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Naturally occurring phytoestrogen effects on twinning rate in Maremmana cattle

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ABSTRACT

Twinning in most cattle herds, occurs with a frequency of 1-2% and the dizygous type twinning accounts for the majority of observations. Twinning rate is known to be affected by age and parity of the dam, seasonal effects, feeds characteristics (protein and phytoestrogens content) and by genetic aspects. The aim of the present study was to assess the influence of naturally occurring phytoestrogens on twinning in Maremmana cattle. The Maremmana herd of Castelporziano farm (Rome) was used as "case study", as in the period from 1996 to 2008 an abnormal rate of twin births for cattle of 12 % (on average) was recorded. During the reproduction season, the herd was divided into 3 groups grazing in 3 different parcels (Malafede [MA], Tiro a Piattello [TP] and Contumaci [CO]). Forages were collected in all parcels in spring 2006 and 2007. After a species identification, forages were dried and grinded to pass a 1 mm mesh. Phytoestrogens (PYE) (Daidzein, Formononetin, Genistein, Biochanin A) and Coumestrol) were extracted by acid hydrolysis and they were quantified by reverse phase HPLC with UV and flurimetric detection. All data were analyzed using the GLM procedure by the Statistica 7 software package (StatSoft Inc., Tulsa, OK, USA). Differences in phytoestrogen's mean content between parcels was declared significant at $P<0.05$. The analysis of forage samples belonging to three parcels showed a significant association ($p<0,001$) between quantity of phytoestogens (Daidzein, Formononetin, Genistein and Biochanin A) in grazed essences and twinning rate. Total Phytohormone level in MA (10114 ±4340mg\kg DM), where twinning rate recorded was on average 12%, was signi+ficantly above the level recorded in field TP, where twinning was absent (2337 ± 1556 mg\kg DM) and CO where twinning rate was 3,5 % (1182 ± 509 mg\kg DM). Individuals showing a higher rate of twin birth were always observed in MA parcel characterized by a high density of Subterranean Clover.Although little is known about enhancing effects of naturally occurring phytoestrogens on twinning rate in cattle, our results suggests that some effects of pasture composition may be observed on Maremmana cattle reproduction in field condition.

INTRODUCTION

Twinning in most cattle herds occurs with a frequency of 1-2% and the dizygous type twinning accounts for the majority of the observations. Twinning rate in cattle is known to be affected by age and parity of the dam, seasonal effects, nutritional aspects, such as proteins, phytoestrogens and others, and genetic aspects. (Gregory *et al*., 1990) As far as the nutritional aspect is concerned, naturally occurring phytoestrogens in feedstuffs are reported to exert some effects on ruminants even though the impacts on twinning rate appear to be controversial. Phytohestrogens (PHY) are a diverse group of natural non-steroidal plant compounds possibly causing estrogenic and/or anti-estrogenic effects in exposed animals (Stopper *et al*., 2005).

Isoflavones are the most investigated class of PHY and, as ascertained by many studies, they have hormonal activity on several animal species (Stopper *et al*., 2005).The main representative isoflavons in plants are genistein (GE), daidzein (DA), biochanin A (BA) and formonotein (FO). Some studies showed that phytoestrogens compete with endogenous steroids so that the balance between estrogenic and anti-estrogenic activity is determined by the proportion of circulating PHY compared to endogenous estrogens (Adams, 1995). Some forage species are particularly rich in isoflavons and they are responsible for reproductive abnormalities in sheep, in cattle, pigs and poultry (Dusza *et al*., 2006). In sheep, the exposure to PHY results in decreased twinning rate and ovulation, hypertrophy of some glands like the thyroid, decreased reproduction (up to -10%) (Burton *et al.,* 2002). In humans, PHY show predominantly anti-estrogenic effects, causing a negative feedback inhibiting the production of FSH and LH from the pituitary gland. The ingestion of clover rich in PHY has been reported to produce serious reproductive problems in cattle (Dusza *et al*., 2006c; Adams, 1995).

The aim of this research was to investigate the effect of naturally occurring PHY on twinning rate in cattle using a Maremmana grazing herd as a case study.

MATERIAL AND METHODS

The Maremmana herd of Natural Reserve (Lazio Region, Italy) was used as a case study. From 1996 to 2008 this herd showed an high twinning rate (range is 5% and 20%). The farm demographic database (1996-2008) was used to collect date and type of births, sex and weight of the calves and reproductive disorders (miscarriage, stillbirth). In the period 2006/2007, 55 samples of forage were sampled in three grazing parcels named “Malafede” (M), “Tiro a Piattello” (P) and “Contumaci” (Q), respectively.

Methods

The twinning rate (TWR) was calculated on per year/herd/parcel basis from 1996 to 2008 (TWR= TWB/TB where TWB = twin births, TB = total births). PHY were extracted from dry and ground forage by acid hydrolysis and a RP-HPLC method was used for detection and quantization (Franke et al., 1994). Quantitative assessment of PHY in pasture samples, was performed against calibrations using the commercial standards: Daidzein (DA)(D7802, Sigma, Germay) Formononetin (Fluka, USA), synthetic Genistein (GE)(Sigma, Germany), (BA) Biochanin A (Sigma, Germany) and Coumestrol (CU)(Fluka, USA). The calibration standards, ranging from 0.086 to 2.896 µg/ml, were obtained by serial dilutions in MetCN/MetCOOH/H_2O (22:8:70). Calibration curves for all the PHY were constructed injecting in duplicate (20 µl) the calibration standars. RP-HPLC analysis was performed using a SpectraSystem HPLC (Thermo Sparation Products, Riviera Beach, FL) equipped with Nova-Pak C18 separative column (150 x 3.9 mm id, 4 µm) (Waters, Milford, MA)

thermostated at 30°C and detecting peaks for GE, FO, DA, BA at λ=260 nm and CU at λ=342 nm. For the peak purity assessment in samples, a 190-500 scan was also performed and spectra for each PHY (standards) were archived.

Statistical analysis

The statistical significance of twinning rate in the herd of Castelporziano (1996 - 2008) was calculated using the χ 2 method. The level of PHY in grazed parcels, M, P and Q were expressed as means ± SEM. Statistical significance was tested by a GLM ANOVA, followed by Fisher post-hoc test ($p<0.05$), using the following model:

$$Y_{ijk} = \mu + A_i + P_j + e_{ijk}$$

Where μ is the global average, A_i is the effect of the grazed parcel (M, P, Q), P_j indicates the effect the year (2006 *vs* 2007) and e_{ijk} is the error term. All statistical analysis were carried out using STATISTICA 7.0 (StatSoft Inc., USA).

RESULTS

Demographic data

From 1996 to 2008 the twinning rate in the Castelporziano herd ranged from 2.9% to 20.0%. (tab. 1). The incidence of twins is highly significant (tab.2).

Table 1: distribution of twin and single births and twinning rate (1996-2008)

	1996	**1997**	**1998**	**1999**	**2000**	**2001**	**2002**	**2003**	**2004**	**2005**	**2006**	**2007**	**2008**
Total births	90	77	88	88	87	97	82	82	104	101	110	70	94
single	*72*	*65*	*72*	*75*	*79*	*89*	*73*	*78*	*95*	*93*	*98*	*68*	*87*
twin	*18*	*12*	*16*	*13*	*8*	*8*	*9*	*4*	*9*	*8*	*12*	*2*	*7*
TWINNING RATE	*20,00*	*15,58*	*18,18*	*14,77*	*9,20*	*8,25*	*10,98*	*4,88*	*8,65*	*7,92*	*10,91*	*2,86*	*7,45*

Table 2: χ 2 test for the distribution of single and twin births in the Castelporziano herd from 1996 to 2008.

	Single births	**Twin births**	**Total births**	χ **2**
Observed value	*1044*	*126*	*1170*	
Expected value	*1135*	*35*	*1170*	*p<0.0001*

Phytoestrogens analysis

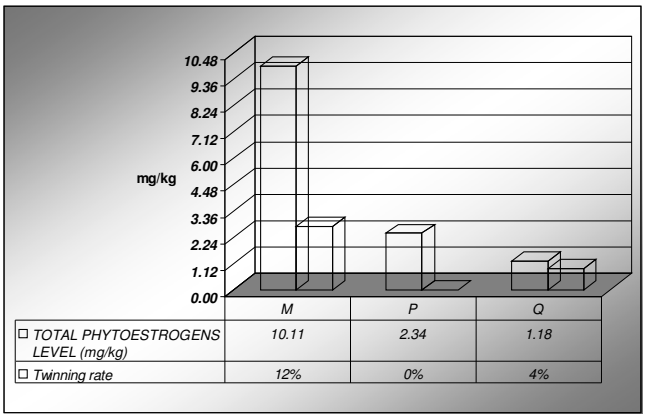
In all forage samples GE, DA, BA and FO. were detected while the Cumesterol was absent. Statistical analysis showed that the amount of all PHY detected in the M parcel was higher ($p<0.01$) than in P and Q parcels. (tab. 3).

Table 3: Total PHY levels of parcels analyzed (g/kg DM) (years 2007 – 2008). ^{a,b} $p<0.05$, ^{A,B} $p<0.01$, LOD: limit of detection

PARCEL	DA	GE	FO	BA	TOTAL
M	*0.27± 0.16*	*4.23 ± 2.90^{a}*	*2.26 ± 1.23*	*3.35 ± 2.18^{a}*	*10.11 ± 4.34^{A}*
P	*<LOD*	*0.65 ± 0.51^{b}*	*1.40 ± 0.89*	*0.29 ± 0.22^{b}*	*2.34 ± 1.56^{B}*
Q	*<LOD*	*0.19 ± 0.07^{b}*	*0.76 ± 0.53*	*0.23 ± 0.12^{b}*	*1.18 ± 0.51^{B}*

In the years 2007-2008 twinning rate in M parcel (12%) was significantly higher than in P (0%) and Q (4%) parcels (fig 1).

Figure 1: Association between the total PHY level and twinning rate



DISCUSSION AND CONCLUSION

The PHY have anti-estrogen or an estrogenic effect resulting reproductive disorders. In particular, GE and DA and their active metabolites, Equolo and Para-ethyl-phenol, causing a decrease in fertility in cattle (*Piotrowska et al., 2006*), Some studies (*Piotrowska et al., 2006*; Adams, 1995; Dusza *et al*., 2006) reported that the negative effect of PHY occur only after the embryo implantation by causing early abortion, while others (Dusza *et al*., 2006) showed that there would be an estrogenic effect at the time of ovulation which would lead to the liberation more oocytes, resulting in a twin pregnancy. Probably due to the grazing, during the breeding season, especially in the parcel M characterized by high concentrations of PHY, caused a positive effect (estrogenic) on cattles' ovulation. The literature also reports an increase in ovulation rate related to the protein content of the diet of mares. For example, in sheep has been seen as the protein level affect the

ovulation rate, high concentrations of protein in the diet result in higher levels of GH and IGF (important growth factors) that can modulate the action of gonadotropins in ovarian (Scaramuzzi *et al*., 2006) but in our case, data on crude protein content of representative samples of all parcels (data not shown) did not differ statistically.

In conclusion, the direct relationship found between quantity of PHY content of the forage grazed parcels and the number of twin births, suggest that there is a positive estrogenic effect of the food component. This result is of special interest in the selection of forage species grown on farms affected by high levels of twins. To the best of our knowledge, this is the first time that a positive association between PHY content of pastures and the twinning rate has been reported for the Maremmana cattle breed in Italy. Further studies are in progress to evaluate other factors (animal genetic, other bioactive compounds in forages, i.e. polyphenolics) possibly affecting the twin birth rate of the studied herd.

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