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ORIGINAL ARTICLE

Grass greenness flush can influence breeding phenology and fertility in equatorial thoroughbred mares in the absence of photoperiod variation

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ABSTRACT

Reproductive phenology is an important trait subjected to natural selection. Current horses in America belong to the Palearctic original populations after being introduced by European colonizers. Photoperiod variation is the main environmental factor for the adjustment of reproductive timing in horses, but is absent in equatorial areas. Here we hypothesize that seasonality of green-grass availability may influence breeding phenology in equatorial regions. We used data of 929 services to mares from 2006 to 2011 in a thoroughbred equine exploitation in Ecuador that experienced strong grass seasonality. Actual births could not be used to infer natural phenology because they were influenced by management decisions. Instead, we used variations in the probability of pregnancy after a service as a measure of the natural tendency of mares to show breeding phenology. We found that although managers tended to schedule pregnancies in two periods within the year, mares were more prone to become pregnant after the increase in grass greenness that takes place at the beginning of the year (February). Our finding has potential applications to improve the success of services and the welfare of animals, by providing green-grass stimuli in the appropriate season.

Key words: *biological rhythms, breeding phenology,* Equus*, mare behavior, racehorses.*

INTRODUCTION

Timing of breeding in ungulates commonly has major effects on reproductive success due to variations in offspring survival and/or mother breeding costs, so that natural selection has expectedly adjusted mother behavior to match the optimal breeding phenology (Kerby & Post 2013).

Modern horses are currently distributed throughout the world, although they originated in the Palearctic temperate region (Vila *et al.* 2001; Jansen *et al.* 2002). As such, they adapted during their evolutionary history to a breeding phenology with births centred in spring and early summer when forage availability peaks (Bronson 1988). In recent times, humans have moved horses to different latitudes, which may have uncoupled their behavioral responses to environmental indicators and the optimal timing for breeding. The main environmental factor known to affect the time of breeding in many mammals including horses is photoperiod (Bradshaw & Holzapfel 2007), which is the duration of the light phase within the circadian cycle. In temperate areas of the northern hemisphere, the photoperiod is increasing during the first months of the year to the maximum in June. Horses are sensitive to light, so that their tendency to start reproduction increases with time exposed to light (Burkhardt 1947; Williams *et al.* 2012), as it has been found for other organisms (Vivien-Roels & Pévet 1983).

When horses were moved to the southern hemisphere, they experienced a similar increase in the photoperiod during the months of October to December, so they readily adjusted their phenology to the forage productivity of spring and summer of the southern latitudes (Marshall 1937). However, in

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areas close to the equator, the photoperiod is almost constant along the year and hence it is useless as a reference for phenology adjustment. Then a question arises on how horses in equatorial areas adjust their breeding phenology, should there be any adjustment at all.

Besides the photoperiod, there are other subsidiary factors that can influence the timing of breeding. In temperate areas, the increase of light phase duration coincides with the exposure to the newly growing herbaceous vegetation. The potential influence of such flush of grass in the reproduction of temperate horses was early suggested (Allen 1978), but to our knowledge its actual role as an external reference for mare breeding phenology has not been studied yet, with the exception of a recent study within this same project on horse reproduction in Ecuador (Carranza *et al.* 2017).

On the other hand, horses are frequently managed so that these natural indicators might be much prevented from playing a significant role in breeding phenology. Food is frequently based on artificial supply and matings are commonly scheduled according to commercial or management interests. The main reason of the industrial interest in foaling time for thoroughbred horses is due to their arrangement at competition. Races are commonly limited to horses that match some predetermined age range. The 'official' inferior limit for birth date for a given age was set as 1 January by the English Jockey Club in 1833 and it was subsequently adopted worldwide. This ranking decision incentivized earlier foaling since earlier foals are more mature at competition (Ginther 1992; Langlois & Blouin 1996). At least within young ages, foals born in the first months may have some developmental advantage over the lately born ones. For instance, in the northern hemisphere foaling typically tends to be in late spring and early summer. Thus, for horses of a given year of age, those born early in the year may have some competitive advantage. In the southern hemisphere, births tend to occur also in late spring and early summer that in this case correspond to September–October. In the southern hemisphere, the ages of horses are arranged in years starting in July rather than in January. Therefore, the interest of managers in southern latitudes is to advance foaling from September–October to approach July as much as possible.

There is no spring close to the equator in the sense of a photoperiod increase, as it is in other latitudes. Thus, in this respect foaling in Ecuador might equally occur in early or late months of the year. In fact, ages of competing horses are arranged in this country by semesters rather than by years (Newsletter of the Hipódromo Nacional de Ecuador). Consequently, managers may be interested in producing foals either shortly after the beginning of January or shortly after the beginning of July, to register them as born in one or another semester of the year.

Here we investigate the phenology of breeding of thoroughbred mares in Ecuador, in absence of variations in the photoperiod. Our hypothesis is that despite the timing of reproductive management practices (specifically, services by stallions), mares may show breeding seasonality if there are seasonal variations in grass greenness as a key environmental indicator.

MATERIALS AND METHODS

Thoroughbred horse farm

The study was carried out in a horse farm in southwestern Ecuador (Santa Elena province) as part of a project on breeding phenology of horses in Ecuador that studied also Peruvian Paso mares (Carranza J, et al., 2017, unpublished data) and Creole mares (Carranza *et al.* 2017). The thoroughbred farm used in this study was located in an area where natural herbaceous vegetation experiences a marked seasonality. Average annual rainfall was 188.94 mm (5 years data, range from 41 to 456 mm) with a dry season between May and December when rains are almost absent. As a consequence, during the dry season herbaceous vegetation dries up.

Mares were maintained in individual boxes. There were ca. 70–90 mares in the farm, in varying numbers depending on years. They were fed with specific horse feed and alfalfa hay. Mares were released together once a day, during several hours in the morning, for spreading in a large paddock area of ca. 20 hectares with spontaneous grasses and forbs that were green or dry depending on seasonal conditions. Mares had free access to all grasses growing in the paddocks. Estrus was inspected visually on the basis of mare behavior. When in estrus, mares were served with stallions selected by the manager, during several consecutive days (1–4) to ensure that services cover ovulation events. When necessary, follicle dynamics and pregnancies were checked by ultrasound spectrometry.

NDVI data

Remote sensing is a widely used tool for the study of vegetation dynamics. From the vegetation indexes, the Normalized Difference Vegetation Index (NDVI) proposed by Rouse *et al.* (1974) is one of the most used today (Pettorelli *et al.* 2005). It has proven to be extremely useful in assessing vegetation phenology (Adegoke & Carleton 2002) and so has relevance as an indicator of green biomass change (Wang *et al.* 2003).

NDVI is based on differences in spectral reflectance in the red (Red) and in the near-infrared (NIR) regions as follows:

$$\mathrm{NDVI} = (\mathrm{NIR} - \mathrm{Red})/(\mathrm{NIR} + \mathrm{Red})$$

These spectral reflectances are themselves ratios that vary from 0 to 1, and hence NDVI values may vary from -1 to 1. Live green plants re-emit solar radiation in the NIR spectral region. Hence, dense, wet and well developed vegetation presents values closer to 1, while vegetation subjected to water stress or soil exposed with sparse vegetation has positive values close to 0. Water surface and most artificial structures (such as buildings) have higher reflectance in Red than in NIR, yielding therefore negative values of NDVI.

We used NDVI in order to assess the temporal variation of photosynthetically active (green) forage available in the paddocks during the study period. Specifically, we used the bimonthly product of the NDVI from Moderate Resolution Imaging Spectroradiometer (MODIS) sensor (MOD13). These NDVI values were computed on 16-day intervals with a pixel size of 250 m from daily satellite overpasses. We used data from years 2005 to 2015 and QGIS software (Quantum GIS Development Team, 2016. http://qgis.osgeo.org) to extract NDVI values for each study field. For the analyses, we averaged the values to a monthly scale in order to simplify the processing, and computed the increment in NDVI (IncrNDVI) as the monthly NDVI value minus the NDVI value of the previous month.

Data management and analyses

We used data on animals between years 2006 and 2011, for which managers registered foaling dates as well as number of mountings and dates for services by individual stallions to mares. For all the period we got data from 178 individual mares. We were interested in the natural tendency of mares to become pregnant, which is assumed to reflect phenology conditions. Although managers scheduled mare services according to their own criteria, services were not always successful to produce pregnancies. Therefore, we could compare the probability for a mare to become pregnant after a service throughout the dates of the year, that is the mare pregnancy rate or fertility. For data analysis we used Generalized Linear Mixed Models as implemented in SPSS v.20 (SPSS Inc., Chicago, IL, USA). To investigate which factors affected 'pregnant' as a binary response variable we used a binomial distribution with Logit link function. Subjects were individual mares nested within stallions and the random effect covariance type was 'variance components'. As fixed effects we first introduced the covariates and their two-way interactions. Thereafter, we used a backward stepwise procedure, removing those non-significant interactions with *P*-values higher than 0.20. All the main fixed effects were kept in the final model except in case of collinearity. We used robust estimation for testing fixed effects and coefficients, and the degrees of freedom were calculated following Satterthwaite's approximation. Collinearity between covariates was treated first by inspection of correlation matrix, and when the Spearman correlation coefficient was higher than 0.60 we chose the variable that resulted in the smaller information criterion values (Akaike and Bayesian criteria).

RESULTS

The distribution of services to mares along the year was bimodal (Fig. 1). As a consequence, the distribution of births showed two peaks matching the beginning of the semesters (Fig. 2). Generalized mixed model with mares nested within stallions as random factor and pregnancy rate as the dependent variable, with NDVI, its increment, meteorological variables (temperature and rainfall), also controlling for the number of mountings per service, resulted in an accuracy of 84.8% to differentiate between successful pregnancies and failures (Table 1). Individual differences between mares contributed significantly to explain the probability of getting pregnant (see Table 1). Fixed effects on the probability of pregnancy included NDVI and its increment. For rainfall, we explored monthly mean rainfall, accumulated rains from all the rainy season (January to May) and accumulated rains at the beginning of the year (January and February), this latter being the one that resulted with lower *P*-values in models but not significant. Also, it is an annual value and hence cannot explain seasonal variations within the year. Model predicted pregnancy rate peaked in February

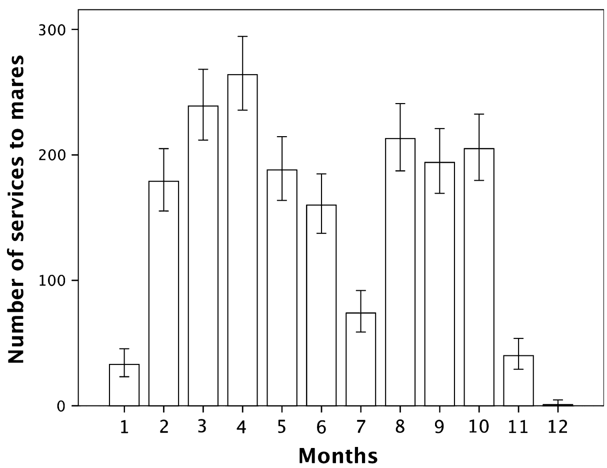

Figure 1 Services to mares along the year. Bars show mean number of served mares and 95% confidence intervals for the years of study. Months are numbered from January to December.

and after a sudden decrease it showed a slow tendency to increase by the end of the year (Fig. 3). There was a strong positive influence of the increment in NDVI, while mean NDVI resulted negative influence (Table 1; Fig. 4). No two-way interactions were significant.

DISCUSSION

Our results have evidenced a positive relationship between the flush of new green grasses and the probability of a mare to become pregnant after a service. This relationship has been found in a farm where mares are fed with feed and alfalfa hay and maintained their physical condition and nutritional requirements at an optimum level throughout the year due to commercial interest of managers. These mares spent most of the day in individual indoor boxes and only used the paddock during part of the day (several hours in the morning). Thus, the observed relationship between pregnancy rates and the presence of new green grasses can hardly be explained by the addition of nutrients or by an increase in body condition of the mares, although we cannot rule out any mechanistic process. Also, highest pregnancy rates did not associate with high NVDI values but with the initial increase of NDVI after the dry season. Therefore, rather than through nutritional elements, our results as a whole point to a potential role of green grasses through the sensorial system, analogous to the effect of changes in the photoperiod.

The effect of new green grasses has previously been reported for other herbivores such as rodents or rabbits (Poole 1960; Wallage-Drees 1983). For rabbits, Wallage-Drees (1983) postulated that the new grasses might act as a trigger for conceptions, which some authors interpreted on the basis of the phenolic substances contained in grasses that are dietary cues for the initiation of reproduction (Cheeke 1987).

The presence of new grasses has been an external factor largely associated with the onset of the

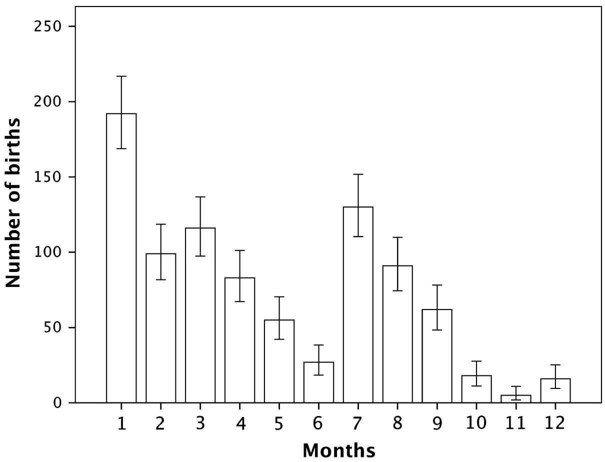

Figure 2 Number of births along the year. Bars show mean and 95% confidence intervals for the years of study. Months are numbered from January to December.

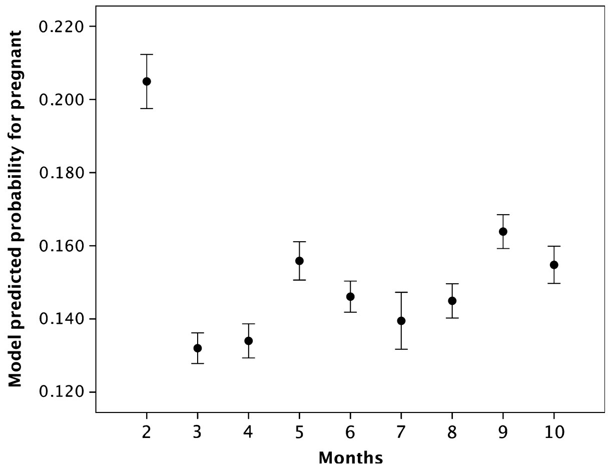

Figure 3 Pregnancy rate after services along the year. Figure shows mean and standard error for the years of study. Months are numbered from February to October. There were no complete data for services to mares in January, November and December.

Table 1 Results of Generalized Linear Mixed Model for pregnancy rate as the dependent variable with mares nested within stallions as random subjects.

Model term	Coefficient	SE	*t*	Sig.	95% CI	
					Lower	Upper
Intercept	−3.544	1.340	−2.645	0.008	−6.173	−0.914
NDVI	−3.617	1.602	−2.258	0.024	−6.761	−0.473
IncrNDVI	2.593	0.994	2.688	0.007	0.721	4.622
Temperature	0.098	0.063	1.554	0.120	−0.026	0.222
Rainfall (Jan + Feb)	0.002	0.001	1.606	0.109	0.000	0.004
Mountings	0.098	0.095	1.027	0.305	−0.089	0.285

Independent factors initially introduced in the model were: Normalized Difference Vegetation Index (NDVI), Incremental NDVI (IncrNDVI), mean temperature, rainfall in January and February, number of mountings per service, and all their two-way interactions. Probability distribution was binomial with Logit link function. Table shows parameter estimates for fixed effects of final model. No interactions were significant. Random effects: Stallions × Mares: Variance ± SE = 0.585 ± 0.295, $Z = 1.981$; $P = 0.048$. $N = 929$ cases. Accuracy of the model = 84.8%. Sig., significance; SE. standard error.

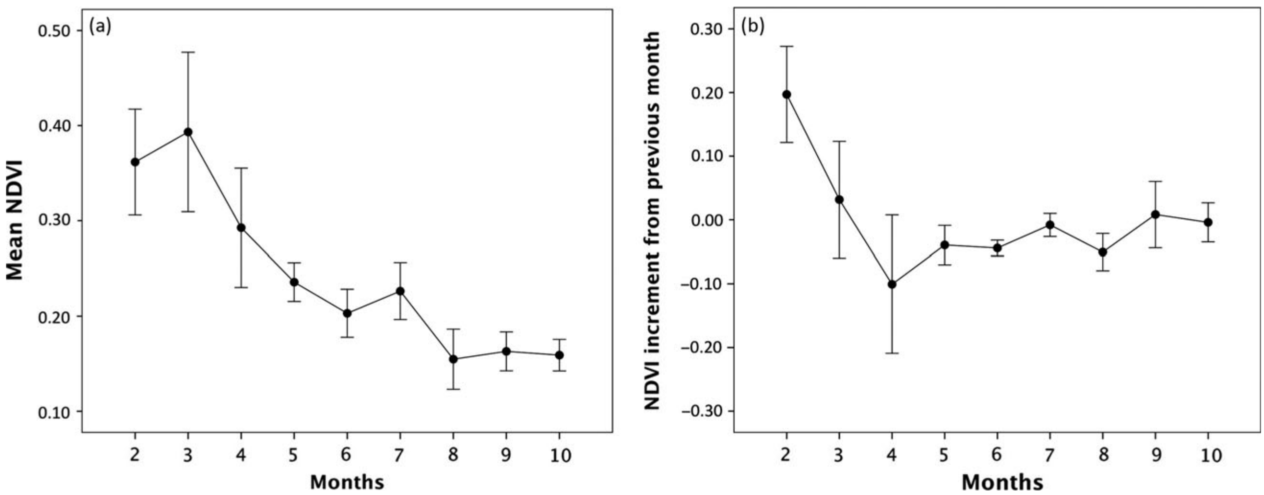

Figure 4 Normalized Difference Vegetation Index (NDVI) variation along the year: mean monthly values (a) and increment from previous month (b) (±SE) for the years of study in paddocks in the study farm. Months are numbered from February to October.

favorable season (spring) for breeding for many herbivores in temperate regions. This fact may have favored its use as a proximal cue triggering the start of reproduction. So far, photoperiod has received most attention as a cue for phenological adjustment of breeding, although other external stimuli, either social (presence of stallion, Wespi *et al.* 2014) or environmental such as green grass, may also play a role. Grass greenness should be a key parameter, especially for those species for which grasses are of central importance in favoring adequate breeding conditions. This is most evident in income breeding species like small mammals. But also, in capital breeders like horses, the presence of new grasses may be a promoting cue due to at least two potential reasons. One of them is related to resources that the mare can obtain to accomplish pregnancy and fetus development, but a second reason in a species with gestation as long as 340 days is that foaling may be quite synchronized with the appearance of new grasses the next year. Thus, it becomes plausible that sensorial perception of new grasses may play a role, especially when mares are subjected to the absence of photoperiod variations in equatorial areas, triggering physiological mechanisms similar to those involved in the regulation based in the perception of light. Even so, poor condition of the mare might prevent the triggering of reproduction by grass greenness, as shown in equatorial free-ranging Creole horses (Carranza *et al.* 2017).

In temperate areas, breeding phenology of the mare is characterized by a winter anovulatory season that starts near the autumnal equinox with the decline in adenohypophyseal secretion of luteinizing hormone (LH) and the consequent cessation of ovarian cycles (Ginther 1992). Ovarian dynamics resumes during spring with the increase in the photoperiod. The mechanism may include effects mediated by peptides like the arginine phenylalanine-amide related peptide (RFRP), since changes in RFRP gene expression and peptide accumulation in the hypothalamus in some species of birds and mammals have been associated with increases in the photoperiod (Tsutsui *et al.* 2000; Ubuka *et al.* 2005; Clarke *et al.* 2008; Revel *et al.* 2008) and recent studies also suggest a role for similar novel neuropeptides in the mare (reviewed in Williams *et al.* 2012). One possible focus of research might address whether in the absence of variation in the photoperiod, sensorial signals related to flushes of grass may associate with gene expression and accumulation in the hypothalamus of specific peptides.

From an applied point of view, it is evident that reproductive efficiency and timing for the thoroughbred industry are of paramount importance, in particular due to the system based on age arrangements for competition counted from the first of January, or in semesters in the case of Ecuador. Currently, almost the only available practice to stimulate ovarian activity in the equine industry is the provision of supplemental lighting. Alternatively, the treatment with native gonadotropin-releasing hormone (GnRH) to stimulate synthesis and secretion of LH may work if correctly applied (see Thorson *et al.* 2014a,b). The relationship between green grass and pregnancy rates evidenced here, opens the possibility for managing the presence of green grasses in paddocks to simulate seasonality, although its efficiency still deserves further research.

Future studies on phenology of pregnancy rates in equatorial mares should focus on the experimental demonstration of the effect of green grasses, their potential effect on ovarian cycles, and on disentangling whether the mechanism can be sensorial or by the intake of some key element in the grass. At the same time, the practical suggestions derived from our results could be experimentally applied to farms in order to evaluate their impact on the phenology and reproductive efficiency of mares.

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