs, which are the first step in the transduction of sound, balance and motion stimuli (Neves *et al.*, 2013). A mature inner ear is a complex labyrinth containing multiple sensory organs and nonsensory structures in a fixed configuration, and any perturbation in the structure of the labyrinth will undoubtedly lead to functional deficits (Wu *et al.*, 1998). It has been observed in studies that the chick embryo begins to hear on the 12th day of incubation (Cohen and Fermin, 1978; Hirokawa, 1978; Rebillard and Pujol, 1983; Whitehead and Morest, 1985a, b). There is some evidence of incubation period interactions between chicken and embryo. One day before the chick hatches, the hen and chick begin to communicate vocally, and this communication increases as the hatching time approaches. It has been observed that the cackling sound of chickens reduces the danger warning. Studies showed that the newly hatched chicks ran toward the box containing a chicken and several chicks, despite they did not see any chicks or chickens. Although chicks may recognize their own hens in different ways, hearing is one of the important factors. The chicks could find their own hens in the dark room even when the own broody hen was replaced with another one. When the brooding was hidden in different ways, the chicks somehow managed to reach their own hens (Edgar *et al.*, 2015). There have been studies on the reactions of chicks to hearing behavior during hatching and after hatching. Making a “clicking” sound or making a hatching sound artificially at certain times during the hatching affects the hatching time and hatching distribution (Vince 1968b, a; Vince *et al.*, 1984; Rumpf and Tzschentke, 2010; Sleigh and Casey, 2014; Tong *et al.*, 2015). Additionally, if the eggs are kept touching each other in the hatching basket, it is stated that the hatching occurs earlier than those that do not touch each other (Vince, 1973). In a study, it was stated that different sound waves triggered the output (White, 1984). Similarly, Veterans *et al.*, (1998) stated that giving an electronic click sound during the hatching period accelerates chick hatching and hatching is completed early.

Sense of Sight

The sense of sight in chickens is relatively better developed compared to other living things. Manipulation of light intensity is an important management tool affecting broiler production and well being (Deep *et al.*, 2010). Lighting is a powerful exogenous factor in control of many physiological and behavioral processes, and light may be the most crucial of all environmental factors to birds (Olanrewaju *et al.*, 2006). The ratio of the eyes on both sides of the head is quite large compared to other living things. They have a viewing angle of 300 degrees. Apart from the ability to distinguish colors, the visual abilities of winged and humans are quite different from each other (Lewis and Morris, 2000). For poultry, light is an essensial part of the sense of sight, both as visual acuity and color discrimination (Manser, 1996). It is also stated in many studies that lighting provides significant improvements in body weight gain in broiler farming. In the first week, sufficient light should be provided in order for the chicks to find the feeder and drinker easily, to learn their place, to stimulate their feeding and drinking behavior and to ensure their activities in the first days, and then it should be reduced (Sarıca and Erensayın, 2009). Light wavelength strongly influences broiler behavior. The human eye can perceive a very narrow region between 400 and 700 nm wavelengths on the scale called electromagnetic spectrum, which is used to describe electromagnetic waves (North and Bell, 1990; Prescott and Wathes, 1999). However, Prescott and Wathes (1999) reported that unlike humans, birds can detect wavelengths up to 360 nm, Holden (1983) 350, Hogsette *et al.* (1997) and Prescott and Wathes (1999a) up to 320 nm. The wavelength (λmax) of human eye is most sensitive up to 555 nm, while that the chicken eye is 562 nm (Lewis and Morris, 2000). There is a superiority in favor of birds in terms of sensitivity to short (400–500 nm) and long (600–700 nm) wavelengths (Prescott and Wathes, 1999). Generally, bright light intensity encourages increased activity, while lower light intensity may cause cannibalism (Olanrewaju *et al.*, 2006).

Effects of Light Wavelength on Chick Development

Wavelenght, intensity, species and location of light play an important role in chick development and performance. Currently broilers are grown under low

light intensity, usually under green or blue light (Rierson, 2011). In the early stages of development, short wavelengths (blue, green) stimulate rapid development, whereas long wavelengths (orange, red) accelerate development and sexual maturity when approaching sexual maturity (Classen, 2003). Many studies show that broilers reared under blue or green light weigh more than those reared under red or white light (Cao *et al.*, 2008; Rozenboim *et al.*, 1999; Halevy *et al.*, 1998; Wabeck and Skoglund, 1974). Chicks exposed to purple or green (415 or 560 nm) light grow better than those exposed to red (>635 nm) or white light (<635 nm) (Foss *et al.*, 1972; Wabeck and Skoglund, 1974; Rozenboim *et al.*, 1999b).

Recent studies have shown that the wavelength or color of light affects the behavior (Manser, 1996), welfare (Manser, 1996; Classen, 2003) and performance (Prayitno *et al.*, 1997; Rozenboim *et al.*, 1999a, 1999b; Classen, 2003) of the birds. Lewis and Morris, (2000) reported that there is a negative interaction between body weight and light wavelength approximately 530–750 nm in broilers, and a decrease of around 50 g in body weight for every 100 nm increase in wavelength. Lein *et al.* (2008) stated that broilers had better weight gain and performance under dim light intensity. This is also supported by previous research (Quentin *et al.*, 2005; Blatchford *et al.*, 2009) that broilers reared under dim light are less active and thus have better body weight gain. Numerous studies have been conducted on the effect of light wavelength on broiler performance (Kondra, 1961). Researchers have often compared white light with blue, red, and green light. Green and blue light is believed to perform better due to their calming effect on broilers (Prayito *et al.*, 1997). It has been reported that short wavelengths (400– 450 nm) generally improve growth and feed efficiency (North and Bell, 1990, Prayitno *et al.*, 1997). Wabeck and Skoglund (1974) found that blue (470 nm) and green (530 nm) light provides the best feed usage in broilers. Similarly, Rozenboim *et al.* (1999a) reported that broilers reared in green (560 nm) light benefited better from feed compared to those reared in white, blue (480 nm) and red (660 nm) light. Smith and Philips (1959) found that broilers reared with green light benefited better from feed than those reared with blue, red and orange light.

Feed Preference of Chicks Under Different Color Light

Rierson (2011) conducted a study investigating the feed preference of broilers under different colors of light. The experiment was carried out with 40 newly hatched Cobb500 male chicks. In the 3.05 m x 1.62 m hen house, 4 feed boxes of 40.5 x 40.5 cm dimensions were placed aside and each box was illuminated with a different color light. The trial lasted 6 weeks; chicks were given powder feed under 4 different colors of light between 1 and 3 weeks, powder and pellet feed under 4 different colors between 4-6 weeks. Blue, green, red and white lights were used as light colors. Rierson (2011) stated that between the 1st and 3rd weeks, the chicks showed a preference for white light and did not prefer to consume feed under blue light. He observed that a small number of chicks were unstable between 1 and 3 weeks. At 4 and 6 weeks, the chicks were found to show a preference for pelleted feed and white light. They did not consume powder feed under green and blue light. Red remained the second color choice as in the first 3 weeks and the number of unstable animals was higher than at weeks 1 to 3. Heshmatollah (2007) discovered that when chickens are given different light intensities, their choices become difficult, but they prefer green light over red, orange, or yellow. The authors also concluded that broiler chicks prefer orange dyed feed at low light levels, but prefer green dyed feed at high light levels. In a study by Rierson (2011) that investigated broiler preferences for different color feeds under different color lights, it was observed that chicks under blue light showed a high preference for red dyed feed. They showed a greater preference for green feed under yellow light and very few chicks were unstable under yellow light. It has been observed that chicks under green light do not prefer blue feed, and the most unstable chicks are those under green light.

Effect of Light Intensity on Broiler Chicks

Prayitno *et al.* (1997) conducted a study on the effect of light color and intensity on behavior and leg problems in broilers. When the red light intensity increases; the time spent on sitting, walking, drinking water, wing stretching and aggressive behavior increased, and the number of chicks showing wing stretching and aggressive behavior increased as the blue light intensity increased. When the red light intensity increases; the time spent on napping, sleeping and pecking decreased, but when the blue light intensity increased, the time spent on these behaviors did not decrease. Prayitno *et al.* (1997) did not find a significant difference between light intensities and light colors in terms of live weight at the end of the experiment. However, they found that body weight gain was higher in blue light than in red light. Feed consumption and feed conversion ratios were the same in all 3 groups. Similar results were obtained with the aforementioned previous studies. Deep *et al.* (2010) conducted a trial on the effects of 4 different light intensities (1 lx, 10 lx, 20 lx and 40 lx) on feed consumption and body weight gain in broilers. In the measurements made on days 0, 7, 14 and 35, it was observed that the body weight gain was not affected by the light intensity. Deep *et al.* (2010) found that the feed efficiency of the animals exposed to 1lx light on the 7th and 14th days was lower than the other groups. According to other studies, it was found that light intensity did not affect feed consumption and feed efficiency (Skoglund and Palmer, 1962; Newberry *et*

al., 1988; Kristensen *et al.*, 2006; Lien *et al.*, 2007; Blatchford *et al.*, 2009).

Many studies have been conducted to investigate the effects of light on performance in birds. In particular, studies on light wavelength are quite numerous and the positive effect of wavelength on feed consumption in broilers has been supported by many researchers. Although the light color does not affect the feed consumption much, it has been stated that the chicks prefer white light more. It can be thought that the chicks prefer the bait because they see it better under white light. In studies with light intensity, it has been determined in many studies that light intensity does not have an effect on feed consumption and feed efficiency.

Sense of Touch

Poultry has a developed sense of touch. In newly hatched chicks, the behaviors of straightening their wings and rubbing on the litter are observed. These behaviors may be an indication of how developed their sense of touch is. The sense of touch is of great importance during the intake of feeds. The beak, which replaces teeth and lips in poultry, is an organ that easily takes up grain feeds, especially on hard ground (Kutlu, 2015). Poultry touch with their beak to recognize an object and try to understand what it is. Chickens become aggressive toward chickens that are later joined to a cage or cage where a social hierarchical order is established. Chickens in a flock need to get to know each other for social order to occur. This ability enables them to recognize only those chickens in the social order and to peck those in their subgroup (Göger *et al.*, 2018). It has been reported that the feed used is powder or pellet, which is a distinguishing feature for chickens and broilers prefer larger particles as age progresses (Portella *et al.* 1988). Feather trimming behavior is quite common in poultry. Since feathers play an important role in water and air insulation, Delius (1988) reports that the bird's aim is to make each feather function better by trimming its feathers with its beak. Clayton *et al.* (1991) argue that feather trimming behavior in poultry reflects a co-evolution between chickens and living parasites. Studies show the importance of beaks for chicks, trying to recognize objects by touching them, choosing feeds and helping them to find and consume the appropriate feed.

Sense of Smell

The olfactory systems (or sense of smell) has received greater study in birds than other specialized chemical senses (McKeegan, 2004). The role of olfaction on feed intake, as indicator for appetite in chickens, is still unclear (Te Pas *et al.*, 2020). Chickens can detect and respond to a wide range of olfactory stimulants (McKeegan, 2004). When the articles belong to 25–30 years ago are examined, it is stated that the sense of smell does not develop in poultry. However, according to recent articles on the sense of smell have shown that the smell of chicks is not so bad.

The number of olfactory receptors is 283 in chicks, 396 in humans, 1130 in mice, and 1948 in elephants (Araneda *et al.*, 2000; Niimura *et al.*, 2014). Smells may be repulsive to or attractive to animals. In a study conducted with 7-day-old chicks, it was observed that the chicks avoided cat odor (Fluck *et al.*, 1996). Likewise, the smell of blood is also repulsive by the chicks. In a study conducted with blood, it was observed that chicks showed blood avoidance behavior, and chicks that were presented with blood and red dye of the same color were visually similar, showed a direct fear reaction when approached the smell of blood, and did not give a negative reaction to the red dye (Jones *et al.*, 1979; Fluck *et al.*, 1996). Chickens raised in a vanilla-scented environment gave fewer fear signals when exposed to the familiar scent of vanilla when moved to another location (Jones *et al.*, 2002).

Early Exposure to Odor

It is widely known from many studies that animals remember the stimuli they were exposed to before birth/hatching. Chickens show various behavioral responses to many artificial and natural odors (Bang and Wenzel, 1985; Wenzel, 1987; Jones and Roper, 1997). Chicks learn odors even after hatching and remember being exposed to the same odor before hatching (Burne and Rogers, 1995; Minguez, 1997), thus avoiding predators (Fluck *et al.*, 1996), learning to feed (Gentle, 1985; Marples *et al.*, 1996) and avoiding harmful substances (Guilford *et al.*, 1987; Burne and Rogers, 1997; Marples *et al.*, 1997).

In the experiment conducted by Sneddon (1998), the exposure of the eggs to strawberry odor between the 15th and 20th days of incubation caused the chicks to spend more time in the strawberry-scented litter. When the taste preferences of the same chicks were investigated, no significant difference was found between strawberry and tasteless water in terms of consumption. In a study by Bertin *et al.* (2010) the eggs were exposed to two different concentrations of orange oil and natural vanilla blend odor during incubation. In the test, in which two forms of known feed with and without smell are presented; the group exposed to high concentration odor preferred odorless bait and this was found to be significant. The group exposed to low concentrations spent more time consuming scented feed.

Exposure to Odors After Hatching

Burne and Rogers (1996) developed a simple method for measuring chicks' responses to odors. Taking the advantage of day-old chicks' tendency to

stare at brightly colored objects, the authors recorded the chicks' responses to plastic beads paired with various fragrances. Observed headshaking and pecking behavior varied at different concentrations of odorants. Porter *et al.* (1999) conducted a study investigating the reactions of chicks to odors after hatching. During the application, the cotton tip of the ear swab was dipped in the scent of strawberry, orange and an odorless ear swab was used for control. It was observed that sleeping chicks shake their heads and bang their beaks together when exposed to the odor. Awake chicks did not show such reactions when exposed to the same odor. In another study by the same researchers (Porter *et al.*, 1999) demonstrate that the behavior of chicks exposed to 3 different scents (mint, lavender, orange) was observed by squeezing the scent from the side of their beaks while they were sleeping. Looking at the test results, it was found that the chicks exposed to the three other stimuli showed different reactions compared to the control group. Chicks showed the highest response to the smell of mint and this reaction was found to be significant. In the study carried out by Porter et al (1999) the chicks reacted the most to the smell of mint. Studies on smell have shown that when chicks are exposed to certain odors before they hatch, they remember those odors and these odors affect some behaviors after hatching. After hatching, chicks respond differently to different odors.

Discussion and Conclusion

Today, behavior has become a science. Knowing the important behavioral characteristics of chickens provides great convenience to for producers in the practices related to their care. The complex behavioral responses of chickens to alterations in the physical and sociological environment should be taken into account by the producers. Regarding to rearing healthy chickens, applications starting from the embryo period are of great importance. Recently, studies on the development of sensory organs in animals have been increasing. Until 25–30 years ago, there was not much research on the development of sensory organs in chicks; however, in recent years, the tendency toward this issue has increased and there are many studies on the development of sensory organs in chicks and their effects in chick performance and behavior. These studies are conducted with day-old chicks, as well as before the chick hatches. Particularly, the senses of hearing, sight and touch are highly developed; it is known that research on the sense of smell is increasing. The practices that the chicks are exposed to during and after hatching has many effects in terms of development and growth. In many studies, it has been observed that sensory organs in chicks have a significant effect on chick behavior and growth. In particular, the effects of the applications exposed during incubation on the chick during the period after hatching have been at significant levels.

Variables such as smells familiar to chicks, light colors and intensities that affect their behavior, sounds in the environment, and feed form affect feed consumption; behavior, growth and development of chicks. However, it is necessary to increase the number of studies on these subject and to contribute to increasing the benefits of broiler production in Türkiye.

Author contributions

All authors contributed equally to the study.

Conflicts of interest

The author declare no conflicts of interest.

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RESEARCH PAPER

LIVESTOCK STUDIES

The Effects of GnRH and hCG Administration on Pregnancy Rate in Postpartum Dairy Cows

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Abstract

This study was designed to determine the effects of GnRH administration alone at the time of artificial insemination (AI) or in combination with hCG 5 days after GnRH injection on conception rates in postpartum dairy cows. Cows in estrus, without any reproductive health problem, between days 70-120 postpartum were randomly assigned to 3 equal groups. Buserelin acetate (10 µg), was administered at the time of AI to Group I (n=40). Group II (n=40) was administered first with Buserelin acetate (10 µg) at the time of AI and then with 1500 IU of hCG on the 5th day after insemination. Group III was maintained for control purposes and did not receive any treatment. Pregnancy diagnosis was performed on day 30 post-AI by transrectal ultrasonography. Conception rates were 80% (32/40), 80% (32/40), and 57.5% (23/40) in Group I, II and III (P<0.05) respectively In conclusion, it was determined that the use of GnRH alone at the time of AI or additionally the use of hCG on the 5th day after AI increased the pregnancy rates in dairy cows between 70-120 days postpartum, however, there was no difference in pregnancy rates between these two treatments.

Introduction

One of the main goals of dairy farming is to ensure that dairy cows conceive in the optimal postpartum period, thus keeping the time between births within economically viable limits. The profitability of dairy production depends on the regular calving of dairy cows every 12 to 13 months. Therefore, the prolongation of this interval has adverse economic implications for dairy farmers (Boulton *et al*., 2017; De Vries, 2006; Jainudeen and Hafez, 2000).

The most common causes of infertility in cows are reported as delayed ovulation, anovulation and early embryonic death (Taponen, 2003). Ovulation induction protocols based on gonadotropin-releasing hormone (GnRH) and human chorionic gonadotropin (hCG) administration are widely used, which prevent ovulation delay and anovulation-related disorders and reduce hormone-related early embryonic deaths (Canadas *et al*., 2019; Kashyap *et al*., 2018; Taponen, 2003). Administration of GnRH analogs at artificial insemination (AI) induces ovulation by initiating a pre-ovulation LH peak and contributes to an increase in the secretory capacity of the corpus luteum (CL). Most previous studies indicate that GnRH analogues increase both the frequency and secretion of LH, increase the differentiation of theca-lutein and granulosa-lutein cells in the CL, can transform small luteal cells into large luteal cells, and thereby increase progesterone secretion and, chance of the survival of the embryo (Besbaci *et al*., 2020; Taponen *et al*., 1999; Willmore and Davis, 2019). It has been reported in some studies that GnRH administration at AI does not affect pregnancy rates (Gümen *et al*., 2011; Perry and Perry 2009). Inducing ovulation by injecting exogenous GnRH before the expected LH wave may leads to shortening of luteal function and adversely affects fertility (Taponen, 2003). Pursley *et al*., (1995) pointed out that induction of ovulation can causes smaller than expected follicles to ovulate. The effect of GnRH on

ovulation depends on many variables such as the presence of the LH wave during administration, the dose of GnRH (Sertkol and Sarıbay, 2017) and the time of administration (Mee *et al*., 1990). GnRH has been reported is capable of inducing the ovulation of follicles larger than 10 mm in diameter in cows (Valenza *et al*., 2012).

Having mainly an LH-like effect, and to a very limited extent FSH-like effect, hCG is used to support CL formation and increase the circulating progesterone level (Schmitt *et al.*, 1996). Administration of hCG during early pregnancy enables both ovulation of the dominant ovarian follicle and formation of an accessory CL. Hence, the level of plasma progesterone increases. hCG has a longer half-life than GnRH and can even induce responsiveness of follicles less than 10 mm in diameter (De Rensis *et al.*, 2010; Rajamahendran and Sianangama, 1992, Santos *et al.*, 2001). In cows administered hCG on day 5 after estrus, plasma progesterone levels rise up to day 13, which in turn increases embryo survival (Rajamahendran and Sianangama 1992, Santos *et al.*, 2001).

In this study, it was aimed to demonstrate the efficacy of GnRH and hCG, which are widely used to increase pregnancy rates in problematic herds, in healthy animals without reproductive, infectious or metabolic abnormalities and in which estrus were successfully detected. For this purpose, the effectiveness of a single dose of GnRH administration at AI and, in addition to this protocol, hCG administration 5 days after AI on conception rate were investigated. Thus, it was aimed to demonstrate the evaluability of these hormones as part of the routine AI protocol in healthy cows.

Material and Method

Animals, Management and Feeding

This study was conducted on a commercial dairy farm that housed animals in semi-open free stall barns and adhered to regular record-keeping rules. From the animals housed in the farm, 120 healthy Holstein cows, aged 3-6 years, with a body condition score of 3-3.25 were included in the study. Prior to the study, it was confirmed by clinical examination that the cows in the study were reproductively and metabolically healthy. The animals included in this study were did not receive any hormonal therapy for estrus induction. Due to the management strategy of the farm unit, estruses were not detected in cows before 70th day pp, since pregnancy is not desired before this day.

Throughout the study, the farm's routine management and nutrition schedule were followed. The cows were housed in free-stall paddocks and fed a total mixed ration (TMR). The composition of TMR is listed in Table 1. The animals were given ad libitum access to water and mineral blocks. Milking was performed twice a day, in the morning and evening, by the farm staff at a fixed system milking parlor. The average milk yield, day in milk (DIM) and lactation number of groups was given in Table 2.

Table 1. Feedstuffs (kg) in the total mixed ration.

Ingredients	Quantity (kg)
Corn silage	21
Wheat straw	4.5
Barley, flaked	1.6
Sunflower meal (28% CP)	3
Cottonseed meal (30% CP)	3.5
Wheat bran	1.3
Limestone	0.25
Salt	0.05
Vitamin mix	0.02

Estrus detection

Visual detection of estrus was done by experienced staff three times a day for at least 20 minutes. Estrus was characterized by cows showed restlessness, attempt to mount other females/ permit them to do so, licking and sniffing of external genitals, or the presence of vulvar mucus (Palmer *et al*., 2012). Cows showing signs of spontaneous estrus underwent ovarian examination by transrectal real-time ultrasonography using a 6–8 MHz linear array transducer (Falko, Pie Medical, Netherlands). Cows standing estrus with a follicle larger than 10 mm diameter which is known to have capacity of ovulate (Sartori *et al*., 2001) were selected for AI.

Groups, Artificial Insemination and Hormonal Treatments

Cows standing estrus were randomly assigned to three equal groups. Group I (n=40) received 10 µg of GnRH alone (Receptal®, 0.004 mg buserelin/mL, Intervet, France, intramuscularly) in AI. Group II (n=40) received 10 µg GnRH (Receptal®, 0.004 mg buserelin/mL, Intervet, France, intramuscular) in AI and 1500 IU hCG (Pregnyl® 3x1500 IU, MSD, Belgium) five days later. Group III (n=40) was maintained for control and did not receive any treatment during or after AI. AI was performed by the same veterinarian approximately 12 hours after the onset of estrus. Estruses did not show seasonal distribution and were observed all year round. However, to minimize the potential impact of the season, the AI was completed each month with approximately equal numbers of animals from each group.

Table 2. Mean values of some milk-related parameters in the groups.

	Group 1	Group 2	Group 3	P
Milk yield	20,31 ± 3,56	19,84 ± 4,11	21,03 ± 6,27	>0.05
Lactation number	2,64 ± 0,83	2,58 ± 0,92	2,61 ± 0,87	>0.05
DIM	93,49 ± 13,15	91,32 ± 11,58	92,63 ± 12,43	>0.05

Pregnancy Diagnosis

Pregnancy diagnosed 30 days after AI by transrectal ultrasonography. Pregnancy (conception) confirmed positive according to visualization of intact embryonic vesicle, embryo, and the heartbeat of embryo during ultrasound imaging. No other examination was done until the parturition. Pregnancy losses were determined by comparing conception and calving records.

Statistical Analysis

The average of the milk yield, lactation number and DIM data of the cows between the groups was determined by the one way ANOVA. The difference between the conception rates in the groups was made with the chi-square test. All statistical analyzes were performed using the SPSS 22.0 software package.

Results and Discussion

The pregnancy rates in groups are shown in Figure 1. Groups I and II significantly differed from the control group (Group III) for pregnancy rates (P<0.05), (Table 3). Single fetus abortion occurred at the second trimester of pregnancy in 2 cows, in Group 1 (1/40) and in Group 3 (1/40).

It was reported that the fertility of cows, which was around 90% in previous studies, decreased to approximately 70% on the 34th day of pregnancy due to embryonic deaths that may occur at a rate of 25% in the first three weeks of pregnancy (Fricke *et al.*, 1993, Mann and Lamming, 2001; Rosenberg *et al.*, 2003).

However, fertility rates have decreased as a result of breeding strategies that have increased milk yield over the years. To achieve the target of one calf per year from one cow, it is recommended to achieve conception within 83 days of postpartum. In this period shortened estrus and ovulation disorders may occur due to the high milk yield. Even if a successful conception is achieved, the pregnancy rate has decreased to 45% today with the effect of embryonic deaths (Walsh *et al.*, 2011). In a recent study, Rethmeier *et al.* (2019) suggested that the target of conception rate should be higher than 35% and the pregnancy rate should be higher than 24% in cow. In a study conducted in Holstein cows in Turkey, it was reported that the pregnancy rate at first insemination was 25.1% in Holstein cows. In the same study, it was reported that low fertility was caused by herd management and adaptation problems, therefore more attention should be paid to estrus detection and

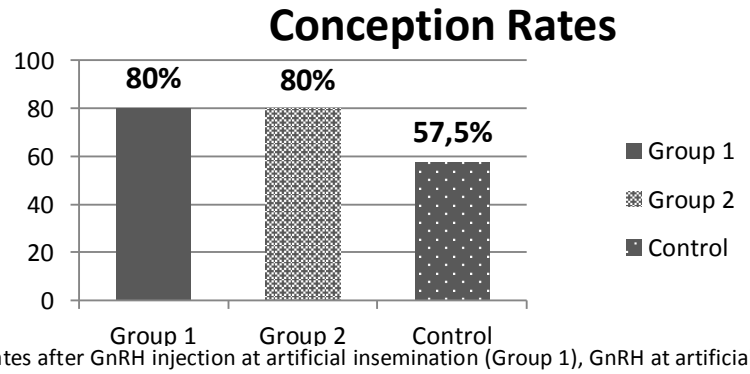

Figure 1. Conception rates after GnRH injection at artificial insemination (Group 1), GnRH at artificial insemination and hCG injection 5 days later (Group 2) and no treatment (Control).

Table 3. Conception numbers in groups.

	Group I	Group II	Group III	P
Conception (x/n)	32/40[a]	32/40[a]	23/40[b]	0.034

a, b: Groups with different superscripts in the same line display statistically significant differences (P<0.05)

insemination activities after the calving (Özdemir *et al*., 2022). In addition to individual milk yield and number of days in milk, average herd milk yield was also shown to be effective on conception. Rearte *et al*., (2018) reported that the pregnancy rate decreased by 1.3% in cows producing 8 kg/day more milk than the herd average in 63 DIM in high-yielding herds, and the pregnancy rate decreased by 14.8% in the same day in low-yielding herds. In the same study, it was reported that such a hazard of pregnancy effect was not observed on milk yield at 100 DIM in high milk yielding animals in herds with low milk yield. In this study, conception rates of all groups were found to be higher than in many other studies. First, the cows were medium to low milk yielding animals and are in the 70-120 DIM period (Table 2). We think that the harmful effects of lactation on conception was begin to decrease in this period. Secondly, the presence of dominant follicles was demonstrated by USG confirmation in cows whose estrus was detected by observation. We think that this double confirmation contributed to the increase in the pregnancy rate. In addition, the fact that the animals included in the study were completely healthy contributed to this result.

Steroid hormones are broken down faster in high-yielding dairy cows due to their high metabolic rate. Thus, it takes time for estradiol and progesterone levels in the bloodstream to increase. A slow rise in blood estradiol level prolongs the induction of the preovulatory LH surge, which both delays ovulation and reduces the quality of the oocyte due to prolonged exposure to LH surges (Wiltbank *et al*., 2012; Yeşilkaya and Erdem, 2021). When GnRH agonists are administered just before or during the LH surge, they can increase the pregnancy rate of cows by enhancing the spontaneous preovulatory LH surge (Morgan and Lean, 1993). GnRH has found wide use in cows due to its ovulation-stimulating effect and subsequent positive effect on luteinization (Rosenberg *et al.*, 2003; Wiltbank *et al.*, 2012). However, the effect of GnRH depends on the diameter of the preovulatory follicle present in the ovary during administration. It has been reported that the mean follicle diameter during ovulation in lactating cows is 17.2±0.5 (Sartori *et al*., 2004). In beef cattle, the pregnancy rate was 53% in the presence of follicles with a diameter of 14.5 mm, and 35% if ovulation was induced in the presence of a follicle diameter of 10.3 mm or less (Perry *et al*., 2005). When ovulation of a small follicle is induced, less estrogen is synthesized; CL volume and the ability to synthesize progesterone are insufficient (Vasconcelos *et al*., 2001). Keskin et al., (2016) reported that cows with a preovulatory follicle diameter between 13.5 and 17.5 have a higher chance of pregnancy. Colazo et al. (2015) reported that an ovulatory follicle diameter of 14 mm and above does not change the predicted pregnancy probability, but a higher rate of late embryonic/early fetal mortality can be observed in cows with an ovulatory follicle diameter greater than 20 mm. In this study, the lowest follicle diameter that could respond to GnRH injection was accepted as 10 mm. Mean follicle diameters of the groups were not noted. We administered GnRH (Buserelin acetate, 10 µg) to group I and group II during AI, and obtained a higher pregnancy rate in these groups compared to the control group (P<0.05). The conception rate, which was particularly low in the control group, may be due to the 10 mm criterion we have chosen. GnRH administration shortens the estrus-LH peak and estrus-ovulation intervals and induces ovulation within 8-12 h post-insemination (Elmore, 1989; Jainudeen and Hafez, 2000; Morotti *et al*., 2021). In this study, there may be animals whose follicle diameters have not yet reached the preovulatory size during AI. Ovulation can be induced in most of the cows in the GnRH administered groups, in accordance with the literature knowledge. However, the ovulation process would take longer time in animals in the same condition in the control group. This possibility may have had an effect on the conception process. However, in order to say that GnRH has an effect on conception, ovulation monitoring should be done, which was not done in this study.

Different pregnancy rates have been reported in studies examining the effects of GnRH analogues administration on pregnancy rates during AI. Shephard *et al.* (2014) reported that GnRH administration at AI increased the pregnancy rate by 11%. Shahneh *et al.* (2008) reported that administration of 15 µg of Gonadorelin during AI increased the pregnancy rate more than twofold compared to the non-administered control (55% vs 25%). Similarly, Iftikhar *et al.* (2009) found that administration of 50 µg Lecirelin acetate during AI increased the pregnancy rate 1.8 times (37.5% vs 68.75%). In the present study, we determined that the pregnancy rate of group I was 22.5% (or 1.39 times) higher than that of the control group (P<0.05). The pregnancy rate we obtained seems to be slightly higher than other studies. This is thought to be due to differences in the GnRH analogue administered, the dose of administration and the individual response of the animals. In this study, we selected healthy cows with moderate milk yield, in good condition, whose estruses were successfully detected, as we aimed to reveal the effects of GnRH and hCG in healthy cows. It can be said that the pregnancy rates obtained in this study reflect the pregnancy rates in optimal conditions.

It has been reported that administration of hCG to support luteal tissue after AI increases pregnancy rates (Ideta *et al.* 2003, Pandey *et al.* 2016). In contrast, some studies found no difference in pregnancy rates (Walton *et al.*, 1990, Niles *et al*., 2019) despite marked increase in serum progesterone concentrations with the administration of hCG in early luteal phase (Shams-Esfandabadi *et al.*, 2007). Zheng *et al*., (2021) reported that injection of hCG 5 days after AI was beneficial to the function luteal tissue and receptivity, although improvement in pregnancy rates was not-significant. In a study in which GnRH (Buserelin) was administered during ovulation in addition to hCG in the later period of the luteal phase (day 12), Paksoy and Kalkan, (2010)

reported that the administration of hCG was ineffective. On the other hand, Zolini *et al*. (2019) applied hCG treatment 5 days after insemination and found that the increase in pregnancy rate was related to the genotype of individual cows. In our study, conception rates of Group I and Group II was the same (P>0,05). It is very difficult to say whether the result obtained here is due to the ineffectiveness of hCG or for other reasons that we did not reveal. In order to make an undisputable comment on this issue, it was necessary to evaluate another group that was not injected with GnRH during AI, but was injected only with hCG. Our results show that both protocols have a significant effect in increasing pregnancy rates in healthy animals.

Conclusion

We conclude that administration of GnRH alone during artificial insemination (AI) and in combination with hCG 5 days after GnRH injection increases conception rates in healthy pp lactating cows. Both protocols can be used to increase the conception rate. The use of hCG alone is recommended to reveal its efficacy on conception rates in future studies.

Author contributions: All authors contributed equally to the study.

Conflicts of interest: The author declare no conflicts of interest.

Ethical approval: This study was conducted according to the 19/02/2020 dated and 2020/02-7 numbered approval of the Local Ethics Board for Animal Experiments. In addition, the authors declared that Research and Publication Ethical rules were followed.

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LIVESTOCK STUDIES

RESEARCH PAPER

The Occurrence of Arsenic, Cadmium, and Lead Residues in Cattle Feed Collected in Kırıkkale, Türkiye: A Preliminary Study

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Abstract

The aim of this study is to investigate the occurrence of arsenic (As), cadmium (Cd), and lead (Pb) residues in 15 cattle feed collected from dairy and beef cattle breeding farms located in 6 different districts (Kırıkkale Province, Yahşihan, Karakeçili, Balışeyh, Delice and Keskin) of Kırıkkale. The quantitative analysis of the As, Cd and Pb levels of the samples was analyzed by ICP-MS. Arsenic and Pb residues were found in all of the collected samples, while Cd residues were found in 13 (86. 67%) of the collected samples. In the samples with heavy metal residues; the mean value ± standard deviation for As, Cd, and Pb were found as 0.1475±0.1060 mg/kg, 0.0382±0.0079 mg/kg and 0.1944±0.1074 mg/kg respectively. The maximum tolerable limit in cattle feed is 2 mg/kg for As, 5 mg/kg for Pb, and for Cd in cattle feed is 1 mg/kg for Cd according to an official announcement (2014/11) of the Ministry of Agriculture and Forestry Turkey. The results of this study showed that detected As, Cd and Pb levels in cattle feeds were below the tolerable limit. Therefore, it is suggested that the heavy metal pollution in terms of As, Cd, and Pb in feed does not pose a risk for cattle farming in Kırıkkale province.

Introduction

The term heavy metal can be used for elements having an atomic mass of 200 or more like mercury, thallium, lead and bismuth. In practice, heavy metals include metals that is clinically cause undesirable effects and potentially dangerous for health (Baldwin and Marshall, 1999). Some metals such as cobalt, copper, chromium, and iron are necessary to maintain many biochemical and physiological processes in living organisms including animals and plants. On the other hand, metals such as As, Cd, Pb, and mercury (Hg) do not play a role in biological functions, and these undesirable substances in animal feeds are called contaminants. These metals threaten public health at high doses, and may even cause organ damage at low doses (Hejna *et al*., 2018). It is known that especially Cd, Pb and less commonly Hg cause kidney damage (Garcia *et al*., 2018). There are also findings reporting that these heavy metals cause cardiovascular diseases and chronic heart diseases (Chowdhury *et al,*. 2018).

The source of heavy metal accumulation in the environment is through natural and anthropogenic sources. Anthropogenic activities such as rapid industrialization, overgrowing urbanization and environmental manipulation lead to environmental pollution with heavy metals. Emissions from rapidly expanding industrial areas, mine residues and the accumulation of heavy metals through the disposal of high metal waste pollute the soil and water (Verma *et al*., 2018).

Arsenic is ubiquitous element, and has many forms on earth. Arsenite and arsenate forms of As are highly toxic to human and animals health. Exposure to As

occurs in many ways. Industrial activities are one of the most important reason of As exposure. Also drinking water and feed may contaminated with As that found in wood preservatives, herbicides, pesticides, fungicides and dyes (Engwa *et al*., 2019; İriş and Çınar, 2019). Cadmium is another highly toxic metals both for humans and animals. It accumulates in the soil and contaminates pastures (Reis *et al*., 2010). Cadmium is released into the environment through coal and waste incineration, disposal of metal-containing products, and the use of phosphate fertilizers (İriş and Çınar, 2019). Some plants can accumulate Cd. Therefore, they can be toxic to animals that consume these plants. While the performance of cattle decreases when consuming 5-10 mg/kg of Cd, it affects the health of animals above 30 mg/kg (Reis *et al*., 2010). Lead is one of the most important contaminants because of its largely use in industrial products. Exposure to lead can occur in many ways such as drinking water, food, cigarettes, industrial processes and domestic sources (Engwa *et al*., 2019). Because of eating habbits cattle reported widely in terms of Pb toxication. Lead toxication mostly affect gastrointestinal, central nervous and hematological systems in living organisms (Thompson, 2007).

Heavy metals can be found as residues in soil, ingestion of plants, sewage wastes, fertilizers and feeds. Apart from these, mine wastes, gasoline with Pb, paints and pesticides are among the causes of metal pollution (Kanbur and Tekeli, 2017). Considering all these reasons, it is important to investigate the level of undesirable substances especially in terms of heavy metals. The aim of this study is to investigate the presence of As, Cd, and Pb metal residues in cattle feed collected from some ruminant farms in Kırıkkale province.

Materials and Methods

Sample Collection

The feeds were collected from local enterprises of dairy and beef cattle breeding farms in Kırıkkale Province. A total of 15 total mixed rations samples were collected from the Districts of Kırıkkale Province (4 samples), Yahşihan (3 samples), Karakeçili (1 samples), Balıseyh (1 samples), Delice (2 samples), and Keskin (4 samples). The feed samples were total mixed rations that were prepared by the farm owners. Environmental differences such as the distance of the enterprises from each other were taken into account in the selection of the enterprises. Feed samples were collected in May, 2021. Containers and bags conforming to standards were used for sampling. The samples were stored at +4^{o}C and analyzed in the Nanolab Special Food Control Laboratory for the necessary analysis as soon as possible.

Sample analysis

The As, Cd, and Pb levels in feed samples were analyzed according to the procedures ISO27085-2009 E modified by Nanolab Special Food Control Laboratory In-house Method- "K.SOP.148"using, inductively coupled plasma mass spectrometry (ICP-MS, AGILENT/7700e, Japan). All chemicals were of analytical reagent quality. Arsenic, Cd, Pb standard stock solutions (1000 mg/L) (Merck) were used (0.05, 0.1, 0.5, 1, 5, 10, 20, 50 µg/L). For calibrations 50, 20, 10, 5, 1, 0.5, 0.1, 0.05 µg/L (ppb), appropriate solutions were made using stock standard solutions.

Table 1. Arsenic, cadmium and lead levels in cattle feed samples (mg/kg).

		Contamination (mg/kg)			
Metal	**Sample number**	**Positive**	**min-max**	**Mean ±SD**	**Samples exceeding legal level**
	n	***n (%)***			***n* (%)**
Arsenic	15	15 (100%)	0.027-0.385	0.1475±0.1060	-
Cadmium	15	13 (86.67%)	0.023-0.053	0.0382±0.0079	-
Lead	15	15 (100%)	0.050-0.403	0.1944±0.1074	-

n: sample number, min: minimum level, max: maximum level

Data were given as mean ± SD. The maximum tolerable value is 2 mg/kg for arsenic, 5 mg/kg for lead, 1 mg/kg for cadmium in catle feed (Republic of Turkey Ministry of Agriculture and Forestry No: 2014/11 Undesirable substances in animal feed)

Approximately 0.5 g of the prepared test sample was weighed into a vessel. Then, 8 ml HNO_3 (%65, Merck) and 2ml H_2O_2 (%30, Merck) were added, mixed, and burnt at microwave oven for 45 minutes. Samples were cooled and diluted to 50 ml using de-ionized water and shaken several times. It was filtered with cellulose asetate filtrate into a 15 ml falcon tube. After filtrated samples were applied to the ICP-MS device. A blank test tube was also prepared with the same procedure as for feed samples. Arsenic, Cd, and Pb heavy metals in test samples were measured at 75, 111, and 208 mass, respectively. The results were calculated according to the calibration.

The data of the study are given as mean ± standard deviation (SD). The evaluation of the data is based on the Republic of Turkey Ministry of Agriculture and Forestry No: 2014/11 Notification of undesirable substances in animal feed.

Results

Arsenic and Pb residues were found in all of the collected samples, while Cd residues were found in 13 (86.67%) of the collected samples. In the samples with heavy metal residues; the mean value±standart deviation for As was found as 0.1475 ± 0.1060 mg/kg ranging from 0.027-0.385 mg/kg. The mean value±standart deviation for Cd was found as 0.0382 ± 0.0079 mg/kg ranging from 0.023-0.053 mg/kg. The mean value±standart deviation for Pb was found as 0.1944 ± 0.1074 mg/kg ranged from 0.050-0.403 mg/kg. The maximum tolerable limit for As in feed is 2 mg/kg, for Pb in feed stuff is 5 mg/kg, for cadmium in cattle feed is 1 mg/kg (The Republic of Turkey's Ministry of Agriculture and Forestry Announcement No: 2014/11). The results of this study showed that the detected values were below the tolerable limit (Table 1).

Discussion

Heavy metals are extensively dispersed into the environment along with industrial wastes, as a result, they bio accumulate in the environment. These metals are known as environmental pollutants that pollute natural environments such as water, air and soil, and have toxic effects on humans through animals, plants and the food chain. Arsenic, Cd and Pb are known as the most exposed heavy metals (Yiğit ve Kabakçi, 2018). The main source of As accumulation in agricultural soil is the use of pesticides containing As (Adriano, 2001). Pesticides are commonly used in orchards. High levels of As have been found in pastures that were previously used as orchards (Willett *et al*., 1993).

Arsenic levels in grain and protein feed mix were reported 10 times higher than those in homegrown feed, probably due to the addition of other ingredients to the feed mix by the feed mill (for example, mineral mixtures containing P) (Li *et al*. 2005). The highest As concentration was reported in mineral mixtures by Li *et al*. (2005). However, acute poisonings with As in cattle are rare (Yiğit and Kabakçi, 2018). In a study conducted in China, the average As content in cattle feed samples was found to be between 0.80-1.38 mg/kg (Zhang *et al*., 2012). Cang *et al*. (2004) reported 0.13 mg/kg average As content in cattle feed in Jiangsu province. Wang *et al.* (2013) determined an average value of 0.3 mg/kg As in 35 dairy cow feeds in Jiangsu province of China. Nicholson *et al.* (1999) reported an average of 0.37 mg/kg in pelleted dairy cattle feed, 0.49 mg/kg in pelleted beef cattle feed, <0.10 mg/kg As in oat-barley mixture collected from the Wales province of England. Li *et al*. (2005) determined As values of 433 µg/kg in dairy feed, 490 µg/kg in dairy feed (substitute) and 450 µg/kg in heifer feed. In the presented study, it was revealed that As levels (0.1475 ± 0.1060 mg/kg) were higher than cattle feeds in the Izmir region declared an average value of 0.046 ± 0.127 mg/kg (Güvercin, 2010), and lower than dairy feeds in Jiangsu city of China with an average value of 0.3 mg/kg As (Wang *et al*., 2013). Arsenic levels of this study did not exceed the legal level recorded as 2 mg/kg.

High concentrations of Cd (up to 10 mg/kg) have been found in forages grown in fields near industrial zinc coating areas where urban sludge is used as fertilizer (Smith, 1986). Cadmium is a human carcinogen associated with lung and prostate cancer therefore it should not be found in feed and feedstuff (Hadjey and Trombetta, 2004). According to the legal restrictions in Turkey Cd in cattle feed should not be more than 1 mg/kg. Nicholson *et al.* (1999) found an average of 0.37 mg/kg Cd in dairy cattle feed, 0.49 mg /kg in pelleted cattle feed, and <0.10 mg/kg in oat barley mixture. Li *et.al*. (2005) determined an average of 51 µg/kg in dairy feed, 159 µg/kg in dairy feed (substitude), and 63 µg/kg Cd in heifer feed. Li *et al*. (2010) totally collected 210 feed samples from poultry, cattle, pig, and sheep feed and declared that over % 88.6 of the samples contain Cd residue. The authors reported that the average value of Cd residue in 71 cattle feed collected from Bejing and Fuxin cities in China was 2.79 mg/kg. Güvercin (2010) collected 216 cattle feed from 3 different areas (Kemalpaşa, Torbalı and Kiraz) in Izmir province and detected an average of 0.07 ± 0.06 mg/kg Cd. Cerit *et al.* (2007) stated that the mean Cd value was 2.016 ± 1.46 mg/kg in 4 cattle feed samples collected from 4 different regions (Bursa-İstanbul Highway, Bursa-İzmir Highway, Uludag University Faculty of Veterinary Medicine, Demirtaş) in Bursa. Wang *et al.* (2013) determined that there is 0.42 mg/kg Cd in 35 dairy cow feeds in Jiangsu province of China. The occurance of Cd in feed may be due to the addition of Zn sulfate, phosphates and Zn oxide supplements to the feed as Cd generally found in these mineral supplements (Li *et al.,* 2010). Thus some resaerchers demonstrated 1–3.6% of Cd in the Zn sulfate additive which shows that phosphate and zinc sulfate were the main sources of Cd in the animal compound feeds (Nong, 2002; Zhong and Jiang, 2005). In the presented study, it was revealed that Cd levels (0.0382

± 0.0079) were lower than cattle feeds in Izmir region that declared an average value of 0.07 ± 0.06 mg/kg (Güvercin, 2010) and in Beijing and Fuxin city of China with an average value of 2.79 mg/kg Cd (Li *et al.,* 2010).

Animal species have different susceptibility to Pb poisoning. Cattle and sheep are considered to be more susceptible to Pb poisoning (Şanlı, 2002). Wang *et al*. (2013) determined an average value of 5.2 mg/kg Pb in 35 dairy cow feeds in Jiangsu province of China. Nicholson *et al.* (1999) found an average value of 2.00 mg/kg Pb in pelleted dairy feed, <1.00 mg/kg Pb in pelleted cattle feed, 1.16 mg/kg Pb in the oat-barley mixture, Cerit *et al.* (2007) detected an average value of 0.902 ± 0.517mg/kg Pb residue in cattle feed. Güvercin (2010) detected an average value of 0.28 ± 0.52 mg/kg Pb, which was higher than the average Pb value of 0.1944 ± 0.1074 mg/kg that was found in this study.

Conclusion

The results of this study showed that the detected values of As, Cd and Pb were below the tolerable limit for cattle feed. For this reason, it has been revealed that heavy metal pollution in terms of As, Cd and Pb do not pose a risk to use cattle feed. Apart from this, it should be taken into account that metal pollution can be encountered in many situations. A more comprehensive analysis should be made not only of feed but also of other nutrients such as water consumed by the animal. When heavy metals are collectible materials, analyzes in terms of metal pollution in the animal's hair, milk, as well as in blood will contribute to the protection of human and animal health.

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LIVESTOCK STUDIES

RESEARCH PAPER

Superovulatin Peformance and Embryo Recovery in South Anatolian Red Cows

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Abstract
South Anatolian Red (SAR) cattle which breeds from Mersin to Şanlıurfa with centered Kilis in the region of South Anatolia, is one of native breeds of Türkiye. The aim of this study is to evaluate the superovulator response and embryo recovery rates after the superovulation protocol applied to the SAR cattle. For this purpose, 10 donors were selected from the conservation herd in the institute. FSH was performed to donors in decreasing doses twice daily over a 4 day period. Before uterine flushing, the ovaries were examined by ultrasound and the structures on them were recorded in order to determine the superovulation response. Each cornu uteri was flushed with foley catheters using a 3-way Y catheter. In the evaluation of embryo recovery, embryos were classified as transferable and non-transferable embryos. The response to superovulation was found as average 7.8 corpus luteum and 2.8 anovulatory follicule for 10 donors. After uterine flushing, 5 UFOs, 3 non-transferable embryos and 2 transferable embryos were obtained from 10 animals. Although the superovulation response was good, the reason for the low embryo recovery rate may be due to the low reproductive performance in SAR cattle. It has been concluded that the hormonal imbalance of these aggressive animals and the difficulties that occur during uterine flushing affect embryo recovery. It was thought that more studies should be done, different superovulation protocols should be tried and OPU technic should be evaluated in order to increase the rate of transferable embryos from SAR cattle. In addition, it was concluded that different techniques should be tried while performing uterine flushing procedures.

Introduction

Animal-originated foods have been one of the fundamental nutritient sources for humankind for ages. Moreover, this condition will maintain as long as human being exists. As a result of the studies, although the productivity increase in the production of animal origin foodstuffs an increase is achieved, some adverse circumstances may also occur. At the beginning of these negativities is the decrease in the sustainable production of local genetic resources. Therefore, the importance of protecting local genetic resources, the essential elements of diversity, increases constantly.

The South Anatolian Red (SAR) breed, which has the best performance in milk yield among the domestic breeds in Türkiye, differs from other breeds in terms of adaptation to warmer and harsher environmental conditions.

This breed is also privileged by having resistance to diseases, especially blood parasites. Formerly, it was reported that the SAR breed was spread over a wide region in the southern region of Türkiye, from Içel in the west to Sanlıurfa and even Hakkari in the east (Göncü, 2005). In 2018 Animal Information System records, the population size of SAR in Türkiye was reported to be smaller than 5500 heads (HBS,2018 October records).

SARs are very noble and elegant-looking cattle. SAR breed has a short neck, an erect head, and prominent and high withers. Moreover, the body is narrow and relatively short. Body color in SAR cattle is generally in combinations ranging from light yellow to dark chestnut red (Yarkın, 1961; Pekel et al., 1990). In SARs, the mean birth weight was variation from 19 to 27 kg. It is reported that an adult's live weight average is 314 kg, but it can

vary between 250 and 424 kg depending on feeding and maintenance conditions (Yarkın, 1961).

Embryo transfer is the process of transferring the embryo or embryos collected from the genital tract of the female donor to one or more synchronized females (Kanagawa et al., 1995; Sağırkaya, 2009). In other words, the transferring of embryos obtained through selected cows and bulls, whose genetic capacity and yield levels have been determined, to carrier cows (Kaymaz, 2015).

One of the most significant techniques to provide rapid genetic progress in dairy cattle and to increase the number of female and male animals with high genetic merits in the herd is embryo transfer applications (Akyol, 2001; Pabuçcuoğlu, 2013, Seidel and Seidel, 1991; Tekeli, 2010). In addition, embryo transfer is one of the leading modern techniques used to increase the success of animal breeding in the most effective way (Bülbül and Dursun, 2005). While only one calf can be obtained from a cow per year, the number of offspring obtained during a year can be increased five times by embryo transfer (Seidel and Seidel, 1991; Tekeli, 2010). The most critical aim of the embryo transfer technique, which consists of a series of biological processes, is to obtain more offspring from high-quality animals, which are healthy and with high genetic capacity, by obtaining high-quality oocytes and embryos (Santos et al., 2008).

The embryo transfer technique is treated by fresh transfer or transfer of frozen embryos that do not have a detrimental effect on embryo viability. The embryo transfer process has become much more accessible through one-step freezing methods using cryoprotectants with high molecular weight (such as sucrose) that do not enter the cell or low molecular weight (Ethylene glycol) that can enter the cell (Palasz and Mapletoft 1996, Massip 2001).

This study aimed to evaluate the success of the superovulation protocol and the embryo recovery rates in SAR cattle. One of the most important reasons for this study is that there has been no previous study on this subject with the SAR local cattle breed. For this purpose, this study will make a great contribution to crossbreeding studies of SAR cattle through the determination of superovulation performance. Thus, crossbreeding studies will be accelerated not only by artificial insemination but also by the embryo transfer method.

Materials and Methods

Ethics Committee approval was obtained for the present study from Adana Veterinary Control Institute Experimental Animals Local Ethics Committee with the decision dated 23.09.2019 and numbered 2019-7/2428.

Donor Selection

The SAR cattle to be used as the donors were selected from the conservated SAR population in the Dogankent Campus of the Eastern Mediterranean Agricultural Research Institute. Ten donors were selected from these cattle in the institute. Before donors were selected, genital organs such as ovaries, uterus and cervix were examined by transrectal ultrasonography using portable the ultrasound (5 MHz, Honda, HS-1001V, Japan). Furthermore, it was determined that they have not any pathological disorders, abnormal uterine structure, and pathological discharge.

Superovulation Protocole

PGF2α (Estrumate, Vet Pharma, Germany) was treated in the cows determined as donors. In 48 hours after PGF2α administrations, animals exhibiting estrus behaviors were detected GnRH (Receptal, Intervet, Germany) was treated in those animals between the 7th and 12th days of the estrus cycle for regression of the dominant follicle, and an ultrasound examination was performed 36-48 hours later after GnRH adminitration. Then, an intravaginal device that releases Progesterone (PRID Delta, 1.55g, Ceva, France) was inserted into the appropriate donors. FSH (Stimufol®, Reprobiol SPRL, Belgium) was received in decreasing doses (100-100 µg, 75-75 µg, 50-50 µg, 25-25 µg) at 12 hours intervals on the 5th day after the PRID was inserted. PGF2α was applied to ensure the regression the corpus luteum at the same time with the 5th and 7th FSH applications. Bulls were utilized to inseminate the cows 12 hours after the last FSH treatment. The superovulation protocol used in the present study was developed taking into account the methods previously described in the literatures (Mapletoft et al., 2002; Merton et al., 2003; Hasler 2004; Bó et al., 2002 and Thangavelu et al., 2007).

Superovulation Success Determination

On the 7th day after the last FSH treatment, uterus flushing was performed in the donors. Before uterine flushing, the number of the corpus luteum (CL) and non-ovulatory (AF) follicles on the ovary was recorded by transrectal ultrasonography using portable ultrasound with a 7.5-MHz linear probe (HS2000, Honda, Japan).

Uterus Flushing

The uterus flushing in donors was performed by using 1000 ml of lactated Ringer's solution (Ringer VIP, Polifarma, Türkiye) containing 0.1% Kanamycin (Kanovet, Deva, Türkiye) and 1% fetal calf serum (FCS) (Sigma, USA). 500 ml of the flushing solution was used for each cornu uteri. After the balloon of the Foley catheter was positioned in the horns of the uteri, each horn of the uteri was filled with 50-100 ml of the solution utilizing a 3-prong Y-catheter and recovered to a sterilized bottle. The fluid, which was given into the cornu uteri and recovered, was collected in sterile bottles. The obtained uterine flushing liquid was examined at the embryo laboratory to find the embryos and evaluate them according to their quality and stages.

Search and Evaluation of the Embryos

The liquid collected in the sterile bottle was filtered by using filters (EMCON filter) (Agtech Zona Filter, Radiated, CAT. #D03, USA) with pores of 70 µm wide for filtration. After washing the filters through holding solution (TCM-199 + 200 mM L-glutamine + 10 mg/ml gentamicin + 20% FCS), the liquid in the filter was taken into 3 Petri dishes (Agtech Square Search Dish, VWR, CAT#D09A, USA). Embryo scans were performed in Petri dishes using heated stereo microscopes (Leica, S8APO, Japan). The embryos were classified according to their quality and developmental stages regarding the evaluation criteria of IETS (Kanagawa et al., 1995; Silva et al., 2009).

Statistical Analysis

SAS software program (SAS Institute Inc., Cary, NC, USA) was utilized for the descriptive statistics in the study.

Results and Discussion

This study was carried out to evaluate the superovulation response and embryo retrieval rate of SAR cattle by FSH hormone treatment twice a day in decreasing doses. Obtained embryos were classified according to the criteria reported by the International Embryo Transfer Society. In terms of response to superovulation in SAR cattle, the mean number of the corpus luteum (CL) per donor was 7.8, and the mean number of novulatory follicle (AF) per animal was 2.8 (Table 1). After the flushing of 10 animals, five (5) unfertilized oocytes (UFO), three (3) degenerated, and two (two) suitable embryos were obtained in the first flushings (Table 2). It was seen that although the superovulation response is quite successful, the success of the embryo recovery rate is low.

Table 1. Response to Superovulation Protocoles

Cow No	Right Ovary		Left Ovary		Total	
	CL	An. Fol.	CL	An. Fol.	CL	An. Fol.
1	2	3	3	1	5	4
2	4	0	4	0	8	0
3	3	1	3	1	6	2
4	3	2	6	2	9	4
5	7	0	3	1	10	1
6	3	2	5	2	8	4
7	3	3	3	2	6	5
8	5	2	1	1	6	3
9	5	1	5	0	10	1
10	3	2	3	2	6	4
TOTAL	**38**	**16**	**36**	**12**	**74**	**28**
MEAN	**3.8**	**1.6**	**3.6**	**1.2**	**7.4**	**2.8**

*CL : Corpus Luteum; An. Fol. : Anovulatory Follicle

Tablo 2. Embryo Recovery after Superovulation

Cow No	Oocyte	Embryo		Total
		Tranferable	Nontransferable	
1	1			1
2			1	1
3				
4			1	1
5		2	1	3
6	2			2
7				
8				
9				
10	2			2
TOTAL	**5**	**2**	**3**	**10**
MEAN	**0.5**	**0.2**	**0.3**	**1**

Considering that the aggressive behavior of these animals can cause hormonal imbalances as well as difficulties in the flushing processes, it was concluded that the aggressive characteristics of SAR cattle may play a role in the low embryo recovery rate. It was thought that more studies that including different superovulation protocols and ovum pick-up (OPU) techniques are needed to increase the transferable embryo recovery rate from SAR animals. In addition, it was concluded that different techniques should be studied for uterine flushing procedures.

Performing a successful superovulation protocol is not only enough for in vivo embryo production. Nutrition, management, and productivity are the other important factors affecting the estrus, superovulation response, and embryo recovery rate (Mapletoft & Bó, 2016). The donors used in this study were from the conservated SAR herd in the institute. Therefore, it was evaluated and fed only for survival rates rather than yield traits.

Tasdemir et al. (2012) reported that the rate of transferable embryo rates for both applications was low in Turkish Native Black cattle, in which they applied FSH in 2 different ways. In the study conducted with Turkish Native Black, the embryo recovery rate was higher than in our study, while the response to superovulation was higher in SAR cattle than those in Turkish Native Black. It is thought that this result may be related to the differences in response to superovulation and ovulation rates of the superstimulated follicles between the breeds.

On the other hand, superovulation success was higher in SAR cattle in this study compared with the study reporting the responses to different superovulation protocols with Turkish Native Black Heifers (Satılmış et al., 2017). Moreover, although the embryo recovery rate was low for both studies, the embryo recovery rate was higher than in our study. It is thought that the difference in superovulation success may be due to the difference in the animal material (heifer-cow) used in the two studies. In addition, it should not be disregarded that the different breeds used in the studies may also have an effect on the results.

It has been reported that excessive stress causes a decrease in the superovulation response or a change in LH increase before ovulation (Bo et al. 2010).. In this study, it should be also considered that manipulations, injections and twice-daily FSH hormone treatment to these animals, which are usualy unmanageable, cause high stress in the aggressive and vicious SAR breed and may have affected the results.

Similarly, superovulation success and embryo recovery rates were evaluated in a study with Tianzhu White Yak cattle, a native breed of China. In the study, the mean number of CLs was 4.75, the number of follicles was 1.13, the number of transferable embryos was 2.50, and the number of non-transferable embryos was 1.38 (Yu et al. 2007). Both in our study and in the of Yu et al.'s (2007) research,, the embryo recovery rate is excessively lower than the world average. On the other hand, it was found that superovulation success was higher in SAR cattle than in Yak cattle. Yu et al. (2007) reported that although they obtained embryos lower in number than the world average, they had a solid understanding of Yak's reproductive physiology through more than 20 years of study, which they claimed as a success. Therefore, this study with SAR cattle is the first study in the literature, and it is an important research area that needs to be studied for many years to obtain more successful results.

It is known that 8 to 12 days after estrus (7 to 11 days after ovulation) second follicular wave starts in animals with two or three follicular waves. It was reported that the second follicular wave day differs between two-wave and three-wave cycles (1 or 2 days before in three-wave cycles) (Bo et al. 2002; Ginter et al. 1989). In this context, it has been clearly shown that superovulation success is higher when super stimulatory treatments are applied as soon as the wave appears (Adams et al. 1994; Bo et al. 2002; Nasser et al. 1993). Initiating the superovulation protocol when a new follicular wave occurs is only suitable for 20% of the estrous cycle (4 or 5 days), while the remaining 80% of the estrus period is not appropriate for an optimal superovulation response. Waiting until the middle phase of the estrus and monitoring oestrus (Bo et al., 2002) are necessary to start the superovulation protocol.

Conclusion

Reproductive characteristics in SAR cattle in this study have not been fully revealed. Moreover, the number of follicular waves in SAR cattle has not been determined yet. Thus, these may have affected the results of the study. For this reason, it was concluded that fully revealing of estrus cycle in the SAR cattle and after the reproductive physiology studies, trying to apply different superovulation protocols suitable for the reproductive characteristics of SAR breed would yield better results.

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Author contributions

All authors contributed equally to this study.

Conflicts of interest

The authors declares that they have no known competing financial or non-financial, professional, or personal conflicts in this paper.

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LIVESTOCK STUDIES

RESEARCH PAPER

An Assessment of the Accuracy of Digital and Optical Brix Refractometers for Estimating Passive Immunity in Beef Calves

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Abstract

This study was aimed at determining and comparing the diagnostic accuracy of digital and optical Brix refractometers (D-Brix and O-Brix, respectively) for the estimation of passive immunity status (<16 and <24g/L) in beef calves. Blood was sampled from 163 one to seven-day-old calves. Immunoglobulin G (IgG) concentrations were estimated with D-Brix and O-Brix refractometers, and measured by the radial immunodiffusion (RID) assay used as the reference test. Correlation coefficients (*r*) were calculated for the results of each method. Youden's J-index was used to select optimal refractometer cut-off values for estimating IgG of <16 and <24 g/L. Overall test performance and refractometer agreement were assessed using AUCs, diagnostic test accuracy, Cohen's kappa coefficient (κ), and Bland-Altman analysis. Positive correlations existed between the RID-IgG concentrations and Brix percentages (*r*=0.903 for D-Brix, r=0.885 for O-Brix), and between the results of the two refractometers (*r*=0.992). The *overall* test performances of the refractometers were excellent (AUC>0.90). For predicting serum IgG concentrations of <16 and <24 g/L, the optimal cut-off values were ≤8.3% and ≤9.4% for the D-Brix refractometer, and ≤8.4% and ≤9.6% for the O-Brix refractometer, respectively. At the optimal thresholds for estimating serum IgG concentrations of <16 g/L, the sensitivity and specificity were 91.89% and 97.62% for the D-Brix refractometer, and 91.89% and 96.83% for the O-Brix refractometer, respectively. At the optimal thresholds for estimating serum IgG concentrations of <24 g/L, the sensitivity and specificity were 88.14% and 80.77% for the D-Brix refractometer, and 86.44% and 80.77% for the O-Brix refractometer, respectively. Cohen's kappa coefficients suggested an almost perfect agreement between the results of the two refractometers for the estimation of IgG of <16 (κ=0.90) and <24 g/L (κ=0.86). In conclusion, digital and optical Brix refractometers could be safely used as monitoring tools for assessing passive immunity status in neonatal beef calves.

Introduction

Calves lack immunity at birth, as the ruminant placenta does not allow the transplacental transfer of maternal immunoglobulins (Ig). Feeding colostrum to neonatal calves within the first 24 h after birth enables the transfer of passive immunity (TPI). An inadequate absorption of colostral immunoglobulins by neonatal calves within this period is referred to as inadequate transfer of passive immunity (ITPI). On the other hand, serum IgG concentrations below the 10 g/L threshold, which mainly serves to reduce mortality, are described as failure of transfer of passive immunity (FTPI). The health status, viability, and performance of newborn calves are closely associated with their passive immunity status (Godden *et al.,* 2019; Lombard *et al.*, 2020; Fischer-Tlustos *et al.,* 2021).

In dairy calves, serum IgG concentrations greater than 10 g/L are traditionally defined as indicating adequate TPI (Godden *et al.,* 2019). In a recent study by Lombard *et al.* (2020), the passive immunity statuses of dairy calves were classified under four strata (poor (<10 g/L), fair (10 – 17.9 g/L), good (18 – 24.9 g/L) and excellent (≥25 g/L)), based on morbidity, mortality and performance criteria. However, currently, there is no consensus on an adequate passive immunity threshold for dairy and beef calves (Waldner and Rosengren, 2009; Chigerwe *et al.,* 2015). Serum IgG concentrations of <16 g/L, <24 g/L and <27 g/L are associated with mortality, morbidity, and growth performance in beef calves (Wittum and Perino, 1995; Dewell *et al.,* 2006; Waldner and Rosengren, 2009). Serum IgG concentrations above 16 g/L are considered to indicate adequate TPI (Waldner and Rosengren, 2009).

Despite being known as the gold standard for the detection of IgG concentrations (Fleenor and Stott, 1981), the radial immunodiffusion (RID) assay has not found common use on the field, given that it is expensive, produces results only after 24 h, is influenced by temperature alterations during incubation, and is required to be performed by experienced staff. Thus, alternative methods have been investigated for the assessment of passive immunity status (Pekcan *et al*., 2013; Deelen *et al*., 2014; Elsohaby *et al*., 2014; 2015; Elsohaby and Keefe, 2015; Hogan *et al*, 2015; 2016; Drikic *et al*., 2018; Topal *et al*., 2018; Akköse *et al*., 2022a). Digital and optical Brix refractometers are efficient, low-cost and practical devices that can be used on the farm to detect ITPI. The Brix scale refers to the sugar concentration of solutions; such that 1% Brix is equivalent to 1 g/dL of sucrose (Ball, 2006). The Brix percentages of biological fluids are correlated with their total solids concentrations. It is also possible to use Brix refractometers in determining colostrum quality (Buczinski *et al*., 2016). While a large number of comparative studies are available on the utility and performance of refractometers in diagnosing ITPI in dairy calves (Buczinski *et al.,* 2021; Akköse *et al*., 2022a:b), there are only very few studies that have evaluated refractometry methods for the assessment of passive immunity status in beef calves (Vandeputte *et al*., 2011; Todd *et al*., 2018; Gamsjäger *et al.,* 2021; Pisello *et al*., 2021; Akköse *et al.,* 2022c). This study aimed at determining and comparing the diagnostic accuracy of digital and optical Brix refractometers for the prediction of different passive immunity statuses in beef calves, and at demonstrating the correlation and agreement level of the results achieved with these refractometers.

Materials and Methods

Animal Material and Collection of Serum Samples

The Local Ethics Board for Animal Experiments of Harran University (HRU-HADYEK) approved the conduct of this study.

This study was conducted between April 2018 – March 2019 at two beef farms located in Turkey. A total of 163 healthy calves, aged 1–7 days and of the breeds Aberdeen Angus and Limousine, were used. Each calf was sampled for blood from the jugular vein, and samples were collected into 10-mL plain tubes (Hema Tube, Ankara, Turkey). The blood samples were centrifuged at 3000 x g for 10 min for the extraction of sera. Serum was stored in volumes of 2 mL under freezing at -20^{o}C until being used for RID analyses after refractometer analyses.

Refractometer Analyses

Refractometer analyses were performed on the farm using fresh serum samples. Brix percentages were measured with a handheld optical Brix (O-Brix) refractometer (ATC HT 113, China) and a handheld digital Brix (D-Brix) refractometer (Atago PAL-1, Tokyo, Japan). The D-Brix refractometer had an automatic temperature compensation function. The D-Brix and O-Brix refractometers were précised to 0.1% Brix and 0.2% Brix, respectively. The scales of the D-Brix and O-Brix refractometers were 0 to 53 and 0 to 32, respectively. Samples of approximately 200 microliters were used for the refractometer analyses. The D-Brix refractometer used led light, and the results were read on the digital panel of the device. The O-Brix refractometer results were read by looking into the eyepiece, while pointing the refractometer at a source of direct light. The white/blue boundary on scale showed the measured value of the O-Brix refractometer. Between samples, the prism of the refractometer was washed in tap water and dried with paper towel. Prior to each series of analyses, the refractometers were calibrated with distilled water.

Radial Immunodiffusion (RID) Analyses

After being thawed at room temperature, the serum samples underwent RID analyses performed with commercial test kits (Triple J Farms, Bellingham, WA, USA). The test kits were stored in a refrigerator at +4 ˚C and removed half an hour before being used. The test kits included 3 reference sera with known IgG concentrations. Each kit had 21 sample wells and 3 control wells. The test procedure was performed as instructed by the manufacturer. Five-µl automatic pipettes were used for the transfer of the reference sera and serum samples to the RID plates. Subsequently, the kits were incubated in their locked cases in a room temperature. After 24 h of incubation, the diameters of the precipitin rings were measured with a 10x peak scale loupe precise to 0.1 mm. The diameters were compared to the standard curve, which was constructed using the IgG concentrations of the reference sera. The reference table provided with the test kit was used to determine the IgG concentrations of the serum samples. Serum samples with precipitin ring diameters that did not fall within the range indicated in the reference table were diluted 1:1 with 0.9% NaCl sterile solution for reanalysis.

Statistical Analyses

The distribution of the D-Brix, O-Brix and RID-IgG results was determined based on skewness and kurtosis values. Values ranging from (–1.5) to (+1.5) indicated normal data distribution. Descriptive statistics were calculated for the IgG concentrations measured by RID assay and Brix percentages obtained by D-Brix and O-Brix refractometers.

The correlation between the RID-IgG concentrations and the refractometer results as well as the correlation between the results of the O-Brix and D-Brix refractometers were determined by calculating Pearson's correlation coefficients.

The accuracy of the refractometers in determining different passive immunity statuses was assessed according to epidemiological diagnostic test characteristics (sensitivity, specificity, positive and negative predictive values), based on receiver operating characteristics curve (ROC) analysis.

Youden's J-index was used for the selection of the optimal thresholds. Sensitivity (Se) was defined as the proportion of calves accurately determined with refractometry as having RID-IgG concentrations of <16 g/L and <24 g/L. Specificity (Sp) was defined as the proportion of calves accurately determined with refractometry as having RID-IgG concentrations of ≥16 g/L and ≥24 g/L. Youden's J-index was calculated using the equation: J = Se + Sp – 1. The maximization of J minimized the total test misclassifications, including the assumption of an equal effect of false negative and false positive results. The positive predictive value (PPV) was defined as the probability of calves, classified by refractometry as having serum IgG concentrations of <16 g/L and <24 g/L in reality also having IgG concentrations of <16 g/L and <24 g/L, respectively. On the other hand, the negative predictive value (NPV) was defined as the probability of calves, classified by refractometry as having IgG concentrations of ≥16 g/L and ≥ 24 m/L, in reality also having IgG concentrations of ≥16 g/L and ≥24 g/L, respectively. A receiver operating characteristics (ROC) curve was constructed for each refractometer at two passive immunity levels (<16 g/L, <24 g/L).

The discrimination ability of the refractometers to estimate passive immunity statuses in beef calves was determined by assessing areas under the ROC curves (AUCs). AUCs pointed out to either excellent accuracy (AUC = 1; Se = Sp = 1), high level of accuracy (AUC > 0.9), moderate accuracy (AUC = 0.7-0.9) or poor accuracy (AUC = 0.5-0.7). Inter-rater agreement (Cohen's kappa coefficient, κ) was calculated to ascertain the agreement level between the results obtained with the two refractometers using the respective cut-off based test dichotomization. Agreement levels were graded as poor agreement (κ < 0.20), fair agreement (0.20 < κ ≤ 0.40), moderate agreement (0.40 < κ ≤ 0.60), substantial agreement (0.60 < κ ≤ 0.80) and almost perfect agreement (κ > 0.80). The level of agreement between the D-Brix and O-Brix results was further assessed by a Bland-Altman analysis (Petrie and Watson, 2013).

Statistical significance was set at p≤0.05. The SPSS 24 statistical package program was used for the statistical analyses.

Results

In total 163 serum samples belonging to Aberdeen Angus (n=144) and Limousine (n=19) calves were subjected to % Brix and RID analyses. Based on skewness and kurtosis coefficients, it was determined that the data were normally distributed. The descriptive statistics of the D-Brix and O-Brix percentages and RID-IgG concentrations are given in Table 1. According to the results of the RID analyses of the blood samples of 163 calves, IgG concentrations were <16 g/L in 37 calves (22.7%), <24 g/L in 59 calves (36.2%), and ≥24 g/L in 104 calves (63.8%).

Table 1. Descriptive statistics of % D-Brix, % O-Brix and RID-IgG concentrations of serum samples taken from beef calves.

Variable	N	Min.	Max.	Mean ± SD	Median	Skewness	Kurtosis
D-Brix (%)	163	6.2	13.1	9.5±1.4	9.7	-0.328	-0.525
O-Brix (%)	163	6.1	12.9	9.6±1.5	10.0	-0.413	-0.619
RID-IgG (g/L)	163	0.0	75.6	27.8±16.3	27.1	0.036	-0.263

Correlations

Positive correlations were determined between the RID-IgG concentrations and D-Brix percentages (*r*=0.903), as well as the RID-IgG concentrations and O-Brix percentages (*r*=0.934). Furthermore, positive correlations were also ascertained between the D-Brix and O-Brix results (*r*=0.993). Scatter-plots showing the correlation between RID-IgG, D-Brix and O-Brix percentages are presented in **Figure 1.**

Diagnostic test characteristics

Sp, Se, PPV, NPV and Youden's J-index calculations were made for digital and optical Brix refractometry, which are used for the estimation of passive immunity status.

While Youden's J-index was used to determine optimal refractometer cut-off values, the Se and Sp values were calculated by ROC analysis. Accordingly, the optimal cut-off values for predicting serum IgG concentrations of <16 g/L and <24 g/L were determined as ≤8.3% and ≤9.4%, respectively, for the D-Brix refractometer, and ≤8.4% and ≤9.6%, respectively, for the O-Brix refractometer (**Table 2**).

The ROC curves constructed for the passive immunity statuses estimated with the D-Brix and O-Brix refractometers are shown in **Figure 2**. In beef calves, the AUC values of the two refractometers for predicting passive immunity statuses of <16 g/L and <24 g/L were determined to be >0.9.

Table 2. Diagnostic test characteristics of the digital and optical Brix refractometers for the estimation of different passive immunity levels.

IgG Conc. (g/L)		Threshold (%)	Sensitivity (%)	95% CI (%)	Specificity (%)	95% CI (%)	PPV (%)	95% CI (%)	NPV (%)	95% CI (%)
D-Brix	<16	≤8.3	91.89	78.1 – 98.3	97.62	93.2 – 99.5	91.9	78.7-97.2	97,6	93.3-99.2
	<24	≤9.4	88.14	77.1 – 95.1	80.77	71.9 – 87.8	72.2	63.4-79.6	92.3	85.6-96.0
O-Brix	<16	≤8.4	91.89	78.1 – 98.3	96.83	92.1 – 99.1	89.5	76.3-95.7	97.6	93.2-99.2
	<24	≤9.6	86.44	75.0 – 94.0	80.77	71.9 – 87.8	71.8	62.9-79.3	91.3	84.6-95.3

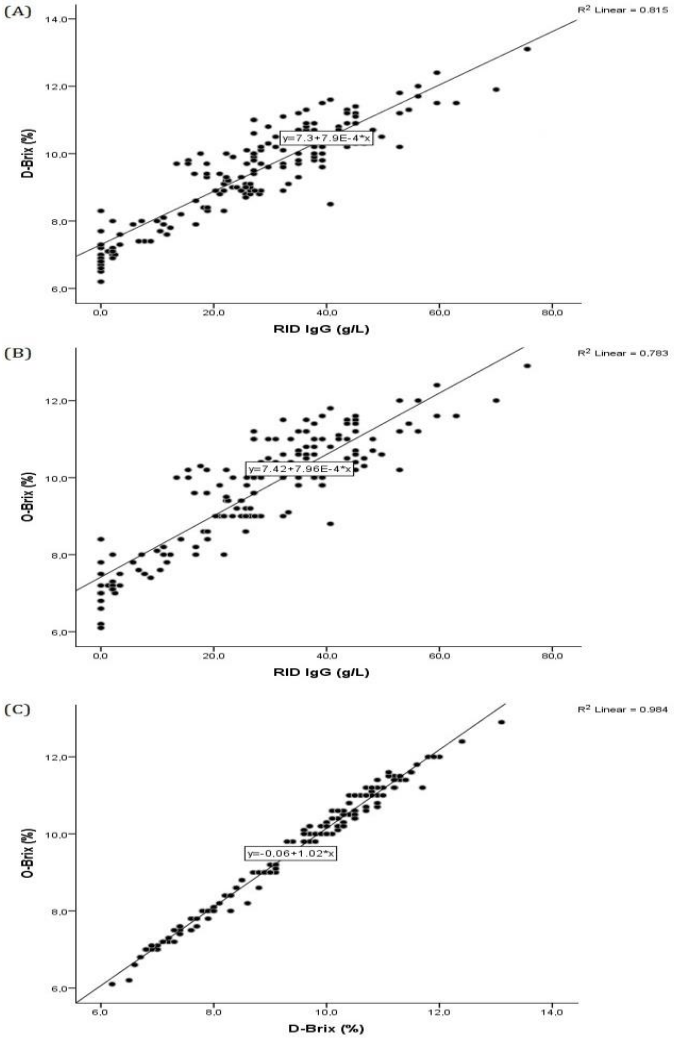

Figure 1. (A) Scatter-plot showing the correlation between the D-Brix percentages and RID-IgG concentrations of beef calves. The solid line indicates the fitted regression equation: Y = 0.00079 (X) + 7.3. (B) Scatter-plot showing the correlation between the O-Brix percentages and RID-IgG concentrations of beef calves. The solid line indicates the fitted regression equation: Y = 0.000796 (X) + 7.42. (C) Scatter-plot showing the correlation between the D-Brix and O-Brix percentages of beef calves. The solid line indicates the fitted regression equation: Y = 1.02 (X) + 0.06. D-Brix, digital Brix refractometer; O-Brix, optical Brix refractometer; RID, Radial immunodiffusion; IgG, immunoglobulin G.

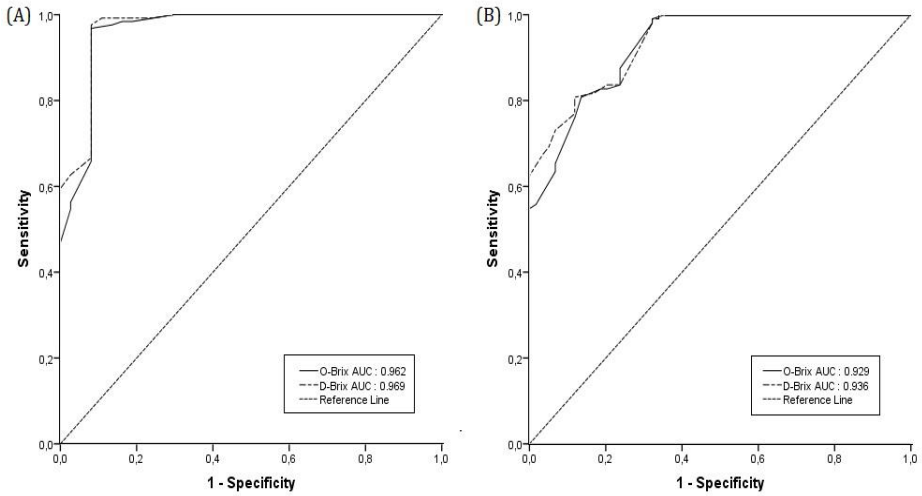

Figure 2. Receiver operating characteristic curves (ROCs) constructed and areas under the curve (AUCs) calculated for the prediction of serum IgG concentrations of <16 g/L (A) and <24 g/L (B) by two different refractometers in neonatal beef calves. AUC, area under the ROC curve; ROC, Receiver operating characteristic curve.

The Agreement between the Refractometers at the selected cut-off values

The results of the D-Brix and O-Brix refractometers for the prediction of IgG concentrations of <16 g/L (κ=0.90) and <24 g/L (κ=0.86) almost perfectly agreed.

This was also evaluated by a Bland-Altman analysis, and it was confirmed that there was no systematic bias, but only a small mean difference of percentage (0.13%) between the D-Brix and O-Brix results **(Figure 3).**

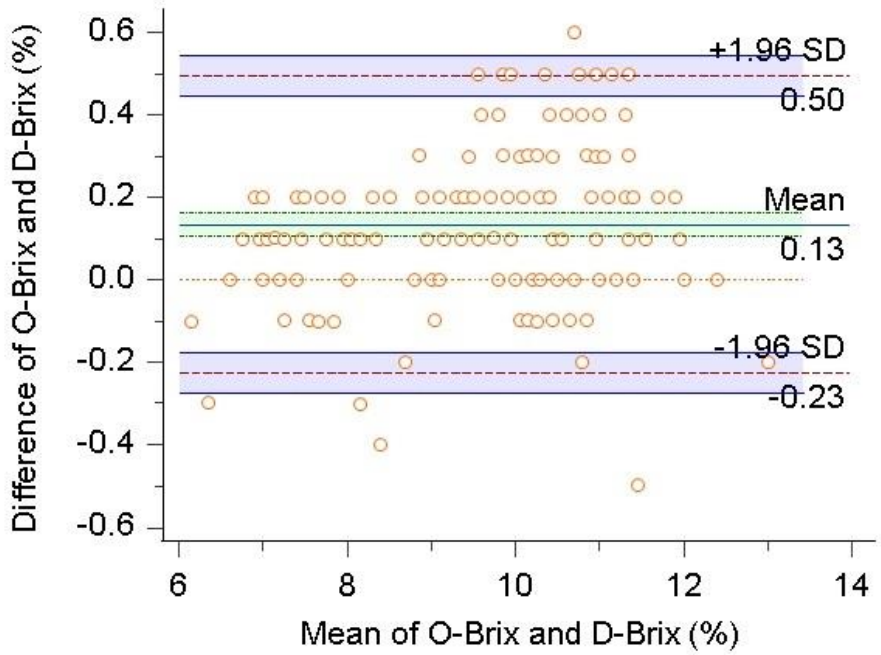

Figure 3. Bland–Altman plot graph illustrating the agreement between the Brix percentages measured by the O-Brix and D-Brix refractometers. The solid line indicates mean differences of O-Brix and D-Brix refractometer measures and dotted lines represent 1.96 SD from the mean difference. The top and bottom solid line on each graph represent the 95% limits of agreement. O-Brix, optical Brix refractometer; D-Brix, digital Brix refractometer, SD, Standard deviation.

Discussion

Farm blindness may falsely normalize calf mortality associated with poor colostrum management (Mee, 2020). There is need for the practical monitoring of the colostral transfer of passive immunity to calves. Our study compared the diagnostic accuracy of optical and digital Brix refractometers in predicting different passive immunity statuses in beef calves. To date, only very few studies have assessed digital Brix percentages indicative of serum IgG of <16 g/L (Gamsjäger *et al*., 2021; Pisello *et al.,* 2021; Akköse *et al*., 2022c) or <24 g/L (Gamsjäger *et al*., 2021; Akköse *et al.,* 2022c). On the other hand, there is no report on optical Brix percentages indicative of serum IgG of <24 g/L.

The optimal cut-off value we ascertained in the present study for the D-Brix refractometer for the estimation of IgG concentrations of <16 g/L was similar to previously reported digital Brix percentages for IgG of <16 g/L (Gamsjäger *et al*., 2021; Pisello *et al*., 2021; Akköse *et al*., 2022c). The cut-off value we determined for the D-Brix refractometer for estimating IgG concentrations of <24 g/L was higher than the digital Brix refractometer cut-off value previously reported by Gamsjäger *et al*. (2021) and lower than the digital Brix refractometer cut-off value previously reported by Akköse *et al*. (2022c). The cut-off value ascertained for the O-Brix refractometer for estimating IgG concentrations of <16 g/L was similar to the optical Brix refractometer cut-off value reported by Pisello *et al*. (2021). The present study proposes, for the first time, a cut-off value for the optical Brix refractometer for estimating serum IgG concentrations of <24 g/L in beef calves. Furthermore, the diagnostic test characteristics at the optimal cut-offs determined for the D-Brix and O-Brix refractometers were similar to those reported in previous studies (Gamsjäger *et al*., 2021; Akköse *et al*., 2022c).

Different cut-off values having been reported in studies could be attributed to the use of different refractometers (Elsohaby *et al.,* 2015), calf age (Topal *et al*., 2018), breed-specific variations (Villarroel *et al*., 2013), and the hydration status of calves. Other influential factors include the automatic temperature compensation capability of the refractor prism and the precision of the refractometer. The refractive index of a material depends on the temperature of the environment, samples or refractometer prism. The precision of the D-Brix and O-Brix refractometers was set to 0.1% Brix and 0.2% Brix, respectively. On the other hand, finding different cut-off values does not imply that these values are truly different. In fact, the best choice for a cut-off value is also related to the uncertainty around it and, thus, to the prevalence and total number of cases of the target condition (Leeflang *et al.,* 2008).

In beef calves, mortality and morbidity rates are related to serum IgG concentrations of <16 g/L or <24 g/L (Wittum and Perino, 1995; Dewell *et al.,* 2006; Waldner and Rosengren, 2009). Serum Brix percentages of ≤8.4% were reported to be associated with significantly higher odds of morbidity and mortality in suckler beef calves (Todd *et al.,* 2018). In a recent study in beef calves, digital Brix refractometer cut-off values of ≤8.4% and ≤8.8% were reported for the estimation of serum IgG concentrations of <16 g/L and <24 g/L, respectively (Gamsjäger *et al.,* 2021). In a study describing ITPI as being associated with a serum IgG concentration of <16 g/L, a cut-off value of ≤ 8.4% was proposed for the digital Brix refractometer (Pisello *et al.*, 2021). Our research team has recently found digital and optical Brix refractometers cut-off values of <8.5% and <10.1% for the estimation of serum IgG concentrations of <16 g/L and <24 g/L, respectively, (Akköse *et al.,* 2022c). These results agree with our findings for the estimation of serum IgG concentrations of <16 and <24 g/L.

AUC is a criterion used for the assessment of the overall accuracy of diagnostic tests and is presented as the mean Se values for all probable Sp values (Lee *et al.*, 2008). In the present study, the AUC values of the refractometers that were used to predict two of the passive immunity statuses were similar to those previously reported by Gamsjäger *et al.* (2021), but were higher than those reported by Pisello *et al.* (2021), when using a threshold of <16 g/L. The AUCs demonstrated that the two refractometers used to determine calves with IgG concentrations of <16 g/L and <24 g/L did not differ for the results they produced. The AUC value having been determined as >90% suggests that the diagnostic tests used in the present study were successful.

Cohen's kappa coefficient suggested an almost perfect agreement between the D-Brix and O-Brix refractometers for the estimation of IgG concentrations of <16 g/L and <24 g/L. Furthermore, the assessment of the agreement between the serum Brix percentages measured by the D-Brix and O-Brix refractometers using Bland–Altman plots demonstrated that there was no obvious systematic bias between the optical and digital refractometers. This suggests that the two refractometers can be used interchangeably. Our findings agree with those reported by Pisello *et al.* (2021) for beef calves. The small mean of 0.13% Brix between the O-Brix and D-Brix refractometers showed that the O-Brix refractometer measured, on average, 0.1 unit (%) higher than the D-Brix refractometer. This little difference could be attributed to the different precisions of the refractometers (Gamsjäger *et al*., 2021), which is 0.1% Brix for the D-Brix refractometer and 0.2% Brix for the O-Brix refractometer.

Being readily available for use on dairy farms to determine IgG concentrations in colostrum and calf serum, two different types of Brix refractometers were compared in this study. Brix refractometers are either

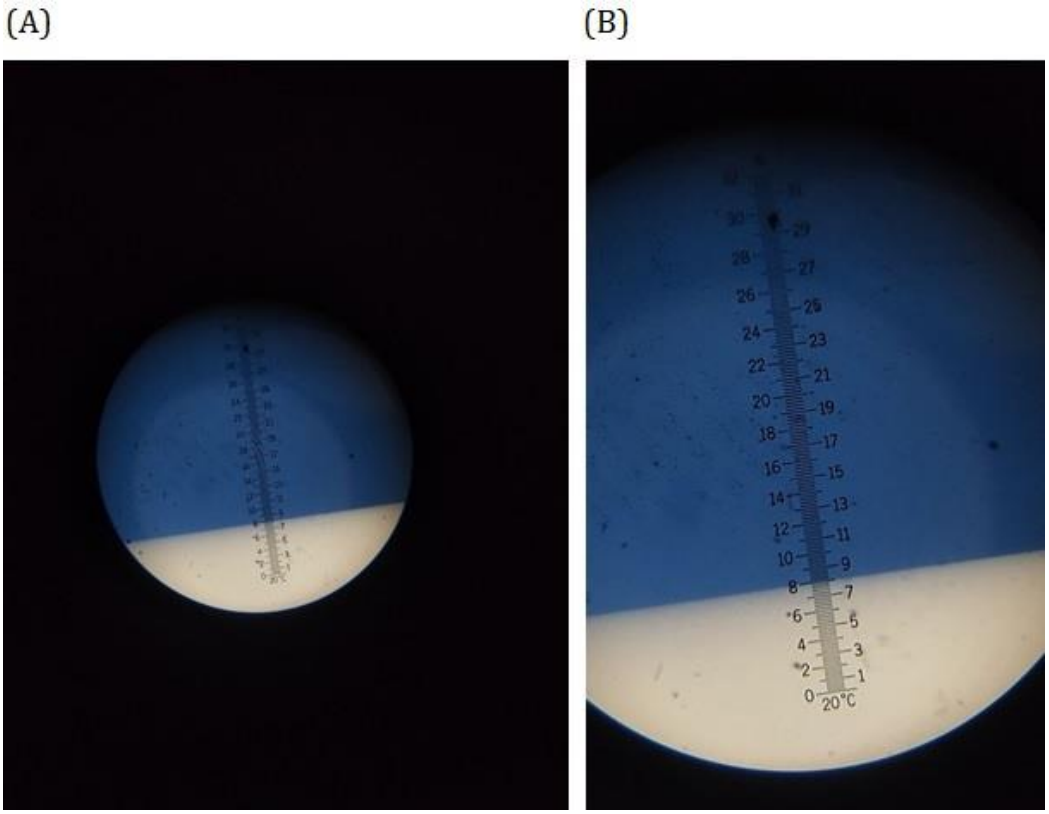

Figure 4. The reading of the optical Brix results through the eyepiece of the refractometer with a scale of 0-30 does not suffice for the clear distinction of the white/blue boundary, (A) and thus, yields results that may vary among individuals. Results that are more accurate can be achieved by reading the scale of the optical Brix refractometer using a magnifier, such as a mobile phone camera (B).

optical (traditional) or digital, the latter being more appealing since it gives a rapid objective measurement of the Brix percent of the sample. The traditional refractometer produces subjective results, as the reading depends on individual eyesight and two persons may report different readings according to how they read the scale **(Figure 4).** The scale of optic refractometers differs for each model or type of refractometer. The commonly used scales of the traditional refractometer are 0 to 10, 0 to 30, and 0 to 50. Logically, the 0 to 10 scale would be expected to yield more accurate readings (MacKenzie, 2021). However, when performing colostral measurements, the refractometer's line on the scale can be read only using a 0 to 30 scale. Owing to its automatic temperature compensation function, higher technology and digital sensor, the digital refractometer offers more than double the resolution of the traditional refractometer, and produces far more accurate readings.

Conclusion

In conclusion, as they do not require special laboratory equipment, digital and optical Brix refractometers can be safely used on the farm as inexpensive and reliable management tools for the estimation of passive immunity statuses after colostrum intake in neonatal beef calves. Owing to a greater objectivity of results and ease of use, the digital Brix refractometer can be preferred over the optical refractometer. Veterinarians and producers can identify calves with ITPI using Brix refractometers, and may choose to monitor the health status of calves under risk. Furthermore, they may use the ITPI prevalence to evaluate the efficiency of the colostrum management strategy of the farm. Farms with a high percentage of tested calves with low Brix% may resort to the adjustment of colostrum management strategies such as inquiring prepartum management strategies of dams, the measurement of IgG concentrations in maternal colostrum or additional colostrum supplementation to calf. On the other hand, farms with a low percentage of tested calves with low Brix% or achieving target Brix% may benefit from the motivation of having confirmed that all is running smoothly on the farm. Therefore, the monitoring of the passive immunity status in beef calves using a Brix refractometer can contribute to both reducing morbidity and mortality rates and preventing economic losses.

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Author contributions

All authors contributed equally to the study.

Conflicts of interest

The author declare no conflicts of interest.

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