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Phenotypic correlations between ovum pick-up *in vitro* production traits and pregnancy rates in Zebu cows

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ABSTRACT. The growth of the Gyr breed in Brazil in terms of genetic gain for milk, along with conditions for market, has led to the use of ovum pick-up *in vitro* production (OPU-IVP) as a leader in biotechnology for the multiplication of genetic material. The aim of this study was to investigate phenotypic correlations between OPU-IVP-linked characteristics and pregnancy rates registered in an embryo transfer program using Gyr cows as oocyte donors. Data collected from 211 OPU sessions and 298 embryo transfers during the years 2012 and 2013 were analyzed and statistical analysis was performed. Estimates of simple Pearson correlations were calculated for NVcoc and PVcoc (number and proportion of viable cumulus-oocyte complexes, respectively); NcleavD4 and PcleavD4 (number and proportion of cleaved embryos on day 4 of culture, respectively); NTembD7 and PTembD7 (number and proportion of transferable embryos on day 7 of culture, respectively); NPrD30 and PPrD30 (number and proportion

of pregnancies 30 days after transfer, respectively); and NPrD60 and PPrD60 (number and proportion of pregnancies 60 days after transfer, respectively). Moderate to moderately high correlations were found for all numerical characteristics, suggesting these as the most suitable parameters for selection of oocyte donors in Gyr programs. NVcoc is proposed as a selection trait due to positive correlations with percentage traits and pregnancy rates 30 and 60 days after transfer.

Key words: Gyr cattle; Oocyte donors; OPU-IVP; Repeatability; Phenotypic correlation

INTRODUCTION

Since the late 1980s, the development of ultrasound-guided follicular aspiration (ovum pick up-OPU) for *in vitro* production of embryos (IVP) has become a well-known technique, developed in both research and commercial production of bovine embryos (Thibier, 2008). In 2011, the total number of transferable embryos produced was 453471, with 85% of these (318119) produced in Brazil, placing this country as the quantitative world leader in bovine IVP (IETS, 2012). In recent years, progressive growth of Brazil's embryo production in dairy breeds directly related to *in vitro* production has been observed. For example, the use of OPU-IVP in Gyr increased more than 600% during this period (Viana et al., 2010a).

Regardless of the significant progress made in recent decades, the IVP technique is still characterized by low relative efficiency (Siqueira et al., 2012). Different studies have reported efficiency of 10 to 40% for embryo production (considering stages of maturation, fertilization, and culture to the blastocyst stage) (Lonergan and Fair, 2008; Rizos et al., 2008) and pregnancy rates between 30 and 40% (Peterson and Lee, 2003).

Phenotypic correlation between traits can only be estimated when measured directly, promoting the discovery of crucial components in the production process that may be modified to enhance productivity of the characteristics of interest (Coimbra et al., 2004). When two economically important traits show positive genetic correlation, selection can be directed to one of these, and this is usually towards the trait that is more easily measured, because ultimately both alternative traits will be improved (Pereira, 2012). In Holstein cows (*Bos taurus taurus*), Merton et al. (2009) found moderate and positive genetic correlation for some IVP traits (number of total and transferable embryos at day 7 of culture); although for other traits, the correlation was zero. The behavior of the observed phenotypic correlations was in line with genetic correlations.

The result of the process of OPU-IVP is measured as the number and percentage of pregnancies per transferred embryo. Therefore, determining phenotypic correlations between related characteristics in OPU-IVP contributes to the understanding of the complex associations that this process presupposes.

MATERIAL AND METHODS

Oocyte donors

The OPU-IVP data used in this study were collected from 50 adult female Gyr donors,

more than 4 years old, located in Rio de Janeiro State, Brazil. All cows were cycling and showed a body condition score between three and four (Edmonson et al., 1989) and were kept only in pastures with shade. A total of 211 OPU sessions and 298 embryo transfers were studied. None of the females was submitted to hormonal treatment before OPU-IVP.

Recovery and oocyte classification

Cumulus-oocyte complexes (COCs) were harvested by OPU. Imaging of ovaries was performed using a portable ultrasonic device with an intravaginal 7.5 MHz sector probe equipped with a needle guide (Scanner 100S, Pie Medical, Maastricht, The Netherlands), and follicles between 2 and 8 mm were punctured using 19-G disposable hypodermic needles with an aspiration vacuum of 60-80 mmHg. Cumulus-oocyte complexes were ranked according to the number of layers of cytoplasm and cumulus cells: I. Viable (GI: more than 3 layers of homogeneous cytoplasm; GII: more than 3 layers of cytoplasm with granules, or less than 3 layers and homogeneous cytoplasm; GIII: less than 3 layers and cytoplasm with granules, or partially denuded and homogeneous or with mild granules cytoplasm) and II. Non-viable (GIV: partially denuded and cytoplasm with grooves, or naked, or expanded, or degenerate).

In vitro maturation, fertilization, and embryo culture

Viable oocytes were matured *in vitro* (IVM) in TCM 199 (Invitrogen-Gibco BRL) with 10% inactivated estrous cow serum and 1.0 µg/mL FSH (Pluset, Serono, Italy), 50 µg/mL hCG (Profasi®, Serono, Italy), 1.0 µg/mL estradiol (Sigma E-2758 St. Louis, MO, USA), 0.2 mM sodium pyruvate (Biochemical 44094), and 83.4 µg/mL amikacin (Instituto Biochimico, Rio de Janeiro, Brazil). After IVM, viable sperm from frozen straws of sexed semen were separated using a Percoll discontinuous density gradient and *in vitro* fertilization (IVF) was performed in TALP-IVF, with 0.2 mM pyruvate, 83.4 mg/mL amikacin, and supplemented with 6 mg/mL bovine serum albumin. *In vitro* culture (IVC) was performed in synthetic oviduct fluid supplemented with 2.5% fetal bovine serum (Cripion, Industria Brasileira, Andradina, SP, Brasil) for 7 days. IVM, IVF, and IVC were carried out in an incubator at 38.5°C in 5% CO_2 in air with high relative humidity. Cleavage rate was assessed 96 h after IVF and blastocyst rate was assessed on day 7.

Transfer of *in vitro*-produced embryos

The pregnancies were produced by transfer of blastocyst grade I embryos. The embryos were transferred non-surgically for *B. indicus x B. taurus* crossbred heifer recipients (previously synchronized with a hormonal protocol of progesterone, estradiol benzoate, cipionate, and cloprostenol) to the uterine horn ipsilateral to the corpus luteum. Pregnancy was diagnosed by ultrasound examination 30 and 60 days after embryo transfer.

Phenotypic correlations

Phenotypic correlations, defined as the correlation between the phenotypes of trait P_1 and trait P_2, were determined and classified as low (<0.2), moderate (0.2 to 0.49), moderately high (0.5 to 0.8), or high (>0.8).

OPU-IVP trait definitions

The phenotypic characteristics were evaluated as follows: 1. Number and proportion of viable cumulus-oocyte complexes (NVcoc and PVcoc, respectively) in classes I, II and III that were successfully matured, fertilized, and *in vitro* cultured; 2. number and proportion of cleaved embryos on day 4 of culture (NcleavD4 and PcleavD4, respectively); 3. number and proportion of transferable embryos on day 7 of culture (NTembD7 and PTembD7, respectively) as a proportion of NVcoc, according to IETS classification as stage 4 (morula), grade 1 (excellent or good), and stages 5 to 7 (early blastocyst, blastocyst and, expanded blastocyst), grades 1 and 2 (excellent and regular); 4. number and proportion of pregnancies 30 days after transfer (NPrD30 and PPrD30, respectively); and 5. number and proportion of pregnancies 60 days after transfer (NPrD60 and PPrD60, respectively).

Pregnancy rates were calculated based on the number of embryos transferred and this parameter was dependent on the number of recipients available in the program.

Many companies dedicated to the *in vitro* production of bovine embryos measure their results in terms of percentages or ratios, thereby evaluating the real performance of the process, with limited obvious numerical effects on many characteristics. Cows with high rates of oocyte production usually produce more embryos at the end of the process; however, this does not always translate into good cleavage, embryos, or pregnancy rates. Achievement of high rates in the above characteristics could considerably improve the low efficiency of the OPU-IVP process reported in the scientific literature (Camargo et al., 2006).

Cows were classified into high, medium, and low efficiency according to the degree of efficiency for the characteristics of interest mentioned above. In order to classify the cows in the OPU-IVP process, the average values reported in the scientific literature (Gyr cows) for each of the variables (Camargo et al., 2007; Pontes et al., 2010; Grázia et al., 2012; Silva et al., 2012; Oliveira et al., 2013) were considered, and these were compared to the results obtained by the institutions where the data were collected. For this classification, only the proportions for all characteristics were considered (Table 1).

Table 1. Classification of Gyr donors according to their efficiency for characteristics of ovum pick-up *in vitro* production (OPU-IVP).

Characteristic	High class (%)	Middle class (%)	Low class (%)
PVcoc	≥80	50-79	<50
PcleavD4	≥80	60-79	<60
PTembD7	≥30	20-29	<20
PPrD30	≥40	30-39	<30
PPrD60	≥35	25-34	<25

PVcoc, proportion of viable cumulus-oocyte complexes; PcleavD4, proportion of cleaved embryos on day 4 of culture; PTembD7, proportion of transferable embryos on day 7 of culture; PPrD30, proportion of pregnancies 30 days after transfer; PPrD60, proportion of pregnancies 60 days after transfer.

Statistical analysis

Statistical analysis was performed using the SAS 9.0 software (2009). Means and standard deviations were calculated with PROC MEANS and estimates for coefficients of Pearson's correlation between traits calculated using PROC-CORR. Repeatability of some traits was obtained from the estimates of the variances using the REML method in the Varcomp procedure.

RESULTS AND DISCUSSION

OPU-IVP and pregnancy rates

A total of 2758 oocytes were collected and 2068 (75.25 ± 20.5%) were considered viable, generating an average of 9.77 ± 6.79 viable oocytes per procedure. The percentage of cleaved oocytes, embryos per cleaved oocyte, and pregnancy rate per OPU-IVP session 30 and 60 days after transfer are shown in Table 2.

Table 2. Means ± standard deviation, minimum, and maximum values for traits of Gyr (*Bos indicus*) donors in the ovum pick-up *in vitro* production (OPU-IVP) program, Rio de Janeiro State, Brazil.

Trait	Mean ± standard deviation*	Minimum	Maximum
NVcoc	9.77 ± 6.79	0	44
PVcoc (%)	75.25 ± 20.5	16.7	100
NcleavD4	6.44 ± 4.89	0	30
PcleavD4 (%)	66.79 ± 33.70	3.8	100
NTembD7	2.61 ± 2.68	0	12
PTembD7 (%)	35.47 ± 34.62	0	100
NPrD30	0.65 ± 1.10	0	7
PPrD30 (%)	28.91 ± 39.59	0	100
NPrD60	0.61 ± 1.06	0	7
PPrD60 (%)	25.73 ± 37.85	0	100

*Descriptive statistics of untransformed OPU-IVP traits. NVcoc and PVcoc, number and proportion of viable cumulus-oocyte complexes; NcleavD4 and PcleavD4, number and proportion of cleaved embryos at day 4 of culture; NTembD7 and PTembD7, number and proportion of transferable embryos at day 7 of culture; NPrD30 and PPrD30, number and proportion of pregnancies 30 days after transfer; and NPrD60 and PPrD60, number and proportion of pregnancies 60 days after transfer.

Of 298 transferred embryos, 139 (28.91%) and 129 (25.73%) led to pregnancies 30 and 60 days after embryo transfer, respectively. The total number of embryos cleaved at day 4 varied from 0 to 30 and the percentage of embryos cleaved varied from 3.8 to 100%.

In the present study, the donor Gyr cows were classified as having average standards in relation to PVcoc and PcleavD4 (75.25 and 66.79%, respectively), and within the high class in relation to trait PTembD7, with 35.47% of developing embryos transferable on day 7 of culture.

Similar results for PVcoc and PcleavD4 were also observed in the studies of Camargo et al. (2006), Pontes et al. (2010), and Oliveira et al. (2013). Table 3 shows the variability of these traits in other studies. However, the value of NVcoc obtained in this study (9.77 ± 6.79) differed to that obtained by Pontes et al. (2010) and Viana et al. (2010b), who reported mean values of 12.1 ± 3.9 and 7.0 ± 1.0, respectively.

Variability among donors of the same breed has been reported previously in several studies (Machado et al., 2006; Pontes et al., 2010), with the sources of variation cited as technician, laboratory, and number and interval between OPU sessions (Abdoon et al., 2001; Camargo et al., 2006; Pontes et al., 2011).

In the present study, pregnancy rates obtained 30 and 60 days after embryo transfer were 28.9 (± 39.5) and 25.73 (± 37.8), respectively. These results may be considered low for day 30 and medium for day 60, with results from Pontes et al. (2010) and Oliveira et al. (2013) showing higher values (>40%) at day 30. The fact that the program included donors without previous OPU-IVP sessions prior to the study (41% of cows) may have influenced the final

outcome for pregnancy rates, consistent with the findings of Vega et al. (2013) who showed the effect of OPU-IVP sessions on pregnancy rates (average of 6.9% for a group of donors with 1 to 5 sessions and 30.9% for a group of oocyte donors with 6 to 13 sessions in *B. indicus* cattle).

Several studies have shown the influence of *in vitro* culture medium on embryonic development (Farin et al., 2001; Khosla et al., 2001; Malekshah and Moghaddam 2005; Camargo et al., 2006) and on pregnancy rates (Hasler, 2000). Abdoon et al. (2001) suggest that factors related to the culture environment are associated with the development of COCs and their further growth to blastocysts.

Table 3. Means ± standard deviation of results for ovum pick-up *in vitro* production (OPU-IVP) traits in Brazilian Gyr cattle from previous studies.

Reference	NVcoc	PVcoc (%)	PcleavD4 (%)	PTembD7 (%)	PPrD30 (%)	PPrD60 (%)	OPU-IVP session
Camargo et al. (2007)	-	-	66.7	29.4	44.0	-	33
Pontes et al. (2010)	12.1 ± 3.9	70.7	68.0	45.0	40.0	-	3778
Silva et al. (2012)	-	58.5	59.6	26.8	-	-	250
Grázia et al. (2012)	16 ± 1.0	61.5	86.6	61.3	-	-	68
Oliveira et al. (2013)	8.94 ± 0.69	74.73	80.97	45.19	47.18	42.95	178
This study	9.77 ± 6.79	75.25 ± 20.5	66.79 ± 33.7	35.47 ± 34.6	28.9 ± 39.5	25.73 ± 37.8	211

NVcoc, number of viable cumulus-oocyte complexes; PVcoc, proportion of viable cumulus-oocyte complexes; PcleavD4, proportion of cleaved embryos on day 4 of culture; PTembD7, proportion of transferable embryos on day 7 of culture; PPrD30, proportion of pregnancies 30 days after transfer; PPrD60, proportion of pregnancies 60 days after transfer.

The PVcoc, PcleavD4, and PTembD7 traits are proportions in relation to the number of viable COCs obtained in the OPU session. The wide variability reported for these traits in previous studies (Table 3) suggests multifactorial participation of influencing factors (donor, technician, and laboratory, among others), and justifies the evaluation of phenotypic correlations between these traits for individual selection.

Camargo et al. (2006) reported low efficiency of OPU-IVP with only 30-40% of oocytes developing to blastocysts. Analysis of the results of 4518 OPU-IVP sessions from previous studies presented in Table 3 shows the value of PTembD7 in donor Gyr to be 43.6% (the weighted average), a result that illustrates the improvement of the technique during the period evaluated.

Phenotypic correlations

The overall goal of the OPU-IVP technique is to increase the number of pregnancies for each embryo transfer; therefore, correlations between pregnancy rates and other characteristics are important for breeding strategies. For example, direct selection using PPrD30 or PPrD60, or indirect selection using other characteristics, such as those suggested by Bosselmann et al. (2005) in the case of embryo transfer produced by multiple ovulation, which included direct selection using the number of transferable embryos or indirect selection for ova flushing.

Phenotypic correlations among NPrD30, PPrD30, NPrD60, and PPrD60 with OPU-IVP traits are shown in Table 4. The magnitudes of phenotypic correlations between traits were favorable, moderate, and moderately high. In general, phenotypic correlations between numerical characteristics were moderate to moderately high.

The observed correlations between NVcoc and NcleavD4 and NVcoc and NTembrD7

(0.71 and 0.42, respectively) indicate that in a batch of embryos the animal with greater production of viable oocytes is 71% more likely to achieve a high number of cleaved oocytes on day 4 and 42% more likely to achieve a greater quantity of transferable embryos on day 7 of culture. In contrast, significant phenotypic correlations between NVcoc and PcleavD4, PTembD7, PPrD30, and PPrD60 were not observed, indicating that the rates cited are independent of NVcoc, suggesting that other components have a greater impact on performance.

Table 4. Phenotypic correlations between ovum pick-up *in vitro* production (OPU-IVP) traits and number and ratio of pregnancies in Brazilian Gyr cows.

	NVcoc	PVcoc	NcleavD4	PcleavD4	NTembD7	PTembD7	NPrD30	NPrD60	PPrD30
NVcoc	-	-	-	-	-	-	-	-	-
PVcoc	0.24**	-	-	-	-	-	-	-	-
NcleavD4	0.71**	0.25**	-	-	-	-	-	-	-
PcleavD4	0.058	0.17*	0.46**	-	-	-	-	-	-
NTembD7	0.42**	0.09	0.65**	0.45**	-	-	-	-	-
PTembD7	0.014	0.04	0.19**	0.50**	0.71**	-	-	-	-
NPrD30	0.14*	0.12	0.34**	0.32**	0.57**	0.49**	-	-	-
NPrD60	0.13	0.12	0.32**	0.31**	0.57**	0.51**	0.98**	-	-
PPrD30	-0.035	0.15*	0.16*	0.39**	0.30**	0.43**	0.67**	0.64**	-
PPrD60	-0.015	0.17*	0.15*	0.35**	0.33**	0.47**	0.65**	0.70**	0.91**

*Correlation is significant at the 0.05 level. **Correlation is significant at the 0.001 level. NVcoc and PVcoc, number and proportion of viable cumulus-oocyte complexes; NcleavD4 and PcleavD4, number and proportion of cleaved embryos at day 4 of culture; NTembD7 and PTembD7, number and proportion of transferable embryos at day 7 of culture; NPrD30 and PPrD30, number and proportion of pregnancies 30 days after transfer; and NPrD60 and PPrD60, number and proportion of pregnancies 60 days after transfer.

There are few studies that describe the phenotypic and genetic correlations in OPU-IVP traits in cows; however, Merton et al. (2009), using Holstein cows, found phenotypic correlations between NVcoc and NcleavD4 and NTembrD7 (0.85 and 0.52, respectively), similar to the results found in this study. The same authors found low heritability for NVcoc, NcleavD4, PcleavD4, and PTembD7 (0.25, 0.19, 0.07, and 0.10, respectively), and the genetic correlations were in line with the phenotypic correlations. They concluded that environment has the greatest influence on phenotypic correlations.

Percentages of phenotypic correlations between numerical and proportional traits within the same stage of embryonic or fetal development were significantly correlated and moderately high to high, e.g., the correlation between PcleavD4 and PTembrD7 was 0.50. In the study conducted by Merton et al. (2009), this phenotypic correlation showed a value of 0.86, indicating that the achievement of high rates of cleavage on day 4 of culture is highly correlated with high rates of embryos on day 7.

On the other hand, moderate correlations between NVcoc and NTembD7 characteristics (0.42) and moderately high correlations between NTembD7 and PTembD7 (0.71), suggest that cows with higher rates of viable oocytes also have higher rates of embryo development to the blastocyst stage on day 7.

Repeatability for some of the features was also calculated. Repeatability is used in genetic breeding as the upper limit of heritability, it is explained by variations provided by the genotype and the permanent alterations caused by the environment (Cruz et al., 2004), and it is defined as the correlation of different records for a particular character, or expression of the same character at different times in the life of the same individual (Ossa Saraz, 2003). In this study, the repeatability was zero or negative in most cases (Table 5), except for NVcoc (0.66),

indicating that the donor cows analyzed have an average probability of repeating the value obtained in the NVcoc variable when subject to additional OPU-IVP sessions.

Table 5. Mean repeatability values of Brazilian Gyr oocyte donors involved in multiple ovum pick-up *in vitro* production (OPU-IVP) programs.

Characteristic	Repeatability
NVcoc	0.66
PVcoc (%)	-0.03
NcleavD4	0.044
PcleavD4 (%)	0.07
NTembD7	0.025
PTembD7 (%)	0.019
NPrD30	0.03

NVcoc, number of viable cumulus-oocyte complexes; PVcoc, proportion of viable cumulus-oocyte complexes; NcleavD4, number of cleaved embryos on day 4 of culture; PcleavD4, proportion of cleaved embryos on day 4 of culture; NTembD7, number of transferable embryos on day 7 of culture; PTembD7, proportion of transferable embryos on day 7 of culture; and NPrD30, number of pregnancies 30 days after transfer.

Characteristics of PTembD7 and NPrD30 showed low or no repeatability (0.025 and 0.03) and moderate phenotypic correlation (0.47), suggesting that environmental correlation is the main source of phenotypic contribution. Therefore, actions focused on improving the environment for embryo development to the blastocyst stage may be translated as an increase in pregnancy rates for these embryos.

A similar situation was shown in multiple ovulation of embryo transfer programs (Ireland et al., 2007), where, in general, a higher number of flushed embryos also resulted in higher numbers of transferable embryos, which corroborates with the moderate correlation between numerical traits found in this study.

The current study showed that OPU-IVP traits could be applied for selection by phenotype to enhance the *in vitro* production of embryos per donor. In conclusion, it is plausible to optimize the process of OPU-IVP by using phenotypic correlations that show moderate, moderately high, and high significance in the selection of oocyte donors. The number of cumulus oocyte-complexes showed a high repeatability (0.66) and moderate and moderately high correlation with proportional traits directly related to pregnancy rates in this program.

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