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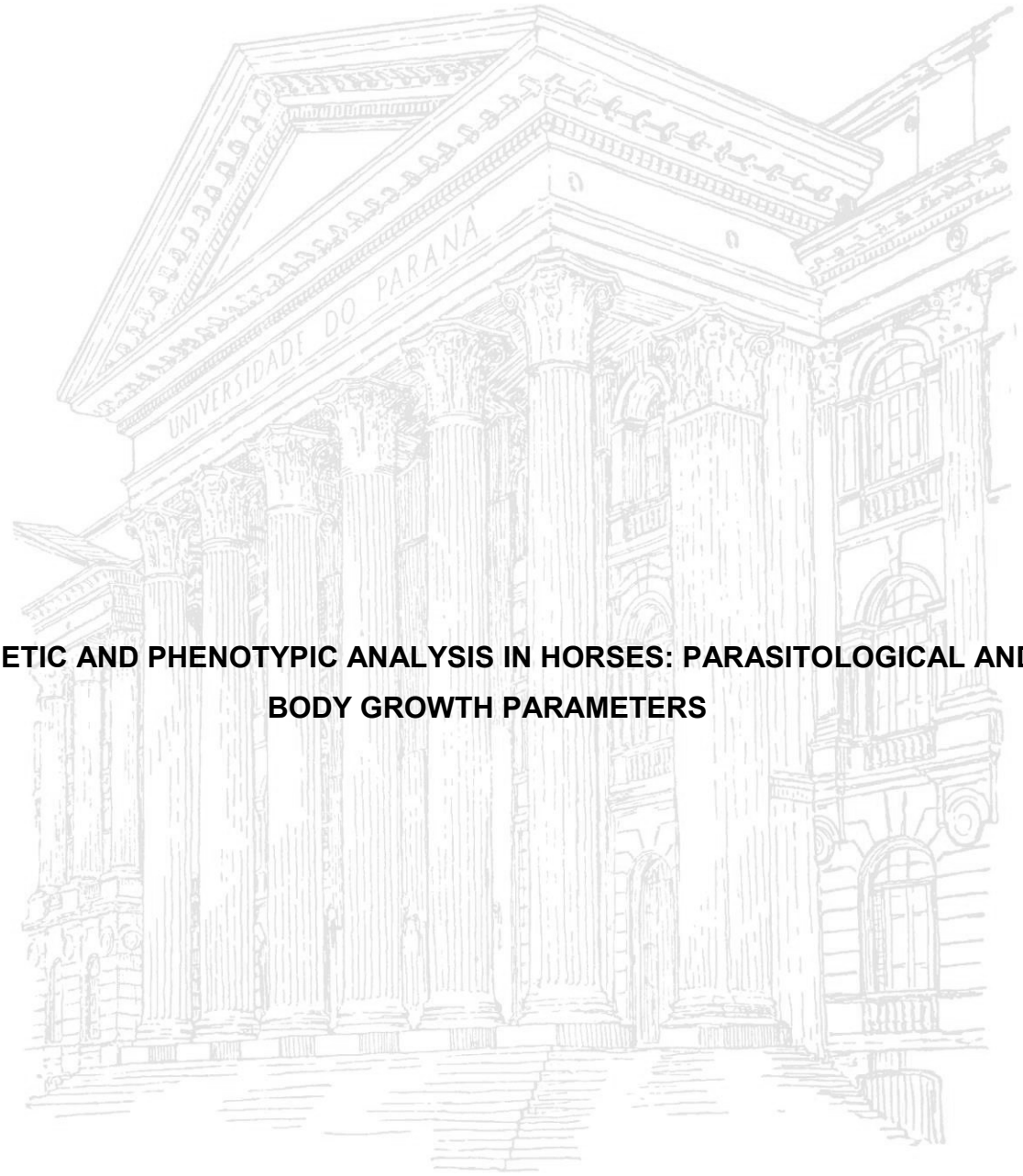

GENETIC AND PHENOTYPIC ANALYSIS IN HORSES: PARASITOLOGICAL AND BODY GROWTH PARAMETERS

CURITIBA

2018

LUCIANA LAITANO DIAS DE CASTRO

GENETIC AND PHENOTYPIC ANALYSIS IN HORSES: PARASITOLOGICAL AND BODY GROWTH PARAMETERS

Tese apresentada como requisito parcial à obtenção do grau de Doutora em Ciências Veterinárias, no Programa de Pós-Graduação em Ciências Veterinária, Setor de Ciências Agrárias, da Universidade Federal do Paraná.

Orientador: Prof. Dr. Marcelo Beltrão Molento

CURITIBA

2018

FICHA CATALOGRÁFICA ELABORADA PELO SISTEMA DE BIBLIOTECAS/UFPR -
BIBLIOTECA DE CIÊNCIAS AGRÁRIAS, DOUGLAS ALEX JANKOSKI CRB 9/1167
COM OS DADOS FORNECIDOS PELO(A) AUTOR(A)

D541g

Dias de Castro, Luciana Laitano
Genetic and phenotypic analysis in horses: parasitological and body growth parameters / Luciana Laitano Dias de Castro. - Curitiba, 2018.
108 f.: grafs.,tabs.

Tese (Doutorado) - Universidade Federal do Paraná. Setor de Ciências Agrárias, Programa de Pós-Graduação em Ciências Veterinárias.
Orientador: Marcelo Beltrão Molento

1. Equino - Parasitas. 2. Parasitologia veterinária. 3. Equino - Doenças. I. Molento, Marcelo Beltrão. II. Título. III. Universidade Federal do Paraná.

CDU 619:636.1

MINISTÉRIO DA EDUCAÇÃO
SETOR SETOR DE CIENCIAS AGRARIAS
UNIVERSIDADE FEDERAL DO PARANÁ
PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
PROGRAMA DE PÓS-GRADUAÇÃO CIÊNCIAS VETERINÁRIAS

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CURITIBA, 21 de Fevereiro de 2018.

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ACKNOWLEDGMENT

I would like to thank my parents and my sister who have always supported me and tried to understand the moments that I missed.

To my supervisor, Prof. Dr. Marcelo B. Molento, who always motivated me, allowing the development of many studies. Thank you for your dedication and support!

To my colleagues at the Laboratory of Parasitic Diseases at UFPR, the Animal Breeding and Biotechnology Group at USP and the Gluck Equine Research Center at the University of Kentucky, who always helped me and made this project possible.

To the professors and employees of the Federal University of Paraná and colleagues from the graduation program, who helped me in achieving this Doctorate degree.

I would also like to express my sincere thanks to the veterinarian Joaquim Antunes and the entire team of the São José da Serra Stud. Thank you for the availability of the place, the animals and also for the help in doing the experiment.

My deep gratitude to the friends I made in these four years of doctorate and who supported me on this path in a new city and to my old friends who always supported and helped me, keeping in touch with the distance.

Thank you all very much!

RESUMO

Ao longo das últimas décadas, os ciatostomíneos (pequenos estrôngilos) tornaram-se os parasitas internos mais importantes em cavalos. Sendo agentes de graves infecções parasitárias e com o controle cada vez mais difícil devido à seleção de populações resistentes à maioria dos grupos de anti-helmínticos disponíveis. Portanto, são necessários estudos para avaliar as infecções parasitárias e a influência dessas doenças no desenvolvimento corporal dos animais; assim como, descobrir os padrões genéticos que podem estar relacionados à sua infeção, apresentando alta ou baixa contagem de ovos por grama de fezes (OPG). O objetivo deste estudo foi realizar análises genéticas e fenotípicas de parâmetros parasitológicos e de desenvolvimento corporal em cavalos de puro sangue inglês (PSI) naturalmente parasitados. A presente tese está dividida em cinco capítulos, apresentados em formato de Introdução, manuscritos e as Considerações Finais. O primeiro artigo comparou duas técnicas de contagem de OPG, McMaster (McM) e Mini-FLOTAC (mF), para a detecção de ovos de nematódeos gastrointestinais de bovinos e equinos, em diferentes locais. Em todos os experimentos em média os valores do desvio padrão e do coeficiente de variação foram significativamente menores para a técnica de mF. Portanto, recomenda-se o uso da técnica Mini-FLOTAC, que é um método com menor variabilidade e maior precisão, especialmente para animais com baixo OPG. O segundo artigo teve como objetivo avaliar o impacto do gênero do animal, mês de nascimento, idade e número de filhos da égua, e contagem de OPG na taxa de crescimento de cavalos PSI. Este estudo também teve como objetivo correlacionar as informações sobre o desempenho dos animais nas corridas associadas aos valores OPG. O peso corporal médio dos animais ao nascer foi 56,5 kg (machos) e 51,9 kg (fêmeas), e aos 18 meses, pesavam em média 435,9 e 441,6 kg, respectivamente. Altura da cernelha média ao nascer foi de 104,36 cm (machos) e 101,19 cm (fêmeas), e aos 18 meses, a altura foi de 154,78 e 153,5 cm, respectivamente. Os dados das corridas mostraram que os animais com maior OPG apresentaram um desempenho menor quando comparados com aqueles com menor OPG, incluindo um número menor de vitórias. Concluindo que animais criados sob um conjunto bem definido de práticas de manejo podem ter poucos ou nenhum sinal de comprometimento devido as infecções parasitárias. O objetivo do artigo 3 foi determinar regiões genômicas associadas aos padrões individuais do OPG dos animais, realizando um estudo de associação genômica ampla entre equinos PSI naturalmente infectados com parasitas gastrointestinais. Neste estudo, regiões candidatas relacionadas ao desenvolvimento e ativação do sistema imune foram encontradas nos cromosomos 17, 21 e 25. Esta tese destacou várias possibilidades para investigar parasitarias gastrointestinais em cavalos, melhorando a compreensão e o controle de infecções por ciatostomíneos. Observou-se que os cavalos criados sob um conjunto bem definido de práticas de manejo podem ter poucos ou nenhum sinal de comprometimento devido a infecções parasitárias.

Palavras-chave: Equino. Ciatostomíneos. Cavalo. Desenvolvimento corporal. Mini-FLOTAC. OPG. GWAS

ABSTRACT

Over the last decades, cyathostomins (small strongyles) have become the most important internal parasites of horses. They are agents of serious parasitic infections and their control is increasingly difficult due to the selection of resistant populations to most of the anthelmintic groups available. Therefore, studies to assess the parasite infections, and the influence of these diseases on body development of animals are necessary. In the same line, it is important to discover the genetic patterns of the animals that may be related to their infection, presenting high or low fecal egg count (FEC). The aim of this study was to perform genetic and phenotypic analysis of parasitological and body development parameters in Thoroughbred horses. The present Thesis is divided in five Chapters, presented in the format of Introduction, manuscripts and Final Considerations. The first article compared two FEC techniques, namely McMaster (McM) and Mini-FLOTAC (mF), for the detection of cattle and horse gastrointestinal nematode eggs, in different locations. On average, in all experiments, the standard deviation and the coefficient of variation values were significantly lower for mF. Therefore, it is recommended the use of the Mini-FLOTAC technique, which is a method with less variability and higher accuracy, particularly for animals with low FEC. The second article had the objective to evaluate the impact of animal gender, month of birth, age of mare, number of offspring and FEC in the growth rate of Thoroughbred horses. This study also aimed to correlate the information on racing careers associated with the FEC values of the animals. The mean body weight at birth was 56.5 kg for males and 51.9 kg for females, and at 18 months', males and females weighed on average 435.9 and 441.6 kg, respectively. The mean withers height at birth was 104.36 cm for males and 101.19 cm for females, and at 18 months, the height was 154.78 and 153.5 cm, respectively. The racing history data showed that animals with higher FEC had a poorer performance when compared to those with lower FEC, including a smaller number of victories. In conclusion, it was suggested that horses raised under a well-defined set of management practices can have little or no signs of impairment due to worm infections. The aim of the article three was to determine genomic regions associated with Strongyle FEC patterns, conducting a genomic wide association study (GWAS) among Thoroughbred equines naturally infected with gastrointestinal parasites. In this study, candidate regions related to immune system development and activation were found in ECA17, ECA 21 and ECA25. This thesis has highlighted several possibilities to further investigate the control of gastrointestinal parasite infections in horses, improving the understanding and control of cyathostomins infections. It was observed that horses raised under a well-defined set of management practices can have little or no signs of impairment due to worm infections.

Key-words: Equine. Horse. Cyathostomins. Body growth. Mini-FLOTAC. EPG. GWAS.

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1 INTRODUCTION

The horse (*Equus caballus*) was domesticated about 6000 years ago (CLUTTON-BROCK, 1999) and have been playing an essential role in the development of many civilizations, humans in many parts of the world have relied on them for thousands of years (OUTRAM et al., 2009). Health and growth of young horses are well known to be influenced by gastrointestinal parasitism (CLAYTON and DUNCAN, 1978). Important gastrointestinal parasites of equines comprise cyathostomins, or small strongyles (LOVE et al., 1999), the tapeworm, *Anoplocephala perfoliata* (PROUDMAN et al., 1998), the large round worm, *Parascaris equorum* (AUSTIN et al., 1990; CRIBB et al., 2006), and *Strongylus vulgaris*, commonly known as the bloodworm (DRUDGE, 1979; NIELSEN et al., 2016).

Cyathostomins are ubiquitous in equine populations worldwide, across different climates and equine management practices (LYONS et al., 1999; CHAPMAN et al., 2003; GAWOR et al., 2006) and may cause reduction in growth rate, weight loss, and poor body condition (UHLINGER et al., 1991; MURPHY and LOVE, 1997; LOVE et al., 1999). Larval stages of these parasites can cause major disease and fatality in affected horses (LOVE et al., 1999; MAIR et al., 2000). During their development, third stage larvae may remain encysted in the mucosa and submucosa of the large intestinal wall, in a state of hypobiosis. To continue development to adults, the larvae emerge from cysts as fourth stage larvae and continue development into the fifth and adult stage within the intestinal lumen. Synchronous mass emergence of these larvae can lead to a clinical syndrome termed larval cyathostominosis characterized by serious injury to the large intestinal walls, with a resultant diarrhea, colic, and mortality rates as high as 50% (ABBOTT, 1998; LOVE et al., 1999; CHAPMAN et al., 2002).

Three anthelmintic classes are licensed for the use against cyathostomins: macrocyclic lactones, tetrahydropyrimidines, and benzimidazoles. However, the excessive use of anthelmintics, as the principal method of parasite control, has led to high levels of anthelmintic resistance in cyathostomin populations across the world (PEREGRINE et al., 2014). Benzimidazole and pyrantel resistant cyathostomins have been reported in at least 14 and 12 countries, respectively, and there are

reports of emerging macrocyclic lactone resistance as well (MOLENTO et al., 2008; PEREGRINE et al., 2014).

The egg reappearance period (ERP) is defined as the duration of time required for fecal egg counts (FEC) to return to 20% of pre-anthelmintic treatment levels (KYVSGAARD et al., 2011). For the macrocyclic lactones, the cyathostomin ERP was initially ~8 weeks for ivermectin and ~16 to 22 weeks for moxidectin (BOERSEMA et al., 1996; MONAHAN AND KLEI, 2002). Several studies performed in recent years have reported dramatically reduced ERPs for both drugs, and strongyle eggs now commonly reappear as early as four weeks post treatment (VON SAMSON-HIMMELSJERNA et al., 2007; MOLENTO et al., 2008; LYONS et al., 2009, 2010, 2011; CANEVER et al., 2013). These shortened ERPs have been associated with resistance in luminal fourth stage larvae (LYONS and TOLLIVER, 2013; BELLAW et al., 2017).

Due to these ever-increasing findings of anthelmintic resistance, veterinary parasitologists have recommended reducing anthelmintic treatment intensity. One widely advocated strategy is the target selective therapy, where treatment decisions are based on individual animal strongyle FEC (HERD et al., 1985; GOMEZ and GEORGI, 1991; KRECEK et al., 1994). Egg counts are determined from all horses within a given population, but only those exceeding a pre-determined FEC threshold receive anthelmintic treatment. However, the use of this method of control for veterinarians and horse-owners has been slow, the first two studies describing the use of selective therapy in horses were in 1991 (GOMEZ and GEORGI, 1991; DUNCAN and LOVE, 1991).

Several studies have illustrated the over-dispersed distribution of parasites among hosts, by documenting how a small number of individuals within a herd are responsible for shedding the large majority of the strongyle egg output (NIELSEN et al., 2006). This has been described as the 20/80 rule, where 20% of the animals in a given herd are responsible for 80% of the total strongyle egg output (KAPLAN and NIELSEN, 2010; RELF et al., 2013). Furthermore, several authors observed that egg shedding levels of individual horses is repeatable over time (DÖPFER et al., 2004; NIELSEN et al., 2006; BECHER et al., 2010; SCHEUERLE et al., 2016). Recently, Kornás et al. (2015) found that some of this individual egg shedding pattern can be explained by genetic variation among horses and estimated that 21% of this phenotype has a genetic inheritable basis. Therefore, this strongyle egg shedding

consistency in horses is a phenotype for which genetic explanations can be sought. However, this is the only study published on this topic thus far, and it did not include any attempt to identify single nucleotide polymorphisms (SNPs) associated with the FEC phenotype in a genome wide approach.

Studies in humans and livestock species, showed the occurrence of million SNPs across the genome of an individual (*Human Genome Project Information*, *The SNP Consortium LTD*, *Bovine Genome Sequencing and Analysis Consortium*, *EquCab2.0 SNP Collection*). Due to the possibility of a relationship between SNPs and genetic diseases, productive and reproductive characteristics in animals and plants, many technologies were developed for their genotyping. Recent technological advances have led to the development of methodology for genotyping tens of thousands of SNPs in a single assay. The genotyping chips of SNPs, containing on average more than 50 thousand SNPs, were generated and validated for human, bovine, equine, ovine, porcine and canine, and are being widely used for genome wide association studies (GWAS).

Currently, the data of complete genome sequences derived from new generation sequencing on Rocha's 454 FLX platforms and Illumina's Solexa platforms, and more recently the Applied Biosystems's SoLid System platform, are available. These technologies allow the identification of a high number of SNPs and variations in the number of DNA copies, and these data can be incorporated in analyses of different methodologies for a better understanding of the genetic architecture of polygenic characteristics. Genome sweeps for thousands of SNPs can be taken simultaneously from an experimental population and from various species of animals using these SNP chips, more quickly and at a lower cost compared to using microsatellites (LEGARRA et al., 2015).

These chips have successfully served, mainly, the identification of SNPs, genomic regions and genes related to coat color (BRUNBERG et al., 2006; REISSMANN et al., 2007), reproduction and fertility (HAMANN et al., 2007; GIESECKE et al., 2009), and performance (HILL et al., 2010; GU et al., 2010). More recently, complex characteristics related to sporting performance (BINNS et al., 2010; SCHRÖDER et al., 2012) and disease resistance (SOLBERG et al., 2004; BROWN et al., 2006; RIOS et al., 2007) have been the subject of research using SNP chips in equines.

Molecular markers based on individual DNA preparations are considered an important way to identify and assess genetic variation in the population. Determination of SNPs associated with strongyle fecal egg count patterns will enable equine managers to predict individuals' phenotypic traits on which to base subsequent parasite control programs, reducing long-term costs of parasite control to better maintain animal welfare. The aim of this thesis was to perform genetic and phenotypic analysis of parasitological and body development parameters in Thoroughbred horses.

This document is formed by the papers: Comparison of McMaster and Mini-FLOTAC fecal egg counting techniques in cattle and horses, written with the data of the analysis of fecal egg counting techniques; by the manuscript, Growth rate and performance of Thoroughbred horses naturally infected with Cyathostomins, which addresses the phenotypic evaluation of the horses in the experiment; and by the manuscript Genome wide association in Thoroughbred horses naturally parasited with cyathostomins, which is a study of genomic association of experimental animals with the traits of interest. At the very end, the document is finish including the Final Considerations based on these findings.

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2 ARTICLE 1 - COMPARISON OF MCMASTER AND MINI-FLOTAC FECAL EGG COUNTING TECHNIQUES IN CATTLE AND HORSES

Paper published in the journal Veterinary Parasitology: Regional Studies and Reports in October 2017 (Appendix A).

ABSTRACT

The aim of this study was to compare two fecal egg count (FEC) techniques; McMaster (McM) and Mini-FLOTAC (mF), for the detection of cattle and horse gastrointestinal nematode eggs, in different locations. Experiment 1: feces were collected from 16 cattle and FEC was performed individually, using mF with the sensitivity of 5 eggs per gram of feces (EPG) and McM with a sensitivity of 50 EPG at Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG and the Laboratory of Parasitic Diseases of the University of Parana – LDP/UFPR. Experiment 2: Fecal samples from 30 horses were analyzed with mF (sensitivity of 5 EPG) and McM (sensitivity of 25 EPG) at the University of Mato Grosso do Sul - UFMS and LPD/UFPR. Experiment 3: feces were collected from 14 foals and FEC was performed using mF (sensitivity of 5 EPG); and McM (sensitivity of 25 EPG) only at the LPD/UFPR. For cattle, the average FEC of mF was 962 at LPD; and 1248 at EPAMIG; for McM it was 1393 at LPD and 1563 at EPAMIG. For horses, the FEC average using the mF was 650 at LPD and 469 at UFMS; and for McM it was 677 at LPD and 554 at UFMS. For foals, the average FEC for mF was 404 and 436 for McM. In all experiments, the standard deviation and the coefficient of variation values were significantly lower for mF. Therefore, it is recommended the use of the Mini-FLOTAC technique, which is a method with less variability and higher accuracy, particularly for animals with low FEC.

Key-words: EPG. Cattle. Horse. Nematode. Diagnosis.

COMPARAÇÃO DAS TÉCNICAS DE CONTAGEM DE OVOS FECAIS MCMASTER E MINI-FLOTAC EM BOVINOS E CAVALOS

RESUMO

O objetivo deste estudo foi comparar duas técnicas de contagem de ovos por grama de fezes (OPG), McMaster (McM) e Mini-FLOTAC (mF), para a detecção de ovos de nematódeos gastrointestinais de bovinos e equinos, em diferentes locais. Experimento 1: fezes foram coletadas de 16 bovinos e o OPG foi realizado, individualmente, utilizando mF com sensibilidade de 5 ovos e McM com sensibilidade de 50, na Empresa de Pesquisa Agropecuária de Minas Gerais – EPAMIG e no Laboratório de Doenças Parasitárias da Universidade Federal do Paraná – LDP/UFPR. Experimento 2: amostras de fezes de 30 cavalos foram analisadas com as técnicas de mF (sensibilidade de 5 ovos) e de McM (sensibilidade de 25 ovos) na Universidade de Mato Grosso do Sul – UFMS e no LDP/UFPR. Experimento 3: foram coletadas amostras de 14 potros e o OPG foi realizado através das técnicas de mF (sensibilidade de 5 ovos) e de McM (sensibilidade de 25 ovos) apenas no LDP/UFPR. Para os bovinos, a média do OPG foi de 962 para mF no LDP e 1248 no EPAMIG; para McM foi 1393 no LDP e 1563 no EPAMIG. Para os equinos, o OPG médio utilizando mF foi 650 no LDP e 469 na UFMS; e para McM foi 677 no LDP e 554 na UFMS. Para os potros, o OPG médio foi 404 para mF e 436 para McM. Em todos os experimentos os valores do desvio padrão e do coeficiente de variação foram significativamente menores para a técnica de mF. Portanto, recomenda-se o uso da técnica Mini-FLOTAC, que é um método com menor variabilidade e maior acurácia, particularmente para animais com baixo OPG.

Palavras-chave: OPG. Bovinos. Cavalos. Nematódeos. Diagnóstico.

2.1 INTRODUCTION

The fecal egg count (FEC) methods permit to determine the number of nematode eggs in livestock (BOSCO et al., 2014; PRESLAND et al., 2005). There are several FEC and these differ in sensitivity, time required to process the samples and the necessary technical knowledge for interpretation. The test can potentially be extended to other situations, where accurate detection of egg number is important; i.e. the fecal egg count reduction test (FECRT) to determine anthelmintic efficacy in a range of parasites (BOSCO et al., 2014).

The most widely used method for FEC was developed in Australia by Gordon and Whitlock (1939), using the McMaster (McM) chamber, originally used to count parasites of sheep. It is known that the McM method lacks sensitivity, particularly at low nematode egg counts (MES, 2003). A modification in the original protocol (i.e. the amount of feces or the volume of flotation solution) suggests that the McM multiplication factor can be changed when analyzing samples from cattle and horses (ROEPSTORFF and NANSEN, 1998).

A more recent technique called FLOTAC has been developed in Italy to increase FEC sensitivity (CRINGOLI, 2006; CRINGOLI et al., 2010). FLOTAC was based on the centrifugal flotation of a sample and the subsequent translation of the top layer of the floating suspension (CRINGOLI, 2006). Despite the high sensitivity, a main limitation of the FLOTAC technique was the complexity of the method, which involved the centrifugation of the sample (KNOPP et al., 2009). To overcome this step, a new simplified apparatus has been developed, named Mini-FLOTAC (mF) (CRINGOLI et al., 2013). A major advantage of this new method is that it can be more easily transferred and carried out in laboratories with limited facilities, due to its simpler protocol. However, a comparative validation field study of this laboratory technique with naturally infected horses and cattle and also the impact of inter-laboratory variability is essential to provide safe and useful information to clinicians and has never been demonstrated. The present study had the aim to compare two FEC techniques, the McM and the mF, in order to detect gastrointestinal strongyles eggs of cattle and horses, in different locations.

2.2 MATERIAL AND METHODS

2.2.1 Experiment 1

Fecal samples, from 16 mix-breed cattle (3/8 Holstein x Zebu) naturally infected with 77.1% *Cooperia* spp., 17.3% *Haemonchus* spp. and 5.7% *Oesophagostomum* spp., with an average age of 180 days old, were collected directly from the rectum for FEC using the mF chamber (Appendix B), mixing 5 g of feces and 45 ml of saturated sucrose solution (density = 1.2) with a sensitivity of 5 eggs per gram of feces (EPG); and the McM chamber using 4 g of feces and 56 ml of saturated sucrose solution (density = 1.2) with a sensitivity of 50 EPG. All individual samples were analyzed in triplicate and the experiment was carried out at the Empresa de Pesquisa Agropecuaria de Minas Gerais - EPAMIG in Lagoa Santa, MG, Brazil. The animals belonged to EPAMIG and were also analyzed at the Laboratory of Parasitic Diseases of the University of Parana – LPD/UFPR in Curitiba, PR, Brazil. Individual fecal samples were sealed in anaerobic conditions and sent to the LPD/UFPR by 1-day courier (1,200 km, Lagoa Santa - Curitiba). The samples were maintained in a thermal box with sufficient ice packs.

2.2.2 Experiment 2

Fecal samples, from 15 adult Quarter Horses and 15 adult Crioulo horses naturally infected with cyathostomins, were collected directly from the rectum for FEC, using mF (sensitivity of 5 EPG) and modified McM (sensitivity of 25 EPG) mixing 4 g of feces and 26 ml of saturated sucrose solution (1.2 density). All samples were analyzed in triplicate and the experiment was carried out at the Federal University of Mato Grosso do Sul - UFMS in Campo Grande, MS and at LPD/UFPR. The Quarter Horse and the Crioulo animals belonged to private farms in Campo Grande, MS and Ponta Grossa, PR, respectively. In order to perform the fecal analysis, the samples were properly packed and shipped as described above, between the two laboratories (1,100 km Campo Grande – Curitiba; and 80 km Ponta Grossa – Curitiba).

2.2.3 Experiment 3

Fecal samples were collected directly from the rectum of 32 Thoroughbred foals naturally infected with cyathostomins (>96%) and 14 samples of animals with low (0 to 200), medium (205 to 400) and high (over 400) FEC were selected for the analyses of mF. For the experiment, the FEC used mF with a sensitivity of 5 EPG and McM with a sensitivity of 25 EPG. All samples were analyzed in triplicates and the experiment was carried out in LPD/UFPR, Curitiba. The fecal material was prepared fresh from animals located in Sao Jose dos Pinhais, PR (25 km, Sao Jose dos Pinhais – Curitiba).

2.2.4 Statistical analysis

The average of eggs per gram of feces, standard deviation (SD) and the coefficient of variation (CV) of EPG values were calculated for each triplicate of each method and from each location. According to the Shapiro Wilk test, the variable EPG did not present a normal distribution. Differences between the techniques and the laboratories were analyzed using a paired Student T test for SD and CV, and paired Wilcoxon test for EPG. The level of significance used was 5% and the statistical analysis was carried out using the Statistica 7.0 software.

2.3 RESULTS

2.3.1 Experiment 1

The average FEC using the mF was 13% lower when performed at LPD/UFPR then in EPAMIG. For McM, the average FEC was 11% lower at LPD/UFPR then EPAMIG. We found no statistical differences on FEC between techniques or locations. Although the SD and CV data were significantly higher ($p<0.05$) for McM in both locations when compared to mF, we observed no statistical difference in the mean, SD and CV when testing the performance of the techniques between the laboratories. The SD of McM from EPAMIG data was approximately 40% higher than the LPD/UFPR (Table 1). The largest variation of CV and SD has

been found for McM in both laboratories, probably by the large multiplication factor used for the FEC (Fig. 1), as the actual EPG did not have major differences.

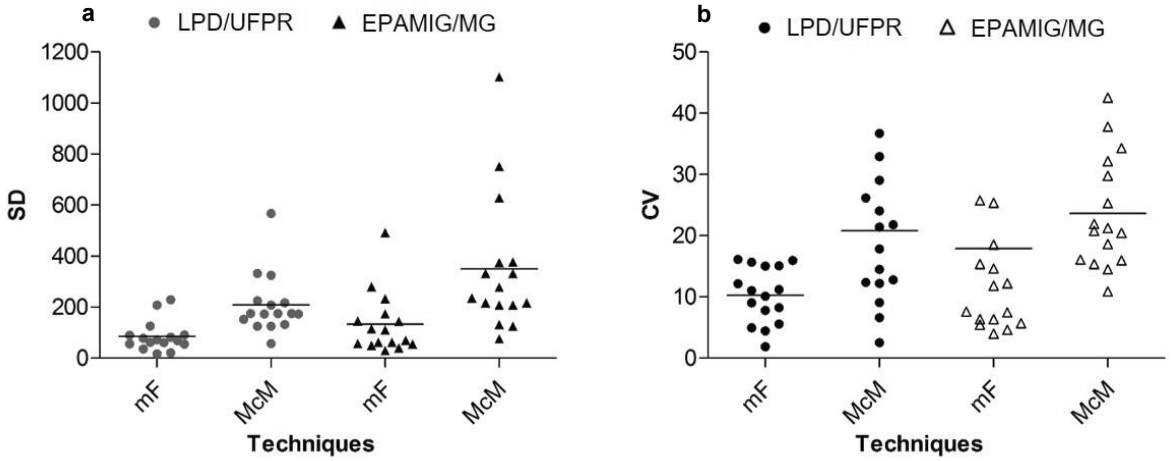
TABLE 1 – MEAN FECAL EGG COUNT (FEC), STANDARD DEVIATION (SD), AND COEFFICIENT OF VARIATION (CV) OF INFECTED CATTLE USING Mini-FLOTAC (mF) AND McMaster (McM) TECHNIQUE AT LPD/UFPR AND EPAMIG.

	LPD/UFPR			EPAMIG		
	FEC	SD	CV	FEC	SD	CV
mF	962[a]	85[a]	10.29[a]	1248[a]	136.66[a]	12.05[a]
McM	1393[a]	209.08[b]	20.83[b]	1563[a]	350.25[b]	23.63[b]
P value	> 0.05	0.007	0.004	> 0.05	0.006	0.0005

Different superscript letters in the same column indicate statistically different values ($p<0.05$).

SOURCE: the author.

FIGURE 1 – IN (**a**) ARE EXPRESSED THE STANDARD DEVIATION (SD) AND IN (**b**) THE COEFFICIENT OF VARIATION (CV) FOR THE ANALYSES OF CATTLE USING Mini-FLOTAC (mF) AND McMaster (McM) TECHNIQUE AT LPD/UFPR AND EPAMIG.



SOURCE: the author.

2.3.2 Experiment 2

The results of this experiment with horses were comparable to the experiment 1 with cattle, suggesting that the egg output from young cattle and adult horses did not have a great amplitude. The FEC average using the mF was 28% lower at the LPD/UFPR then at UFMS. For the McM, the average had the same 28% difference from the LPD/UFPR and UFMS. The FEC average in the UFMS was significantly lower for both techniques when compared to the UFPR ($p<0.03$). Evaluating the variation within the technique between the laboratories, we observed no statistical difference in the SD and CV. Analyzing the techniques, both at the

LPD/UFPR and UFMS, the mean of the triplicates of the SD ($p<0.03$) and the CV ($p<0.03$) were significantly higher in the McM technique (Table 2).

TABLE 2 – MEAN FECAL EGG COUNT (FEC), STANDARD DEVIATION (SD), AND COEFFICIENT OF VARIATION (CV) OF INFECTED EQUINES USING Mini-FLOTAC (mF) AND McMaster (McM) TECHNIQUES AT LPD/UFPR AND UFMS.

	LPD/UFPR			UFMS		
	FEC	SD	CV	FEC	SD	CV
mF	650[a]	68.41[b]	13.85[b]	469[a]	64.62[b]	22.55[b]
McM	677[a]	113.79[a]	23.38[a]	554[a]	113.72[a]	27.15[a]
P value	> 0.05	0.029	0.004	> 0.05	0.006	0.027

Different low case letters in the same column indicate statistically different values ($p<0.05$).

SOURCE: the author.

2.3.3 Experiment 3

In this experiment, the average FEC for mF was 404 and 436 for the modified McM. For these, we observed a high SD due to selection of samples with low, medium and high EPG. The average FEC between the techniques and to each category were not statistically different (Table 3). Analyzing the animals with low, medium and high FEC we observed that the average between the techniques was significantly different only for the group with medium FEC. The largest variation of SD and CV was found in the group with low EPG, since the McM presented higher values than mF for these two measures ($p<0.05$) (Table 3). Figure 2 shows that the average SD for the McM (123.76) was significantly higher than the mF (74.43), $p=0.0034$. The same happened with the CV, where the value for McM was 48.02 and the Mini-FLOTAC was 28.32 ($p=0.037$).

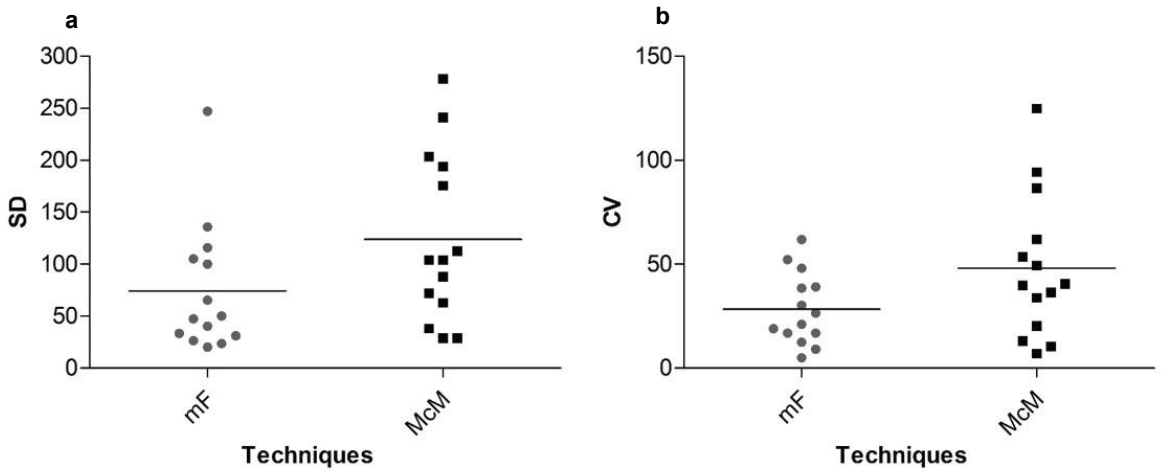
TABLE 3 – MEAN FECAL EGG COUNT (FEC), STANDARD DEVIATION (SD), AND COEFFICIENT OF VARIATION (CV) OF FOALS WITH LOW (0 TO 200), MEDIUM (205 TO 400) AND HIGH (OVER 400) FEC USING Mini-FLOTAC (mF) AND McMaster (McM) TECHNIQUES.

	FEC			SD			CV		
	mF	McM	p	mF	McM	p	mF	McM	p
Low	93[a]	87[a]	> 0.05	28.80[a]	71.17[b]	0.0107	35.38[a]	83.47[b]	0.043
Medium	286[a]	360[b]	0.005	68.91[a]	91.89[a]	> 0.05	24.95[a]	26.31[a]	> 0.05
High	941[a]	967[a]	> 0.05	138.36[a]	229.32[a]	> 0.05	23.69[a]	30.85[a]	> 0.05

Different letters in the same line indicate statistically different values ($p<0.05$)

SOURCE: the author.

FIGURE 2 – IN (**a**) ARE EXPRESSED THE STANDARD DEVIATION (SD) AND IN (**b**) THE COEFFICIENT OF VARIATION (CV) WITH FECAL EGG COUNTS FOR EQUINES USING Mini-FLOTAC (mF) AND McMaster (McM) TECHNIQUE AT LPD/UFPR.



SOURCE: the author.

2.4 DISCUSSION

In livestock production, FEC have a major objective; that is to decide which anthelmintic to use, after a drug efficacy test. As we do not have a gold standard parasite egg count analytical method, we should use a technique with the lowest multiplication factor (5 instead of 25) to improve the diagnostic sensitivity, to better identify animals with zero EPG, measure accurately drug efficacy, and to improve the individual selection of animals for target selected treatments. Conversely, in our study we observed that McM might be used to make simple and fast decisions about the treatment of cattle or horses, due to the similar results of mF. However, for the use of selective therapy and anthelmintic efficacy assessment, we recommend the use of mF, because of the lower SD and CV, as it uses a lower multiplication factor and the mF chamber has more area for counting.

Comparing with small ruminants, cattle always have smaller FEC (TSOTETSI et al., 2013) and the same occurs after anthelmintic treatment to any species. Our data revealed that horses with low FEC have the largest variation of CV and SD using McM, indicating that this technique is less precise. Similar observations were reported by Neves et al. (2014) when performing the FECRT using the McM and FLOTAC methods. Furthermore, a relatively small number (20%) of horses are responsible for shedding the majority of nematode eggs into the environment; where 80% of strongyle egg-shedding horses have low EPG (KAPLAN and NIELSEN,

2010; WOOD et al., 2012). Therefore, the identification of these high egg-shedders with a precise technique is fundamental to reduce pasture contamination, and to decrease the number of anthelmintic treatments of the other horses.

For the FEC techniques, the flotation solutions have a fundamental role in determining the analytic sensitivity and the accuracy of any copromicroscopic technique, either qualitative or quantitative, including the FLOTAC, mF and McM (CRINGOLI et al., 2010). Rinaldi et al. (2011) observed that the best flotation solution to identified nematode gastrointestinal eggs in sheep using FLOTAC, was the saturated sodium chloride solution with density of 1.2 and the sucrose and potassium iodomercurate (1.25). So, in order to identify gastrointestinal nematode eggs of cattle and horses, we used the saturated sucrose solution with the same density (1.2) of the sodium chloride solution.

In our results, the low variation of SD and the CV indicated that the mF technique was more accurate, as well as, more sensitive, as it was observed in the research conducted by Noel et al. (2017) in horses. This may be because the minimum detection limits were 5 EPG for mF and 25 to McM. Due to these advantages, the mF technique is a more suitable method to be used with pooled samples, such as the study by Kenyon et al. (2016) that observed a strong correlation between the individual assessments and the fecal pools from sheep using mF. This strategy has the advantages of decreasing the costs and the number of animals involved in the evaluation tests for anthelmintic efficacy, facilitating its applicability.

The suggestion to use mF was possible due to the similar results of FEC, SD and CV among the three laboratories in Brazil. The differences observed between the locations are to be expected as many factors (long distances, storage conditions, material degradation) can be important for validation/standardization studies, even using identical protocols, as observed by Von Samson-Himmelstjerna (2009). Although all personnel were properly trained, some variations were also anticipated, as well as, environmental and laboratory conditions, such as consumables, water, and the solutions. This comparative validation field study with naturally infected horses and cattle was able to determine the impact of inter-laboratory variability, providing useful information to other laboratories in Latin America and elsewhere.

2.5 CONCLUSION

Based on the present results, we suggest the use of the Mini-FLOTAC technique, which is a method with higher accuracy, especially when animals have low FEC, avoiding the false-negative results. This technique may be widely used to monitor FEC variability along the year when using the target selective treatment; assess anthelmintic efficacy; and to choose drugs for rotation of cattle and horses.

Acknowledgment

The authors wish to thank the personnel from the Gruta Azul, JB and HSJS Studs for their valuable assistance. The authors recognize the great contribution made by Dr. Giuseppe Cringoli for developing such a neat apparatus called Mini-FLOTAC. The authors wish to express their gratitude to UFPR, UFSM and FAPEMIG for their financial support.

Conflict of interest statement

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

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3 ARTICLE 2 - GROWTH RATE AND RACE PERFORMANCE OF THOROUGHBRED HORSES NATURALLY INFECTED WITH CYATHOSTOMINS

ABSTRACT

Cyathostomin infections can cause a reduction to body development of foals. However, the actual effect of the disease associated with this parasite family remains unclear. The objective of this study was to evaluate the impact of animal gender, month of birth, age of mare, number of offspring and fecal egg count (FEC) in the growth rate of Thoroughbred horses. This study also aimed to correlate the information on racing careers associated with FEC values. We analyzed 31 naturally infected Thoroughbred foals, from birth to 21 months of age. Body weight (BW) and withers height (WH) were conducted monthly, FEC was measured from 9-21 months of age, and performance was determined by races held from Dec/2015 to Mar/2017, when the animals had on average 2.5 to 3.5 years old. The mean BW at birth was 56.55 kg for males and 51.9 kg for females, and at 18 months' males and females weighed on average of 435.89 and 441.65 kg, respectively. The mean WH at birth was 104.36 cm for males and 101.19 cm for females, and at 18 months, the height was 154.78 and 153.5 cm, respectively. All animals were positive for cyathostomins and the FEC ranged from 389.15 to 1635.2. Cyathostomin FEC did not have a significant correlation with BW or WH. The number of offspring per mare influenced the WH of the animals at birth ($p = 0.035$) and at 6 months of age ($p = 0.026$). The racing history data showed that animals with higher FEC had a poorer performance when compared to those with lower FEC; including a smaller number of victories. The study provides evidence regarding the expected growth rate of Thoroughbred foals, and the racing performance associated to FEC. We, conclude that horses raised under a well-defined set of management practices can have little or no signs of impairment due to worm infections.

Key-words: Internal parasites. Fecal egg count. Body weight. Withers height.

TAXA DE CRESCIMENTO E RENDIMENTO DE CAVALOS PURO SANGUE INGLÊS NATURALMENTE INFECTADOS COM CHYATHOSTOMINEOS

RESUMO

As infecções por Ciatostomíneos podem causar redução do desenvolvimento corporal de potros. No entanto, o real efeito da doença associada a esta família de parasitas ainda não está claro. O objetivo deste estudo foi avaliar o impacto do gênero do animal, mês de nascimento, idade e número de filhos da égua, e contagem de ovos por grama de fezes (OPG) na taxa de crescimento de cavalos de Puro Sangue Inglês (PSI). Este estudo também teve como objetivo correlacionar as informações sobre o desempenho dos animais nas corridas associadas aos valores OPG. Analisamos 31 potros PSI naturalmente infectados, desde o nascimento até os 21 meses de idade. O peso corporal (PC) e a altura da cernelha (AC) foram aferidos mensalmente, o OPG foi realizado quando os animais estavam com 9 a 21 meses de idade e o desempenho foi determinado pelos registros das corridas de dez/2015 a mar/2017, quando os animais apresentaram em média 2,5 para 3,5 anos de idade. Em média o PC dos animais ao nascer foi 56,55 kg para machos e 51,9 kg para fêmeas, e aos 18 meses, pesavam em média 435,89 e 441,65 kg, respectivamente. Em média a AC ao nascer foi de 104,36 cm para machos e 101,19 cm para as fêmeas, e aos 18 meses, a altura foi de 154,78 e 153,5 cm, respectivamente. Todos os animais foram positivos para a infecção com cyathostomins com OPG variando de 389,15 a 1635,2. O OPG não teve correlação significativa com PC ou AC. O número de filhos por égua influenciou a AC dos animais no momento do nascimento ($p = 0,035$) e aos 6 meses de idade ($p = 0,026$). Os dados das corridas mostraram que os animais com maior OPG tiveram um desempenho menor quando comparados com aqueles com menor OPG; incluindo um número menor de vitórias. O estudo fornece evidências sobre a taxa de crescimento esperada de potros PSI e o desempenho desses animais associado as corridas, concluindo que animais criados sob um conjunto bem definido de práticas de manejo podem ter poucos ou nenhum sinal de comprometimento devido as infecções parasitárias.

Palavras-chave: Parasitas internos. Contagem de ovos fecais. Peso corporal. Altura da cernelha.

3.1 INTRODUCTION

In Brazil, the horse population is about 5 million (IBGE, 2015). The horse industry has been of great commercial importance since 1825, and the related market is worth about US$ 7.5 billion, employing 3.2 million people (ALMEIDA and SILVA, 2010). The breeding and maintenance of Thoroughbred horses, generates US$ 76 million, employing 2,830 people (LIMA, 2015; LIMA and MAZZA, 2014). In 2013, the four main racetracks in Brazil, Gávea in Rio de Janeiro; Cidade Jardim in São Paulo; Cristal in Porto Alegre and Tarumã in Curitiba, provided a general betting movement of US$ 122 million (LIMA, 2014).

In horses, the body development is associated with environmental and genetic factors, the influence of which determine their athletic potential (FITZHUGH, 1976). Therefore, it is important to provide adequate nutritional and sanitary conditions to foals, as well as, to have an efficient breeding program. Parasites are the main cause of gastrointestinal infections in grazing animals, and one of the most important pathogenic parasites, is the cyathostomins (LYONS et al., 1999; CHAPMAN et al., 2003; GAWOR et al., 2006). Currently, many horse farms are organized in small areas, resulting in high animal density associated with the increase incidence of parasite infections since their first weeks of life (MOLENTO, 2005).

Cyathostomins infections may cause a reduction in growth rate, weight loss, weakness, diarrhea and even fulminant episodes of colic (MURPHY and LOVE, 1997; MAIR et al., 2000). Due to the ever-increasing finding of anthelmintic resistance, veterinary parasitologists have recommended reducing anthelmintic treatment intensity, and one widely advocated strategy is based on treatment decisions of individual fecal egg count (FEC) (GOMEZ and GEORGI, 1991; KRECEK et al., 1994).

Nutrient requirements and allowances for growing animals have been established based on their age, body weight (BW), expected mature BW and growth rate based on average daily weight gain (INRA, 2011). Although information regarding the development of Thoroughbred horses has been described (KAVAZIS and OTT, 2003; BROWN-DOUGLAS et al., 2005; JONES and HOLLANDS, 2005; KOCHER and STANIAR, 2013), little is known about the body measurements of

animals raised in Brazil. The objective of this study was to evaluate the impact of animal gender, month of birth, age of mare, number of offspring, and FEC from birth until 21 months of age in the growth rate of Thoroughbred horses. We also aimed to correlate the information on their early racing careers, associated with FEC values.

3.2 MATERIAL AND METHODS

3.2.1 The study farm and animals

The study was performed with 31 Thoroughbred foals (20 fillies and 11 colts) from birth until 21 months of age. All animals were from a breeding farm located in São Jose dos Pinhais in the state of Paraná, Brazil (latitude 25°40'28.5" S and longitude 49°12'23.6" W), according to Köppen's climate classification this region has a humid subtropical (Cfb) climate. The animals were born from July to October 2013. The animals were maintained on ryegrass (*Lolium multiflorum*) and white clover (*Trifolium repens*) pasture during winter, and 'bahia grass' (*Paspalum notatum*), dallis grass (*Paspalum dilatatum*) and native pasture during summer. In addition, the horses received 2 kg of oat grain (*Avena sativa*), mineral salt and alfalfa hay (*Medicago sativa*) twice a day, equivalent to 3% of their BW. Water was provided ad libitum. All foals were weaned at 6 months of age, when they were separated into lots of males and females. The protocol for this study was approved by the Ethical Committee for Animal Experimentation of UFPR (number 026/2014), appendix D.

3.2.2 Parasitological and growth rate parameters

Measurement of animal weight and withers height (WH) was conducted monthly from birth until 21 months of age. The BW was measured using a mechanical weight scale, and height was measured with the aid of a hypsometer. In order to determine the fecal egg count (FEC), individual fecal samples were collected directly from the rectum from the 9th to the 21st month of age. The samples were analyzed at the Laboratory of Parasitic Diseases of the Federal University of Parana, using the Mini-FLOTAC technique (BARDA et al., 2013; DIAS DE CASTRO et al., 2017), where each observed egg was multiplied by 10. Identification of the genus of the parasites was performed after coproculture (BEVILAQUA et al., 1993).

3.2.3 Parasite control program

Deworming of horses was conducted according to their age, foals were dewormed with fenbendazole at 45-day intervals from 2 to 6 months of age; after which animals received anthelmintic treatment every 2 months (mixture of ivermectin and pirantel pamoate 5x; and moxidectin 1x).

3.2.4 Racing performance

As the animals received the same treatment regime throughout the period, it was possible to discriminate then based on their individual FEC for all experiment period. According to these values, the animals were ranked and split in high (n=5) and low (n=5) FEC and the number of races and victories of the groups was correlated with their individual average of FEC and growth rate measurements. After the field-monitoring period, the racing performance of the animals was assessed from the records of the Brazilian Stud Book (http://www.abcpcc.com.br) for races held from December 2015 to March 2017.

3.2.5 Statistical analysis

According to the results of the Shapiro–Wilk test, none of the variables presented a normal distribution. Correlation between all variables was analyzed using Spearman's rank correlation coefficient. Comparison of some variables was carried out using the Mann–Whitney test (only two categories) or the Kruskal-Wallis test (three or more categories). The level of significance was set at 5%, and all analyses were performed using Statistica 7.0 (Informer Technologies, USA). To evaluate the effect of the sex, month of birth, age and number of offspring of the mares in relation to the development of the foal, it was analyzed data collected at birth, 6, 12 and 18 months of age.

The mature BW of the animals was calculated using the following equation (NRC, 2007):

Percent mature BW = a + ((100 - a)(1 - e-ct))

where a is the percent mature BW at birth (considering 580 kg as the adult weight), c is the constant, t is the age in months, and e is 2.7183.

3.3 RESULTS AND DISCUSSION

The average BW at birth was 56.55 ± 5.84 kg for males and 51.9 ± 7.6 kg for females. At 6 months, the foals were 4.3-times heavier compared to their weight at birth, with an average of 246.55 ± 38.51 kg and 241.95 ± 15.77 kg for males and females, respectively. At 12 months, males weighed 354.4 ± 25.98 kg and females weighed 338.76 ± 14.12 kg, reaching an average of 435.89 ± 19.24 kg and 441.65 ± 22.54 kg, respectively, at 18 months, which was 7.6-times their initial weight (Figure 1). Colts were heavier than fillies from birth until 14 months of age, and again at 21 months of age; females were heavier from 15 to 20 months, but there was no significant difference for sex ($p > 0.05$).

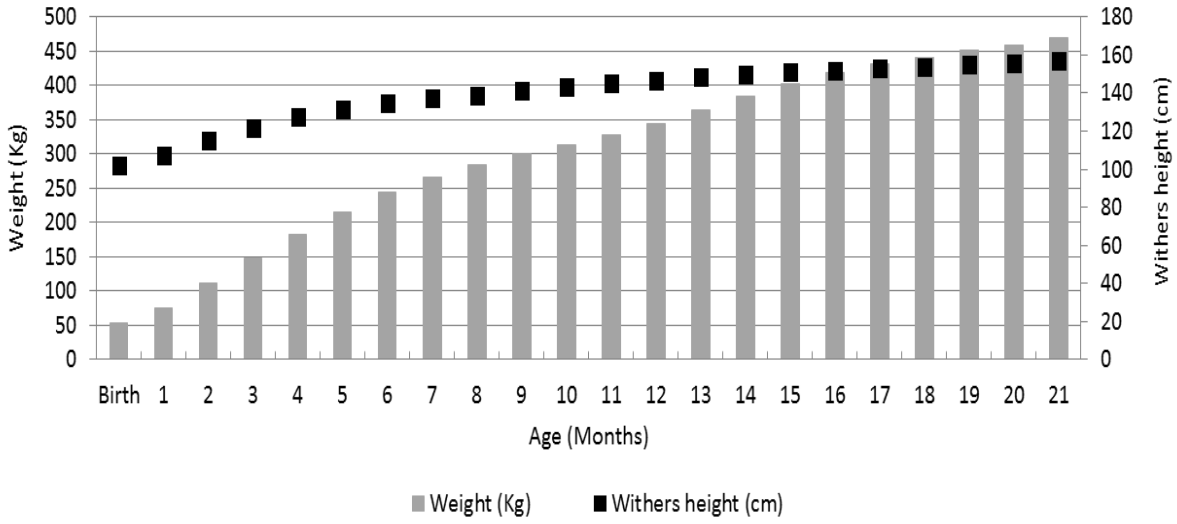
The difference in BW between colts (332 ± 18.61 kg) and fillies (316.44 ± 18.11 kg) became greater (15.56 ± 1.5 kg) from 9 to 12 months of age. This coincided with the puberty period for Thoroughbred foals, which has been reported to occur from 7 to 13 months of age in foals born in the Southern Hemisphere, when body masses range from 277 to 409 kg (BROWN-DOUGLAS et al., 2004). Nogueira et al. (1997) reported that the weight gain of Brazilian Thoroughbred foals increased dramatically in the months following puberty due to an increase in hormone levels.

The average WH at birth was 104.36 ± 2.66 cm for males and 101.19 ± 4.25 cm for females. At 6 months, the average height was 135.77 ± 4.41 cm for males and 134.6 ± 3.99 cm for females. At 12 months, the average height was 148.25 ± 2.84 cm for males and 145.88 ± 3.61 cm for females. At 18 months, the mean withers height was 154.78 ± 2.22 cm and 153.5 ± 3.8 cm for males and females, respectively, representing 1.5-times their initial height (Figure 1). Colts were taller than fillies during all periods; however, this difference was only significant at birth ($p = 0.017$).

Considering that the average BW and WH of an adult Thoroughbred horse is 550 kg and 162 cm for males and 500 kg and 160 cm for females (NRC, 1989), respectively, the animals in this study were born at approximately 10.19% of their mature weight and 63.53% of their adult height, which increased to 46.39% and 83.85% at 6 months, 65.49% and 91.08% at 12 months, and to 83.78% and 95.59% at 18 months of age, respectively. Our results were similar to those reported by

Garcia et al. (2011) for Thoroughbred foals from Bagé, Brazil, and Hintz et al. (1979), which reported that foals from Oshawa, Canada, were 46.0, 67.0 and 80.0% of the mature Thoroughbred weight and 83.0, 90.0 and 95.0% of the mature height at 6, 12 and 18 months of age, respectively.

FIGURE 1 - AVERAGE BODY WEIGHT (Kg) AND WITHERS HEIGHT (cm) OF THOROUGHBRED FOALS FROM BIRTH TO 21 MONTHS OF AGE IN A STUD FARM IN BRAZIL.



SOURCE: the author.

According to a 2007 report by the National Research Council (NRC), the animals in the study were born at approximately 9.22% of their mature weight, reaching 42.88% at 6 months, 64.06% at 12 months and 77.38% at 18 months of age. These values were lower than those calculated by the NRC in 1989, and these differences may be associated with the evolution of mature weight of the animals. The mature BW was considered to be 500 kg for mares and 550 kg for males in 1989, which has increased to 580 kg in the most recent NRC report published in 2007.

Regarding the month of birth, 22.58% (n = 7) were born in July, 25.81% (n = 8) in August, 29.03% (n = 9) in September and 22.58% (n = 7) in October. Despite the different birth periods, the month of birth had no significant effect on body development ($p > 0.1$) (Table 1). These results are different to those of Pagan et al. (2006), who reported that Thoroughbred foals born in January, February and March had lower BW during the first month than those born in April and May in a study conducted in Kentucky, USA. The authors reported that the effect of birth month

remained for the second, third and fourth months ($p < 0.05$), but the differences in BW were no longer apparent by the fifth month.

TABLE 1 – AVERAGE (± STANDARD DEVIATION) OF WEIGHT AND WITHERS HEIGHT OF THOROUGHBRED FOALS AT BIRTH, 6, 12, AND 18 MONTHS OF AGE IN BRAZIL.

	Measures*	July	August	September	October	P**
Birth	W (kg)	56.29 ± 8.46	55.00 ± 2.98	52.50 ± 5.46	49.50 ± 11.88	0.72
	WH (cm)	104.14 ± 3.29	102.50 ± 1.51	102.60 ± 2.50	99.00 ± 7.40	0.52
6 M	W (kg)	237.29 ± 46.66	245.50 ± 11.53	248.10 ± 12.89	240.50 ± 27.93	0.83
	WH (cm)	135.43 ± 6.30	135.25 ± 2.92	135.50 ± 3.44	133.50 ± 4.47	0.79
12 M	W (kg)	352.33 ± 31.61	338.13 ± 9.01	338.60 ± 13.75	350.00 ±25.19	0.34
	WH (cm)	147.75 ± 4.24	146.38 ± 3.16	146.95 ± 3.32	145.67 ± 4.37	0.61
18 M	W (kg)	443.80 ± 7.95	441.13 ± 21.60	443.80 ± 14.25	428.33 ± 35.72	0.67
	WH (cm)	154.80 ± 3.49	154.25 ± 3.33	154.20 ± 3.26	152.17 ± 3.97	0.48

* W: weight (kg); WH: withers height. ** Kruskal Wallis test.

SOURCE: the author.

Jones et al. (2005) found no significant association between the growth rate and month of birth of 200 Thoroughbred foals in the first 200 days of age in a study conducted in the UK, suggesting that the condition of the pasture in winter was sufficient to meet the energy requirements of the mare and support the foal's growth. The same situation was observed in our study, due to the excellent winter grazing on the farm, and consequently, abundant milk production by the mares. Another important factor to consider was the use of foster mares when complications with the foals' mother occurred during birth or when the mares were sent for reproduction in the first weeks of parturition.

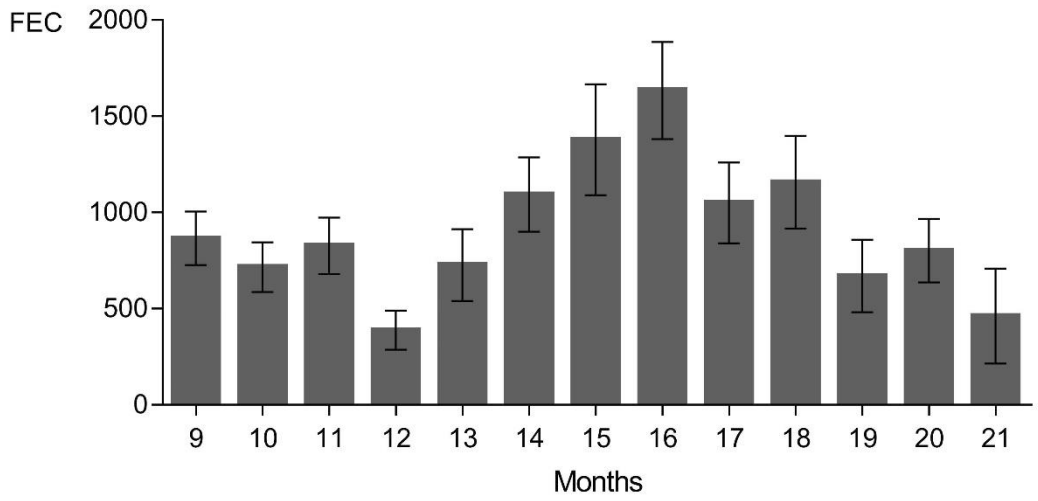
According to the National Institute of Meteorology, the average monthly temperature for the experimental period was 15.06 °C, ranging from 12.91 to 17.31 °C. The relative humidity was on average 78.85%, ranging from 75.65 to 81.96%. The monthly precipitation ranged from 39.40 to 197.00 mm^3, with the month of August presenting the lowest precipitation over the experimental period. The constant climate conditions may have also had a positive effect on milk yield of the mares and pasture quality, which directly may have influenced the foal development, reducing the effect of birth month on the suckling animals.

The age of the mares ranged from 5 to 20 years (10.22 ± 4.12), and the number of offspring ranged from 1 to 15 (5.06 ± 3.46). According to the Spearman

correlation, the age of mares did not influence the development of the animals ($p > 0.05$). On the other hand, the number of offspring influenced the weight of the animals at birth (coefficient = 0.385, $p = 0.03$) and at 6 months of age (coefficient = 0.392, $p = 0.026$). Therefore, our results show that the lower the number of offspring per pregnancy for each mare, the greater the weight of foals during the periods.

All animals tested positive for gastrointestinal parasites, and the average FEC from the 9th to the 21st months of age ranged from 389.15 to 1635.2 (average 904.82 ± 353.15; Figure 2). The majority (97.1%) of the identified larvae were cyathostomins, whereas less than 3% were large strongyles. The animals were treated with anthelmintics every 2 months beginning at 6 months of age. Although this treatment strategy is accepted as a standard procedure for foals worldwide, this suppressive regime has contributed to the selection of multidrug-resistant isolates in Brazil (MOLENTO et al., 2008). In the present case, cyathostomins FEC differences did not have significant correlations between the growth rate (BW and WH) of the animals ($p > 0.05$).

FIGURE 2 - MONTHLY AVERAGE OF FECAL EGG COUNT (FEC) FROM THOROUGHBRED FOALS FROM THE 9th TO THE 21st MONTH OF AGE.



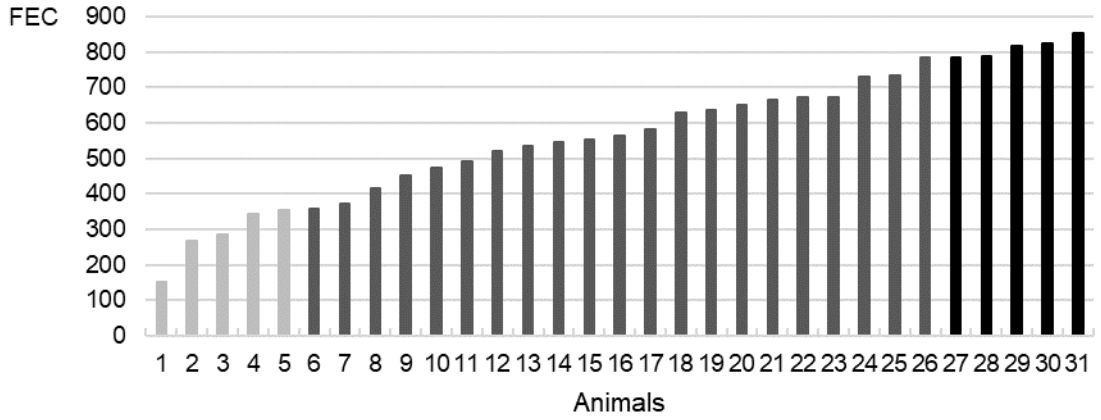
SOURCE: the author.

Nielsen et al. (2010) observed no direct linear relationship between strongyle FEC and adult worm burden in a study conducted in Kentucky, USA, as a higher FEC did not necessarily mean the presence of more worms in Thoroughbred foals from birth until 2 years of age. This fact may also be associated with the results of the

present study, as during most of the period, the animals had medium to high FEC, but their body development was similar to the reported in the literature. Other studies have observed similar results to those reported in the current research. Fritzen et al. (2010) observed that young foals and animals from 1 to 3 years old that had been naturally infected with cyathostomins showed subclinical signs with little developmental impairment in North Rhine-Westphalia, Germany. Furthermore, field studies indicate that although cyathostomins infections are ubiquitous in horses kept on pasture, most infections are subclinical and do little harm to adult animals (KLEI and CHAPMAN, 1999; LOVE et al., 1999).

As the animals received the same treatment regime throughout the period, it was possible to discriminate animals based on their individual FEC. We observed that the individual average FEC of 11 equines was below 500, ranging from 125 to 491, as 20 animals had FEC above this value, ranging from 520 to 852 (Figure 3).

FIGURE 3 - INDIVIDUAL FECAL EGG COUNT (FEC) AVERAGE OF 31 THOROUGHBRED FOALS DURING ALL EXPERIMENT. LIGHT GRAY COLUMNS ARE THE FIVE ANIMALS WITH THE LOWEST INDIVIDUAL FEC; BLACK COLUMNS, ANIMALS WITH THE HIGHEST INDIVIDUAL FEC.



SOURCE: the author.

According to the Brazilian Stud Book, the animals with the lowest individual FEC (n=5), ranging from 152.75 to 356.05, took part in a total of 27 races. These animals had five victories, with one being a repeat winner at Gávea, Rio de Janeiro State, and were placed in the top fifth place 15 times. Conversely, the animals (n=5) with the highest individual FEC (average of 813.76) ($p<0.001$) took part in only 11 races, always finishing after the top five position. The high and low egg-shedders showed similar values for the others variables, with no significant difference between the BW and WH among the groups ($p > 0.05$; Table 2). The horses were trained in 4

different training centers, and were only put on track, to demonstrate their promising carriers, after their athlete conditions were met. We suggest that their parasite history, hereby measured by FEC, can influence on their early racing performance.

TABLE 2 – DESCRIPTION OF THE INDIVIDUAL AVERAGE FECAL EGG COUNT (FEC) ± STANDARD DEVIATION, MONTH OF BIRTH, GENDER, WEIGHT AND WITHERS HEIGHT AT 18 MONTHS OF THE LOW (1 TO 5) AND HIGH (6 TO 10) EGG SHEDDERS.

Animal	FEC	Birth	Gender	Weight (kg)	Withers height (cm)
1	152.75 ± 284.04	July	F	450	157
2	266.25 ± 649.25	October	M	448	154
3	284.75 ± 358.84	September	F	454	153
4	343.00 ± 587.25	July	M	446	155
5	356.05 ± 539.66	October	F	444	155
Mean	280.56[a]			448.4[a]	154.8[a]
6	785 ± 1229.58	July	F	442	155
7	788.25 ± 1475.98	September	M	414	155
8	817.78 ± 1050.71	October	F	425	151
9	825.50 ± 1134.68	September	F	451	157
10	852.25 ± 1162.05	July	F	448	158
Mean	813.76[b]			436[a]	155.2[a]

SOURCE: the author.

3.4 CONCLUSION

The results presented here provide important information about the expected growth rate of Thoroughbred foals. Factors such as sex, month of birth and mare age did not influence BW and WH of the animals. We, also, conclude that horses raised under a well-defined set of management practices can have little or no signs of impairment due to worm infections, whilst parasite load was not determined on pasture or in the animals. However, low FEC levels (below 400) during the foaling period influences the racing performance of the animals. This long-term experiment may be an essential piece of information that shall be used by the horse industry worldwide.

Conflict of interest

The authors have no conflicts of interest regarding the data or results presented in this manuscript.

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4 ARTICLE 3 - GENOME WIDE ASSOCIATION IN THOROUGHBRED HORSES NATURALLY PARASITED WITH CYATHOSTOMINS

ABSTRACT

Over the last decades, cyathostomins have become the most important internal parasites in horses. They are agents of serious parasitic infections and their control is increasingly difficult due to the resistance to most of the anthelmintic groups available. However, a small number of horses within a herd are responsible for eliminating a majority of eggs and this phenotype can be explained for genetic source. Genomic structural variation is an important and abundant source of genetic and phenotypic variation. The aim of this study was to identify genomic regions associated with Strongyle fecal egg count (FEC) individual patterns and hematological parameters performing a genomic wide association study (GWAS) among Thoroughbred equines naturally infected with gastrointestinal parasites. During 12 months, the packed cell volume (PCV) and differential leukocyte counts of 90 Thoroughbred horses were measured bimonthly and individual FEC, monthly. All the animals were genotyped using Illumina Equine 70K BeadChip panel, that contains 65,157 SNP markers. The variance components were estimated using a single trait model by single step genomic BLUP procedure. The five genomic regions with highest percentage of additive genetic variance explained for each trait (top 5) were further explored to identify candidate genes. A total of 33, 21, 30, 21, and 19 genes for FEC, PCV, eosinophils count, neutrophils count, and lymphocytes count traits were identified, respectively. In this study, candidate regions related to immune system development and activation were found in ECA17, ECA 21 and ECA25. Top 5 markers region explained 2.86%, 2.56%, 2,73%, 2,33% and 2.37% of the genetic variation for FEC, PCV, eosinophils count, neutrophils count, and lymphocytes count, respectively.

Key-words: GWAS. Horse. FEC. SNP. EPG.

ASSOCIAÇÃO GENOMICA AMPLA EM CAVALOS PURO SANGUE INGLÊS NATURALMENTE PARASITADOS COM CYATHOSTOMINEOS

RESUMO

Ao longo das últimas décadas, ciatostomíneos tornaram-se os parasitas internos mais importantes em cavalos. Sendo agentes de infecções parasitárias graves e sendo se controle cada vez mais difícil devido à resistência à maioria dos grupos anti-helmínticos disponíveis. No entanto, um pequeno número de animas é responsável por eliminar a maioria dos ovos e este fenótipo pode ter explicações de origem genética. A variação estrutural genômica é uma fonte importante e abundante de variação genética e fenotípica. O objetivo deste estudo foi identificar regiões genômicas associadas aos padrões individuais de contagem de ovos por grama de fezes (OPG) e parâmetros hematológicos, realizando um estudo de associação genômica ampla (GWAS) entre cavalos Puro Sangue Inglês (PSI) naturalmente infectados com parasitas gastrointestinais. Durante 12 meses, o hematócrito (HT) e a contagem diferencial de leucócitos de 90 cavalos PSI foram medidos bimestralmente and e OPG individual, mensalmente. Todos os animais foram genotipados utilizando o painel *Illumina Equine 70K BeadChip*, que contém 65.157 marcadores SNPs. Os componentes de variância foram estimados usando um modelo de característica única pelo procedimento genômico BLUP de passo único. As cinco regiões genômicas com maior porcentagem de variância genética aditiva explicada para cada característica (top 5) foram exploradas para identificar os genes candidatos. Um total de 33, 21, 30, 21 e 19 genes para as características OPG, HT, contagem de eosinófilos, de neutrófilos e de linfócitos foram identificados, respectivamente. Neste estudo, regiões candidatas relacionadas ao desenvolvimento e ativação do sistema imune foram encontradas nos cromosomos 17, 21 e 25. Marcadores da região Top 5 explicam 2,86%, 2,56%, 2,73%, 2,33% e 2,37% da variação genética aditiva para os fenótipos OPG, HT, contagem de eosinófilos, de neutrófilos,e de linfócitos, respectivamente.

Palavras-chave: GWAS. Cavalo. OPG. SNP.

4.1 INTRODUCTION

Cyathostomins are ubiquitous in equine populations worldwide, across different climates and management practices (CHAPMAN et al., 2003; GAWOR et al., 2006) and may cause reduction in growth rate, weight loss, and poor body condition (UHLINGER et al., 1991; MURPHY AND LOVE, 1997; LOVE et al., 1999). Larval stages of these parasites can cause major pathology and can be fatal in affected horses (LOVE et al., 1999; MAIR et al., 2000). Three anthelmintic classes are licensed to control cyathostomin infections: macrocyclic lactones, tetrahydropyrimidines, and benzimidazoles. However, the excessive use of anthelmintic as the principal method of parasite control has led to high levels of anthelmintic resistance in cyathostomin populations across the world (KAPLAN et al., 2004; MOLENTO et al., 2008; CANEVER et al., 2013). Benzimidazole and pyrantel resistant cyathostomins have been reported in at least 14 and 12 countries, respectively, and there are reports of emerging macrocyclic lactone resistance as well (PEREGRINE et al., 2014).

Due to these ever-increasing findings of anthelmintic resistance, veterinary parasitologists have recommended reducing anthelmintic treatment intensity, and one widely advocated strategy is selective therapy, where treatment decisions are based on individual animal strongyle fecal egg count (FEC) (HERD et al., 1985; GOMEZ AND GEORGI, 1991; KRECEK et al., 1994). Parasite eggs must be determined from all individual horses within a given population, but only those exceeding a pre-determined FEC threshold receive anthelmintic treatment, as a small number of individuals within a herd are responsible for eliminating a majority of eggs (NIELSEN et al., 2006). This has been described as the 20/80 rule, where 20% of the animals in a given herd are responsible for 80% of the total strongyle egg output (KAPLAN AND NIELSEN, 2010; RELF et al., 2013).

Previous study in equine, using an indirect method based on microsatellites, investigated a possible association between major histocompatibility complex (MHC) gene (*DRB*) and low strongyle FEC, however the correlation was weak (r^2=0.07) (COLEMAN et al., 2009). More recently, Kornás et al. (2015) reported that some of this individual egg shedding patterns can be explained by genetic variation among horses and estimated that 21% of this phenotype has a genetic inheritable basis.

Therefore, this strongyle egg shedding consistency in horses is a phenotype for which genetic explanations can be search. However, these were the only two equine studies published on this topic thus far, and none of them included any attempt to identify single nucleotide polymorphisms (SNPs) associated with the FEC phenotype in a genome-wide approach.

Genomic markers such as SNP can be used in genome wide association studies (GWAS), a well-established strategy that has identified thousands of markers spread across the equine genome related to important economic traits (HU et al., 2016). Research in sheep and cattle has documented a genetic background for this phenomenon, but this has not been established in horses. The aim of this study was to identified genomic regions associated with Strongyle FEC individual patterns performing a genomic wide association study (GWAS) among Thoroughbred equines naturally infected with gastrointestinal parasites.

4.2 MATERIAL AND METHODS

4.2.1 Location and animals

The phenotypic data for this study were collected through monthly observations from 90 Thoroughbred horses from a breeding farm, located in São José dos Pinhais, PR, Brazil, latitude 25°40'28.5"S and longitude 49°12'23.6 "W, from September 2014 to November 2015. The animals were divided into three categories according to their age, keeping 30 horses per group (brood mares, foals born in 2014; and yearlings born in 2013). This study was approved by the Ethical Committee for Animal Experimentation of the Federal University of Parana, Brazil under the number: 026/2014.

4.2.2 Phenotypic Data

The packed cell volume (PCV) and differential leukocyte counts of the 90 Thoroughbred horses were measured every two months during 12 months. In order to determine the FEC, individual fecal samples were collected directly from the rectum monthly during 12 months. The samples were analyzed at the Laboratory of Parasitic Diseases of the Federal University of Parana, using the Mini-FLOTAC

technique (BARDA et al., 2013; DIAS DE CASTRO et al., 2017), where each observed egg was multiplied by 10. Identification of the genus of the parasites was performed after coproculture (BEVILAQUA et al., 1993).

Deworming of horses was conducted according to their age, foals were dewormed with fenbendazole at 45-day intervals, from 2 to 6 months of age; after which animals received anthelmintic treatment every 2 months (association of ivermectin 0.04% and pirantel pamoate 38.3%); adult animals were controlled through their FEC bimonthly, and anthelmintic treatment (association of ivermectin 0.04% and pirantel pamoate 38.3%) was given to those with FEC above 600. The FEC values were selected with a minimum of 30 days between any anthelmintic treatments, such that anthelmintic treatment would have no influence the data collection.

For the statistical analyses, animals were grouped in contemporary groups (CG) formed by the year of birth, keeping variability within groups. They were also classified in three age classes: up to 6 months; from 6 to 24 months; and above 24 months of age. Animals with less than five repeated measures were excluded from the analysis.

4.2.3 Genotypic data and Quality control

Individual blood samples were collected in vacuum tubes with anticoagulant. The DNA was extracted with Biopur-Plus MiniSpin extraction kit (Biopur, Brazil) according to the manufacturer's protocol. After this procedure, the DNA concentration (ng/μL) and their purity were determined by Q-bit (Invitrogen, USA).

All 90 animals were genotyped using Illumina Equine 70K BeadChip panel (Illumina Inc., USA) comprising 65,157 markers distributed throughout the genome. Quality control procedures were applied removing makers with call rate < 0.90, minor allele frequency < 0.05, and deviation of Hardy–Weinberg Equilibrium ($p < 10^{-6}$) were excluded from the dataset. Animals with call rate < 0.90 were also excluded. After quality control, genotypes of 89 animals for 51,320 SNPs were included in the GWAS analysis.

4.2.4 Data Analyses

As FEC did not follow a normal distribution, the data was transformed using the function $f(z) = 2[(z + 0.375)]^{1/2}$, according to Anscombe (1948). The other traits were kept in original scale.

GWAS was performed using Bayesian methodology using the package GIBBS1F90 (MISZTAL et al., 2002). A single-trait model was considered to predict the individual genomic breeding values:

$$y = Xb + Za + Wpe + e \qquad \text{i]}$$

where y is the vector of observed phenotypes; b is the vector of fixed effects (CG and age class); a is the random vector of additive genetic effects; pe is the vector of random permanent environmental effects and non-additive genetic effects; and e is the vector of residual effects. X, Z and W are incidence matrices relating y values to fixed, animal and permanent environmental effects, respectively. It was assumed that $a \sim N(0, G\sigma_a^2)$, where G is the genomic relationship matrix estimated as in Vanraden (2008); $pe \sim N(0, I\sigma_{pe}^2)$ and $e \sim N(0, I\sigma_e^2)$, where I is an incidence matrix. The analyses consisted of 55,000 interactions, burn-in period of 5,000 and thinning each 20 sample.

SNPs effects were derived from the breeding values, as:

$$a = Mu \qquad \text{[ii]}$$

where M is a matrix relating genotypes of each locus and u is a vector of SNP marker effects. All analyses were performed with BLUPF90 family software (MISZTAL et al., 2002). The results were reported considering the proportion of total genetic variance explained by consecutives 15 SNPs makers.

The genomic covariance between traits was obtained by two-trait analyses, considering the same model applied for the GWAS analyses.

4.2.5 Gene Search and Functional Enrichment

The five genomic regions with highest percentage of additive genetic variance explained for each trait (top 5) were further explored to identify candidate genes. The lists of the genes were obtained from Ensembl Genome Browser database, based on the equine genome reference EquCab2, with *Biomart* tool (http://www.ensembl.org), appendix E, and also the National Center for Biotechnology Information (NCBI) database, with *Map Viewer* tool (https://www.ncbi.nlm.nih.gov), appendix F. The identified genes were added in OMIM (http://www.omim.org/) and *GeneCards* (http://www.genecards.org/) platforms to identify biological mechanisms and functions involving these genes as well as to highlight the most relevant regions that are putatively associated with the traits. QTLs previously described at the Animal QTL Database (http://www.animalgenome.org/cgi-bin/QTLdb/index) in the top 5 regions were also described, and the biological pathways were searched on Panther Gene List Analysis (http://www.pantherdb.org/).

4.3 RESULTS AND DISCUSSION

The animals were parasite-positive and the average FEC of all observations ranged from 0 to 5670, with an average of 700.79 and standard deviation (SD) of 854.19 (Table 1). All identified larvae were of cyathostomins and corresponded to more than 98% of the total number of larvae, where less than 2% were from large strongyles. The average of PCV ranged from 22 to 56, with average of 38.15 (± 4.46). These values were similar to other studies with Thoroughbred horses in UK (ALLAN and ARCHER, 1973), USA (HARVEY et al.,1984) and Turkey (ULUISIK et al., 2013). Through differential leukocyte count it can be observed a higher percentage of neutrophils (61.67 ± 8.37) followed by lymphocytes (35.02 ± 8.13) and eosinophils (2.76 ± 2.43), which was similar to Uluisik et al. (2013) that observed an average of 60.80 (± 1.13) for neutrophil, 30.40 (± 1.21) for lymphocyte and 4.20 (±0.37) for eosinophil for yearlings.

TABLE 1 - DESCRIPTIVE ANALYSIS OF THOROUGHBRED HORSES SELECTED PHENOTYPES, NUMBER OF ANIMALS (N), NUMBER OF OBSERVATION, MEAN, MINIMUM (MIN), MAXIMUM (MAX), STANDARD DEVIATION (SD) AND STANDARD ERROR (SE).

Trait*	N	Observations**	Mean	Min	Max	SD	SE
FEC	89	675	700.79	0	5670	854.19	32.88
PCV	89	438	38.15	22	56	4.46	0.21
Neutrophil (%)	89	440	61.67	36	89	8.37	0.40
Lymphocyte (%)	89	440	35.02	9	63	8.13	0.39
Eosinophil (%)	89	437	2.76	0	15	2.43	0.12

*Traits: Fecal egg count (FEC), packed cell volume (PVC), Neutrophil, Lymphocyte and Eosinophil.
**Total number of observation for the phenotype trait.

SOURCE: the author.

4.3.1 Fecal egg count trait

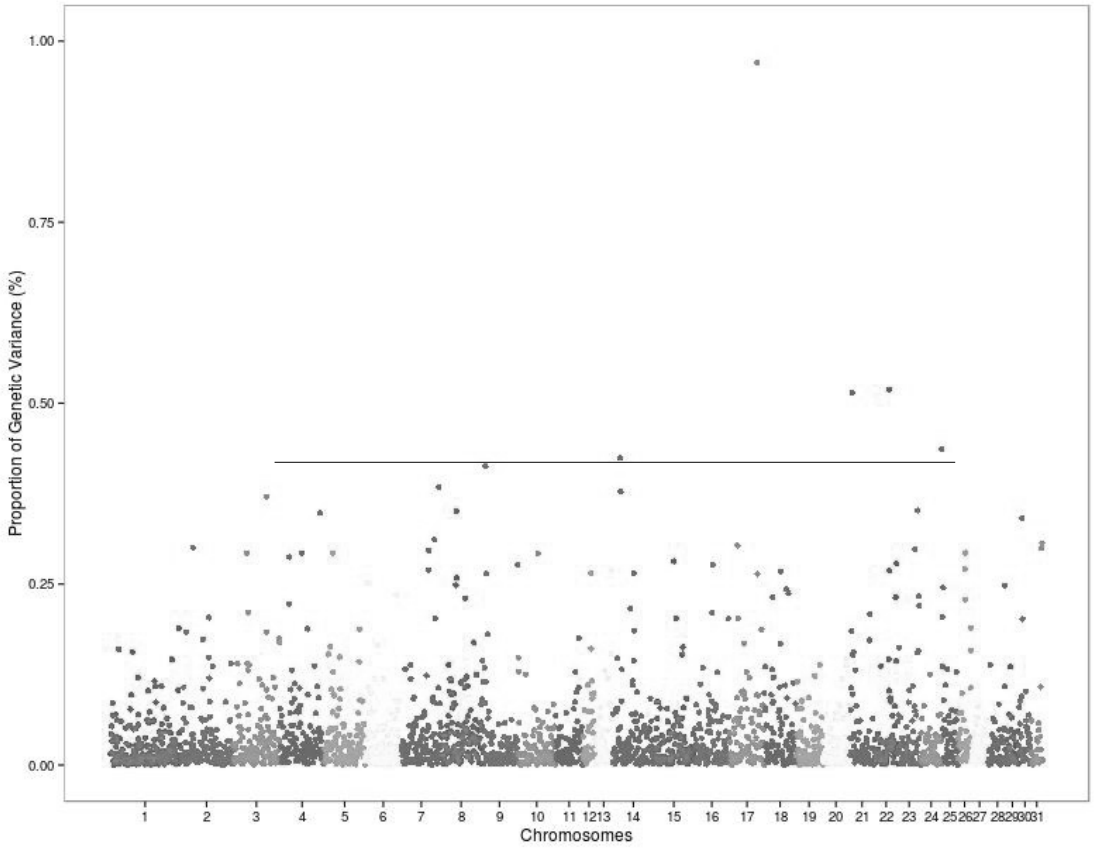
Helminth parasites are typically dispersed in their hosts, relatively few individuals are infected with the majority of the associated parasite population (ANDERSON and MAT, 1978; CALABRESE et al., 2011). In equine, Strongyle nematodes in the large intestine are the most important parasites and have a high prevalence (KAPLAN and NIELSEN, 2010). This prevalence can be observed with the Strongyle FEC in horses, the majority of individuals shedding a relatively low number of eggs (WOOD et al, 2013). For the FEC trait, we observed five regions that were associated on five chromosomes and 33 genes were identified. In the region of chromosome 17 (ECA17) 5 genes were identified, in ECA22 were identified 3 genes, in ECA21 only 1 gene, in the ECA25 the highest number of genes was observed (n=23) and in the ECA14 only 1 gene (Table 2 and Figure 1).

Chromosome 17 has the region with the highest value of additive genetic variance explained and the gene *ABCC4* were identified in this genomic region. Van de Ven et al. (2008), using a cultured human dendritic cell (DC) lines and human skin explants, showed that *ABCC4* contributed to the migratory capacity of DCs from peripheral organs to lymph nodes and this migration is important in the initiation of a T cell-mediated immune response. An experiment using *Trichinella spiralis* and *Nippostrongylus brasiliensis* have demonstrated that the population of mucosal mast cells expands during T-cell dependent responses to these parasites (MAYRHOFER and FISHER, 1979). In equines, large intestinal mucosal mast cells (PICKLES et al. 2010), CD4+ T helper cells, eosinophils (COLLOBERT-LAUGIER et al., 2002) and

mucosal Th2 cytokines (DAVIDSON et al. 2005) increased during cyathostomin infection.

In ovine, the major histocompatibility complex (MHC) has been consistently associated with nematode resistance (SCHWAIGER et al., 1995; DUKKIPATI et al., 2006). The expression of MHC products on cell membranes is necessary for antigen presentation to T lymphocytes by macrophages or DCs (HOHENHAUS and OUTTERIDGE, 1995). Thus, *ABCC4* contributed to migration of DCs toward draining lymph nodes and therefore has a role in the adaptive immune response to parasites.

FIGURE 1 - MANHATTAN PLOT OF THE ADDITIVE GENETIC VARIANCE EXPLAINED BY WINDOWS OF 15 ADJACENT SNPS FOR FECAL EGG COUNT (FEC) TRAIT. SNPS IN THE TOP 5 REGION ARE ABOVE THE BLACK LINE.



SOURCE: the author.

TABLE 2 – CHROMOSOME AND GENOMICS REGIONS ASSOCIATED WITH STRONGYLE FECAL EGG COUNT (FEC), PERCENTAGE OF ADDITIVE GENETIC VARIANCE, CANDIDATE GENES AND DESCRIPTION.

Chromosome	Genomic region (bp)	% additive genetic variance explained	Candidate genes	Description*
ECA17	65192507-65722749	0.97	*DNAJC3*	DnaJ heat shock protein family (Hsp40) member C3
			UGGT2	UDP-glucose glycoprotein glucosyltransferase 2
			DZIP1	DAZ interacting zinc finger protein 1
			CLDN10	claudin 10
			ABCC4	ATP binding cassette subfamily C member 4
ECA22	31272127-31783982	0.52	*CHD6*	chromodomain helicase DNA binding protein 6
			U6	U6 spliceosomal RNA
			PTPRT	protein tyrosine phosphatase, receptor type T
ECA21	6060362-6579939	0.51	*CD180*	CD180 molecule
ECA25	644299-1149014	0.44	*HINT2*	histidine triad nucleotide binding protein 2
			CREB3	cAMP responsive element binding protein 3
			TESK1	testis-specific kinase 1
			TMEM8B	transmembrane protein 8B
			GBA2	glucosylceramidase beta 2
			OR13J1	olfactory receptor family 13 subfamily J member 1
			CD72	CD72 molecule
			SIT1	signaling threshold regulating transmembrane adaptor 1
			CCDC107	coiled-coil domain containing 107
			C25H9orf100	Equus caballus chromosome 9 open reading frame 100 ortholog (C25H9orf100), mRNA.
			RGP1	RGP1 homolog, RAB6A GEF complex partner 1
			CA9	carbonic anhydrase 9
			MSMP	microseminoprotein, prostate associated
			NPR2	natriuretic peptide receptor 2
			TPM2	tropomyosin 2 (beta)
			TLN1	talin 1
			FAM166B	family with sequence similarity 166-member B
			RUSC2	RUN and SH3 domain containing 2
			RNase_MRP	RNase MRP
			ARHGEF39	Rho guanine nucleotide exchange factor 39
			UNC13B	unc-13 homolog B
			SPAG8	sperm associated antigen 8

			FAM221B	family with sequence similarity 221-member B
ECA14	16191238- 16740057	0.42	*U6*	U6 spliceosomal RNA

* NCBI

SOURCE: the author.

Another gene from chromosome 21 found in the top 5 region was *CD180*, also known as *RP105*. This gene is related to the immune system among diverse species, *RP105* is a cell surface molecule consisting of extracellular leucine-rich repeats (LRR) and a short cytoplasmic tail. The extracellular LRR is associated with a molecule called MD-1 and form the cell surface receptor complex, RP105/MD-1. It belongs to the family of pathogen receptors, Toll-like receptors (TLR). RP105/MD1, by working in concert with TLR4, controls B cell recognition and signaling of lipopolysaccharide (LPS), so *RP105* may also contribute to the inductive phase of B-cell activation in response to innate immunity (MIURA et al., 1998; SCIOR et al., 2013).

In our study, it was identified in the top 5 region the *CD72* gene from ECA25, this gene appears to be the receptor for *CD100*, which is expressed on the surface of activated Natural Killer (NK) cells as a homodimer, mediates the killing of target cells by binding to *CD72*. *CD100* increases NK cytotoxicity by enhancing the adhesion between NK cells and their targets, this increased adhesion leads to a more efficient killing and enhanced IFN gamma secretion (KUMANOGOH et al., 2000; TAYLOR et al., 2012). Also in ECA25, it was identified the *TMEM8B* gene, that upregulated the MHC when is expressed (LI et al. 2001). Studies with ovine provide evidence that the MHC on chromosome 20 and interferon gamma (IFN) on chromosome 03 are involved with host resistance (BISHOP and MORRIS, 2007; DOMINIK, 2005), and in study with cattle infected by *Ostertagia* sp., the immune response is upregulated by cytokines IFN gamma (GASBARRE et al., 2001).

Based on the identified genes in the top 5 genomic regions, it was possible to search biological pathways reported by other studies. The biological pathways identified for the gene *TLN1* was the integrin signaling pathway (Pathway Accession P00034); for the gene *NPR2*, Gonadotropin releasing hormone receptor pathway (P06664); for the gene *CREB3*, Transcription regulation by bZIP transcription factor (P00055); and for the gene *CREB3*, Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026). The descriptions of these pathways are in the supplementary data (Table S1). Among these pathways searched from the genes identified by the GWAS analysis, none of them is connected directly with FEC trait according to the data described in the literature (Panther Gene List Analysis).

In the top 5 genomic regions identified in this study, besides the biological pathways, the QTLs reported by other authors in the QTL Animal Database were

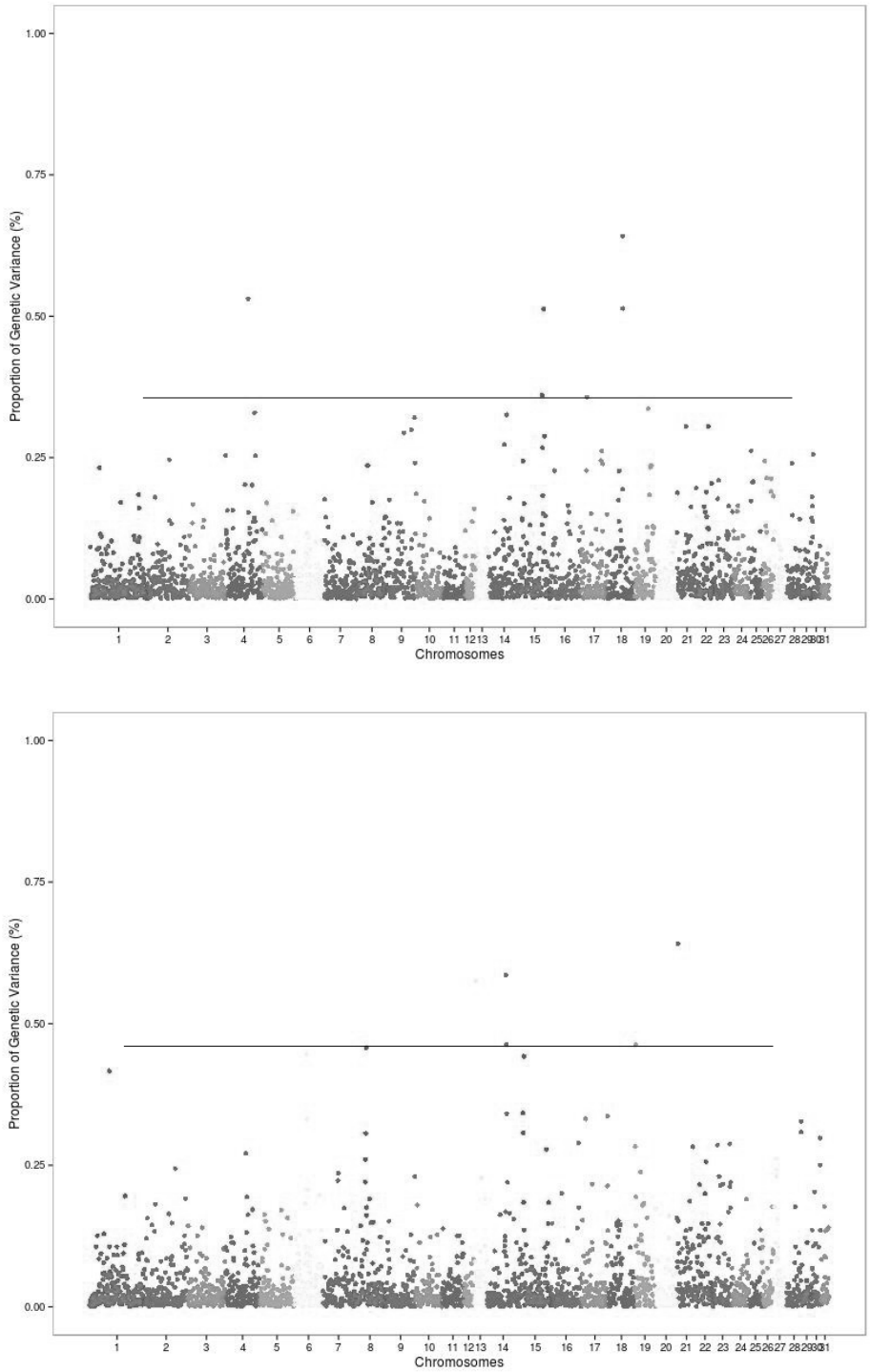
also surveyed. In chromosome 25 (Region: 644.299 to 1.149.014 bp) was described the QTLs OSTD (Osteochondritis dissecans) and OSTEO (Osteochondrosis) (WITTWER et al., 2007). In chromosome 14 (Region: 16.191.238 to 16.740.057 bp), the QTLs OSTEO (Osteochondrosis) (MCCOY et al., 2016) and NAVBM (Navicular bone morphology) (DIESTERBECK et al., 2007).

Horses acquire incomplete resistance to cyathostomin infection and this phenomenon is still not fully understood. The *ABCC4* (ECA17), *CD180* (ECA21), *CD72* (ECA5) and *TMEM8B* (ECA25) genes, identified in our study, are described to have functions related to the body's immune and defense response. They were associated with the function of dendritic cell migration, Toll-like receptors controling B cell recognition, natural killer cells cytotoxicity, interferon gamma secretion and major histocompatibility complex expression. An improved understanding of the equine immune response to cyathostomin infection is a prerequisite to developing more rational and novel strategies for their control.

4.3.2 Hematological traits

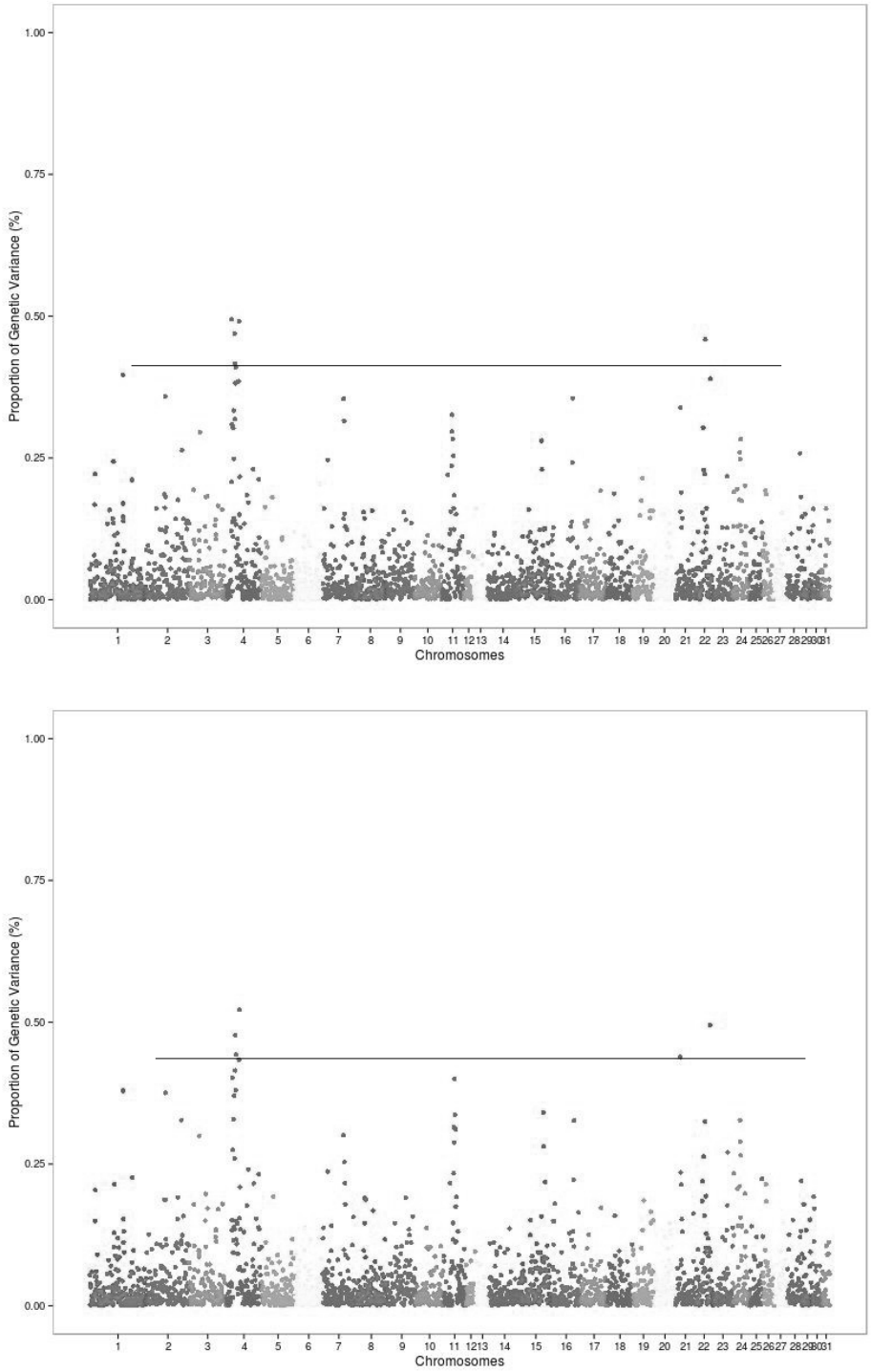
Blood cells make essential contributions to oxygen transport, hemostasis, and innate and acquired immune responses (JESEN, 2009; JENNE et al., 2013). However, GWA studies for hematological parameters have been less reported than for fecal egg count. The SNPs windows regions which accounted the top 5 value of the genetic additive variance were used to search for candidate genes. A total of 21, 30, 21 and 19 genes for PCV, eosinophils count, neutrophils count, and lymphocytes count traits were identified, respectively (Figure 2 and 3). For the PCV trait, the chromosome 18 (ECA18), region with the highest value of additive genetic variance explained, 5 genes were identified, in ECA4 were identified 2 genes and in ECA15, 14 genes (Table 3 and Figure 2A).

FIGURE 2 - MANHATTAN PLOT OF THE ADDITIVE GENETIC VARIANCE EXPLAINED BY WINDOWS OF 15 ADJACENT SNPS FOR (A) PACKED CELL VOLUME, (B) EOSINOPHILS COUNT TRAIT. SNPS IN THE TOP 5 REGION ARE ABOVE THE BLACK LINE.



SOURCE: the author.

FIGURE 3 - MANHATTAN PLOT OF THE ADDITIVE GENETIC VARIANCE EXPLAINED BY WINDOWS OF 15 ADJACENT SNPS FOR (A) NEUTROPHILS COUNT, (B) LYMPHOCYTES COUNT TRAIT. SNPS IN THE TOP 5 REGION ARE ABOVE THE BLACK LINE.



SOURCE: the author.

TABLE 3 – CHROMOSOME AND GENOMICS REGIONS ASSOCIATED WITH PACKED CELL VOLUME (PCV), PERCENTAGE OF ADDITIVE GENETIC VARIANCE, CANDIDATE GENES AND DESCRIPTION.

Chromosome	Genomic region (bp)	% additive genetic variance explained	Candidate genes	Description*
ECA18	43664672-44240915	0.64	*FIGN*	protein fidgetin, microtubule severing factor
ECA4	69554511-70230095	0.53	*DOCK4*	mRNA dedicator of cytokinesis 4
			ZNF277	zinc finger protein 277
ECA18	45371122- 45972142	0.51	*SCN2A*	sodium voltage-gated channel alpha subunit 2
			CSRNP3	cysteine and serine rich nuclear protein 3
			GALNT3	polypeptide N-acetylgalactosaminyltransferase 3
			TTC21B	tetratricopeptide repeat domain 21B
ECA15	71303173-71955686	0.51	*ITSN2*	intersectin 2
			FAM228A	family with sequence similarity 228-member A
			FAM228B	family with sequence similarity 228-member B
			PFN4	profilin family member 4
			TP53I3	tumor protein p53 inducible protein 3
			SF3B6	splicing factor 3b subunit 6
			FKBP1B	FK506 binding protein 1B
			WDCP	WD repeat and coiled coil containing
			MFSD2B	major facilitator superfamily domain containing 2B
			UBXN2A	UBX domain protein 2A
			ATAD2B	ATPase family, AAA domain containing 2B
ECA15	65972384-66493958	0.36	*CAPN13*	calpain 13
			LCLAT1	lysocardiolipin acyltransferase 1
			LBH	limb bud and heart development

* NCBI

SOURCE: the author.

For the eosinophils count trait, 30 candidate genes were associated on five chromosomes. In the ECA21, region with the highest value of additive genetic variance explained, 15 genes were identified, in ECA14 were identified 5 genes, in ECA13 were identified 7 genes and in ECA19 only 3 genes (Table 4 and Figure 2B). It was observed on ECA14 the *EPB41L4A* gene that are related to regulation of the interaction between the cytoskeleton and plasma membrane, with highest expression in brain, liver, thymus, and peripheral blood leukocytes (ISHIGURO et al., 2000). In this same chromosome, it was identified the *YTHDC2* gene that expression was upregulated by tumor necrosis factor alpha (TNF-alpha) which is a multifunctional proinflammatory cytokine secreted predominantly by monocytes/macrophages that has effects on lipid metabolism, coagulation, insulin resistance, and endothelial function (SHIRAI et al., 1985; MOROHASHI et al. 2011). Another gene associated with immune response was *LPAR2*, located on ECA21, Goetzl et al. (2000) reported that CD4+ T cells and B cells express *LPAR2*.

For the neutrophils count trait, 21 candidate genes were associated on two chromosomes. In the region of chromosome 4 (ECA4), region with the highest value of additive genetic variance explained, 15 genes were identified, and in ECA22 were identified 6 genes (Table 5 and Figure 3A). For the lymphocytes count trait, 19 candidate genes were associated on three chromosomes. In the region of chromosome 4 (ECA4), region with the highest value of additive genetic variance explained, 14 genes were identified, in ECA22 were identified 1 gene, and in ECA21, 4 genes (Table 6 and Figure 3B). Among the associated windows, 9 genes were common for neutrophils and lymphocytes count traits, located on ECA4.

In the genomic regions associated with hematological patterns, the *CAPN13*, *FKBP1B*, *ITSN2*, *ATP13A1*, *BLCAP*, *HGF*, *VOPP1*, and *PEG10* genes are involved in a variety of cellular processes including apoptosis, cell division, motility and growth, protein folding and trafficking, clathrin-mediated endocytosis, manganese homeostasis and cell proliferation and differentiation. It was also observed genes related to chromatin function (*ATAD2B*), spermiogenesis and oogenesis (*YJEFN3*), cell interactions and host-pathogen recognition (*CASD1*).

TABLE 4 – CHROMOSOME AND GENOMICS REGIONS ASSOCIATED WITH EOSINOPHILS COUNT, PERCENTAGE OF ADDITIVE GENETIC VARIANCE, CANDIDATE GENES AND DESCRIPTION.

Chromosome	Genomic region (bp)	% additive genetic variance explained	Candidate genes	Description*
ECA21	3805989-4523943	0.64	*ATP13A1*	ATPase 13A1
			GATAD2A	GATA zinc finger domain containing 2A
			GMIP	GEM interacting protein
			LPAR2	lysophosphatidic acid receptor 2
			NDUFA13	Uncharacterized protein
			PGBD2	piggyBac transposable element derived 2
			SH3BP5L	SH3 binding domain protein 5 like
			YJEFN3	YjeF N-terminal domain containing 3
			ZNF672	zinc finger protein 672
			ZNF692	zinc finger protein 692
			MAU2	MAU2 sister chromatid cohesion factor
			TSSK6	testis specific serine kinase 6
			CILP2	cartilage intermediate layer protein 2
			TRNAL-CAA	transfer RNA leucine (anticodon CAA)
			TRNAE-CUC	transfer RNA glutamic acid (anticodon CUC)
ECA14	58144753-58716055	0.59	*MCC*	mutated in colorectal cancers
			YTHDC2	YTH domain containing 2
ECA13	7010566-7703789	0.58	*CYP3A89*	Equus caballus cytochrome P450 3A89 (CYP3A89), mRNA.
			CYP3A93	Equus caballus cytochrome p450 3A93 (CYP3A93), mRNA.
			CYP3A94	cytochrome p450 3A94
			CYP3A95	Equus caballus cytochrome p450 3A95 (CYP3A95), mRNA.
			CYP3A96	Equus caballus cytochrome P450 3A96 (CYP3A96), mRNA.
			CYP3A97	cytochrome P450 3A97
			RCC1L	RCC1 like
ECA14	59493316-59873032	0.46	*EPB41L4A*	erythrocyte membrane protein band 4.1 like 4A
			SNORA13	Small nucleolar RNA SNORA13
			SNORA25	Small nucleolar RNA SNORA25
ECA19	3023312-3382528	0.46	*NMD3*	NMD3 ribosome export adaptor
			OTOL1	otolin 1
			SPTSSB	serine palmitoyltransferase small subunit B

* NCBI
SOURCE: the author.

TABLE 5 – CHROMOSOME AND GENOMICS REGIONS ASSOCIATED WITH NEUTROPHILS COUNT, PERCENTAGE OF ADDITIVE GENETIC VARIANCE, CANDIDATE GENES AND DESCRIPTION.

Chromosome	Genomic region (bp)	% additive genetic variance explained	Candidate genes	Description*
ECA4	17504389-17799979	0.49	*TNS3*	tensin 3
ECA4	37858768-38702247	0.49	*PON1*	paraoxonase 1
			PEG10	paternally expressed 10
			COL1A2	collagen type I alpha 2 chain
			CASD1	CAS1 domain containing 1
			SGCE	sarcoglycan epsilon
			PPP1R9A	protein phosphatase 1 regulatory subunit 9A
			U6	U6 spliceosomal RNA
ECA4	24364724-25216249	0.47	*VOPP1*	vesicular, overexpressed in cancer, prosurvival protein 1
			SEC61G	Sec61 translocon gamma subunit
			EGFR	epidermal growth factor receptor
			LANCL2	LanC like 2
			5S_rRNA	5S ribosomal RNA
ECA22	27808920-28303696	0.46	*BLCAP*	bladder cancer associated protein
			CTNNBL1	catenin beta like 1
			NNAT	neuronatin
			RPRD1B	regulation of nuclear pre-mRNA domain containing 1B
			TTI1	TELO2 interacting protein 1
			VSTM2L	V-set and transmembrane domain containing 2 like
ECA4	26341606-26936480	0.42	*HGF*	hepatocyte growth factor
			CACNA2D1	calcium voltage-gated channel auxiliary subunit alpha2delta 1

* NCBI

SOURCE: the author.

TABLE 6 – CHROMOSOME AND GENOMICS REGIONS ASSOCIATED WITH LYMPHOCYTES COUNT, PERCENTAGE OF ADDITIVE GENETIC VARIANCE, CANDIDATE GENES AND DESCRIPTION.

Chromosome	Genomic region (bp)	% additive genetic variance explained	Candidate genes	Description*
ECA4	37858768-38702247	0.52	*COL1A2*	collagen type I alpha 2 chain
			CASD1	CAS1 domain containing 1
			SGCE	sarcoglycan epsilon
			PEG10	paternally expressed 10
			PPP1R9A	protein phosphatase 1 regulatory subunit 9A
			PON1	paraoxonase 1
			U6	U6 spliceosomal RNA
ECA22	41575807-42135530	0.50	*DOK5*	docking protein 5
ECA4	24364724-25216249	0.48	*SEC61G*	Sec61 translocon gamma subunit
			EGFR	epidermal growth factor receptor
			LANCL2	LanC like 2
			VOPP1	vesicular, overexpressed in cancer, prosurvival protein 1
			5S_rRNA	5S ribosomal RNA
ECA4	27842695-28769871	0.44	*SEMA3E*	semaphorin 3E
			SEMA3A	semaphorin 3A
ECA21	15292316-15923414	0.44	*GPBP1*	GC-rich promoter binding protein 1
			MIER3	MIER family member 3
			SETD9	SET domain containing 9
			MAP3K1	mitogen-activated protein kinase kinase kinase 1

* NCBI

SOURCE: the author.

4.4 CONCLUSION

Candidate regions related to immune system development and activation were found in this study. This candidate regions and candidate gene information can help us understand why some horses tend to acquire more parasites than others, providing an opportunity to identify horses in need of more elaborate parasite control efforts. This, as a consequence, would reduce the anthelmintic use, the overall costs, selection for parasite resistance, and also the production losses linked to clinical signs and surgery the use of it.

Several SNP markers have been shown to be associated with FEC, PCV, eosinophils count, neutrophils count, and lymphocytes count traits. The top 5 marker regions explained 2.86; 2.56; 2,73; 2,33 and 2.37% of the genetic variation for FEC, PCV, eosinophils count, neutrophils count, and lymphocytes count, respectively.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors wish to acknowledge the veterinarian Joaquim Antunes and the Stud team for their technical support and the Laboratory of Parasitic Diseases team for carrying out the EPG analysis.

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TABLE S1 - METABOLIC PATHWAY ASSOCIATED WITH GENES CANDIDATES FOR TRAIT NEMATODES INFECTION IN THOROUGHBRED HORSES, PERCENTAGE OF ADDITIVE GENETIC VARIANCE, CANDIDATE GENES AND DESCRIPTION.

Gene	ID Pathway	Pathway name	Description*
TLN1	P00034	Integrin signalling pathway	The integrin signalling pathway is triggered when integrins in the cell membrane bind to extracellular matrix components. Downstream events include actin reorganization and activation of MAPK and other signalling cascades.
NPR2	P06664	Gonadotropin releasing hormone receptor pathway	The GnRH receptor (GnRHR), expressed at the cell surface of the anterior pituitary gonadotropin is critical for normal secretion of gonadotropins LH and FSH, pubertal development, and reproduction. The signaling network downstream of the GnRHR and the molecular bases of the regulation of gonadotropin expression have been the subject of intense research. The murine LbetaT2 cell line represents a mature gonadotrope, and therefore is an important model for the study of GnRHR signaling pathways, and modulation of the gonadotrope cell by physiological regulators. In order to facilitate access to the information contained in this complex and evolving literature, we have developed and curated a comprehensive knowledgebase of the GnRHR signaling in the LbetaT2 cell in the form of a process diagram (Fink et al., 2010; PMID: 20592162). Positive and negative controls of gonadotropin gene expression, which included GnRH itself, hypothalamic factors, gonadal steroids and peptides, as well as other hormones, were illustrated. The GnRHR signaling pathway is being updated yearly, based on the latest publications in the field in addition to experts' suggestions. The pathway map was curated using CellDesigner ver.4.1 (http://celldesigner.org/). For simplification purposes, activating reactions, including those linking transcription factors to genes, were depicted with "State Transition" black filled arrows. Inhibitory reactions were colored in red. The size and color of each module were configured by us. Briefly, round angle green squares signify cell signaling proteins, whereas green and yellow circles represent small molecules and ions, respectively; round angle blue squares symbolize transcription factors, and round angle purple squares strictly correspond to nuclear receptors; yellow/green rectangles designate genes, on which some response elements were represented as small white squares.
CREB3	P00055	Transcription regulation by bZIP transcription factor	bZip General Transcription Factors for RNA Polymerase II The basic-leucine zipper (bZIP) transcription factors of eukaryotic are proteins that contain a basic region mediating sequence-specific DNA-binding followed by a leucine zipper region required for dimerization. This pathway represents the CRE-BP/ATF subfamily of bZIP.
CREB3	P00026	Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway	Gi alpha and Gs alpha mediated pathways are G-protein receptor activated pathways. A number of activated receptors can bind to and activate the associated heterotrimeric G-protein consisting of either Gi alpha or Gs alpha. The activated, GTP-bound G alpha subunit dissociates from its cognate beta gamma subunit and is free to effect downstream signaling molecules. In the case of Gi alpha, G-protein inwardly rectifying potassium channels are activated and adenylyl cyclase is inhibited. In contrast, Gs alpha activates adenylyl cyclase. The countering effects of Gi alpha and Gs alpha regulate the overall downstream effect of multisourse input from various associated G-protein coupled receptors.

* NCBI
SOURCE: the author.

5 FINAL CONSIDERATIONS

The search for better tools for the control of gastrointestinal parasite infections in horses is constant in breeding centers, due to the concern with the health of the animals and the problems related to the economic impact of some parasitosis. Due to the intensive use of commercial compounds, Strongyle parasites were selected for resistance, reducing the effectiveness of their control. Consequently, we need to develop new management methods to protect horses, by controlling parasite infection. This thesis has highlighted several possibilities to further investigate this problem, that hold promise for improving the understanding and control of cyathostomins infections in equines.

There are numerous tests that can be used for faecal egg count in horses. In this work a more modern and less widespread technique was presented for horses. Proving its accuracy for the identification of animals that need treatment and also for the evaluation of the anthelmintics effectiveness in the fecal egg count reduction test in the field. Also, the Mini-FLOTAC technique may be widely used to monitor FEC variability along the year when using individual sampling, adopting the target selective treatment in horse farms.

This Thesis contributed to establish a body growth reference data to the national Thoroughbred hosres. In addition, the results indicate that the development of foals in the experiment were similar to those in other countries in different continents. We also conclude that horses raised under a well-defined set of management practices can have little or no signs of impairments due to worm infections, whilst parasite load was not determined on pasture or in the animals. However, low FEC levels (below 400) during the foaling period influences the racing performance of the animals.

Through the GWAS technique, we determined the candidate regions that may be related to immune system development and activation in the chromosome 17, 21 and 25. These candidate regions and candidate gene information can help us to understand why some horses tend to acquire more parasites than others. It also provides an opportunity to identify horses in need of a more elaborate parasite control, reducing parasite selection, anthelmintic costs, and other production losses. As there is no such a thing as a perfect research (in this case due to limited time, money and sample size of animals), it should be recognized that the present work

presents limitations. GWA studies, especially for quantitative traits, need to have large populations, involving hundreds of individuals to discover and define strong genomic associations. However, such studies are prohibitively expensive, considering the resources typically available for horse research. Thus, as future prospects, more animals will be evaluated to increase the phenotypic and genotypic database.

The increase of the equine herd and the demand of animals of high performance needs to be supported by a more genetic-driven breeding programs. The development of high resolution genetic maps shall be a valuable tool for the isolation of genes and markers associated with economically important traits. In addition, this wouls also be used to enable researchers to develop specific diagnostic tests and to develop preventive management and treatment measures. The SNPs derived gene function annotation that were used in the present study identified candidate genes and their association with FEC in equines. However, a complementary study associated with the validation of GWAS candidate genes, like expression analyses, re-sequencing of genes, and haplotype blocks, may be strongly considered in the future.

Initially, we may propose a quantitative real-time PCR (qPCR) under contrasting groups of animals that are genetically different (low and high FEC; artificial infection levels; diferent breeds). These groups can be selected by means of the predicted breeding values for FEC. Other methods to validate or refine the identified candidate genes in future studies can be done directly by a re-sequencing approach. The advent of next-generation sequencing (NGS), and the decrease in the whole-genome sequencing associated costs, allow us to sequence specific genome regions (related to genes of interest) of a few contrasting individuals and to access, for example, by alignment algorithms, causal polymorphisms underlying these regions.

The optimal racehorse should have a correct conformation, willingness to run fast, be resistant to diseases and injuries, it should mature early in life and it should be durable. For some of these traits a large fraction of the phenotype is controlled by genetics. As such, even though we now about a handful of genes that influence horse performance, there is much more left to discover and force the genetics community to think on a genome-wide scale.

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- **Oral Presentations**

1. Selective therapy to control cyathostomins in Thoroughbred mares, **AAVP 62nd Annual Meeting, 2017**, Indiana/US, 2017.

2. Selective therapy for parasite control as a tool to identify tolerant animals, **X International Equine Infectious Diseases Conference**, Buenos Aires/AR, 2016.

3. Identificação morfométrica de larvas infectantes de ciatostomíneos (Nematoda: Cyathostominae) de equinos PSI, **42° Conbravet e I congresso sul-brasileiro da anclivepa**, Curitiba/PR, 2015.

4. Avaliação da câmara Mini-FLOTAC e McMaster para quantificação de ovos de nematódeos gastrintestinais em equinos, **XVIII Congresso Brasileiro de Parasitologia Veterinária**, Gramado/RS, 2014.

APPENDIX A – COMPARISON OF MCMASTER AND MINI-FLOTAC FECAL EGG COUNTING TECHNIQUES IN CATTLE AND HORSES PUBLISHED IN VETERINARY PARASITOLOGY: REGIONAL STUDIES AND REPORTS

Veterinary Parasitology: Regional Studies and Reports 10 (2017) 132–135

Contents lists available at ScienceDirect

Veterinary Parasitology: Regional Studies and Reports

journal homepage: www.elsevier.com/locate/vprsr

Short communication

Comparison of McMaster and Mini-FLOTAC fecal egg counting techniques in cattle and horses

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ARTICLE INFO

Keywords:
EPG
Cattle
Horse
Nematode
Diagnosis

ABSTRACT

The aim of this study was to compare two fecal egg count (FEC) techniques; McMaster (McM) and Mini-FLOTAC (mF), for the detection of cattle and horse gastrointestinal nematode eggs, in different locations. Experiment 1: feces were collected from 16 cattle and FEC was performed individually, using mF with the sensitivity of 5 eggs per gram of feces (EPG) and McM with a sensitivity of 50 EPG at Empresa de Pesquisa Agropecuária de Minas Gerais - EPAMIG and the Laboratory of Parasitic Diseases of the University of Parana – LDP/UFPR. Experiment 2: Fecal samples from 30 horses were analyzed with mF (sensitivity of 5 EPG) and McM (sensitivity of 25 EPG) at the University of Mato Grosso do Sul - UFMS and LPD/UFPR. Experiment 3: feces were collected from 14 foals and FEC was performed using mF (sensitivity of 5 EPG); and McM (sensitivity of 25 EPG) only at the LPD/UFPR. For cattle, the average FEC of mF was 962 at LPD; and 1248 at EPAMIG; for McM it was 1393 at LPD and 1563 at EPAMIG. For horses, the FEC average using the mF was 650 at LPD and 469 at UFMS; and for McM it was 677 at LPD and 554 at UFMS. For foals, the average FEC for mF was 404 and 436 for McM. In all experiments, the standard deviation and the coefficient of variation values were significantly lower for mF. Therefore, it is recommended the use of the Mini-FLOTAC technique, which is a method with less variability and higher accuracy, particularly for animals with low FEC.

1. Introduction

The fecal egg count (FEC) methods permit to determine the number of nematode eggs in livestock (Bosco et al. 2014; Presland et al. 2005). There are several FEC and these differ in sensitivity, time required to process the samples and the necessary technical knowledge for interpretation. The test can potentially be extended to other situations, where accurate detection of egg number is important; i.e. the fecal egg count reduction test (FECRT) to determine anthelmintic efficacy in a range of parasites (Bosco et al. 2014).

The most widely used method for FEC was developed in Australia by Gordon and Whitlock (1939), using the McMaster (McM) chamber, originally used to count parasites of sheep. It is known that the McM method lacks sensitivity, particularly at low nematode egg counts (Mes 2003). A modification in the original protocol (i.e. the amount of feces or the volume of flotation solution) suggests that the McM multiplication factor can be changed when analyzing samples from cattle and horses (Roepstorff and Nansen 1998).

A more recent technique called FLOTAC has been developed in Italy to increase FEC sensitivity (Cringoli 2006; Cringoli et al. 2010). FLOTAC was based on the centrifugal flotation of a sample and the subsequent translation of the top layer of the floating suspension (Cringoli 2006). Despite the high sensitivity, a main limitation of the FLOTAC technique was the complexity of the method, which involved the centrifugation of the sample (Knopp et al. 2009). To overcome this step, a new simplified apparatus has been developed, named Mini-FLOTAC (mF) (Cringoli et al. 2013). A major advantage of this new method is that it can be more easily transferred and carried out in laboratories with limited facilities, due to its simpler protocol. However, a comparative validation field study of this laboratory technique with

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http://dx.doi.org/10.1016/j.vprsr.2017.10.003
Received 22 March 2017; Received in revised form 21 June 2017; Accepted 8 October 2017
Available online 10 October 2017

APPENDIX B – DESCRIPTION OF THE MINI-FLOTAC TECHNIQUE

Mini-FLOTAC

Veterinary Parasitology and Parasitic Diseases
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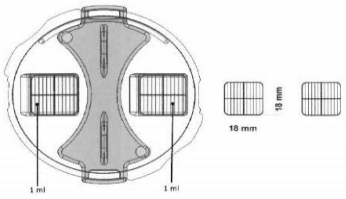
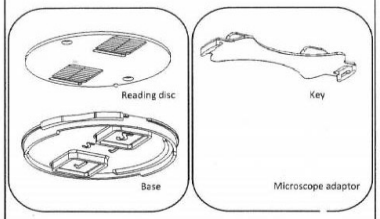
Faculty of Veterinary Medicine, University of Naples Federico II
Via della Veterinaria, 1 - 80137 Naples, Italy
Tel. +39 0812536283 – cringoli@unina.it

Mini-FLOTAC



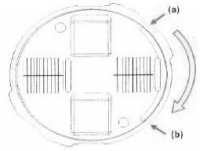
Components Accessories



ASSEMBLY

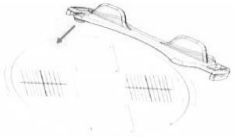
Place the lower side of the Reading disc onto the upper side of the Base, so that the raised portion of the reading disc enter the base slot.

ASSEMBLY



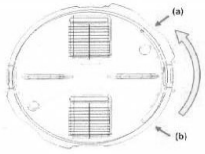
Turn only the Reading disc clockwise until the raised portion of the Reading disc stop further movement (b)

ASSEMBLY



Place the Key on the assembly so that the two raised portions on the underside of the Key fit into the two holes on the Reading disc

ASSEMBLY

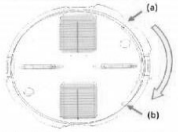


The Key is used to turn the Reading disc counter – clockwise (about 90°) until the Reading disc stop further movement (a)

FILLING

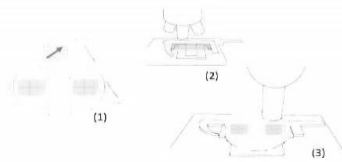
Using the filling holes, the flotation chambers can be filled with the faecal suspension using a pipette until a little meniscus is formed. In order to avoid formation of air bubbles, the chambers must be filled with the Mini-FLOTAC apparatus inclined towards the technician.

TRANSLATION



After 10 minutes, the Key is used to turn the reading disc clockwise (abouth 90°) until the Reading disc stop further movement (b).
Thus, the top parts of the two floated suspension (i.e. the parts which contain the parasitic elements) have been traslated 90° and are now completly separated from the rest of the flotation chambers (i.e. the parts which contain the faecal deb·is).
Turn firmly with one movement!

READING



1 - Remove the ke
2 - Attach the Microscope adaptor to the microscope
3 - Place the Mini-FLOTAC apparatus on the Microscope adaptor with the ruled grid No 1 on the right.

Mini-FLOTAC BASIC TECHNIQUE

1 - Weigh 5 grams of fresh faeces taken from a larger amount of faecal material thoroughly homogenized (preferably in liquid phase).

2 - Add 45 ml of the chosen flotation solution (FS) (dilution ratio = 1:10). If less than 5 grams of faeces are available, use the final dilution ratio 1:10.

3 - Homogenize the suspension thoroughly.

4 - Filter the suspension through a wire mesh (aperture = 250 µm).

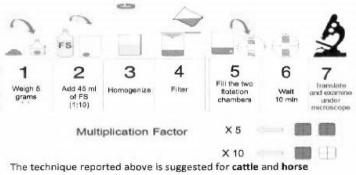
5- Homogenize the suspension and fill the two flotation chambers of the Mini-FLOTAC.

6 - Close the Mini-FLOTAC and wait for 10 min.

7 - Translate the top parts of the flotation chambers and read under the microscope.

The analytic sensitivity of the Mini-FLOTAC basic technique is: 5 EPG, 5 LPG, 5 OPG, and 5 CPG.

Mini-FLOTAC BASIC TECHNIQUE



The technique reported above is suggested for **cattle** and **horse**

For **human** and **dog:**
1 - Weigh 2 grams of fresh faeces and add 2 ml of formalin 5% (dilution ratio = 1:1) and homogenize
2 - Add 36 ml of flotation solution (dilution ratio = 1:20)
3-7 - See the technique reported above

The analytic sensitivity of the Mini-FLOTAC basic technique is: 10 EPG, 10 LPG, 10 OPG, and 10 CPG.

APPENDIX C – CERTIFICATE OF APPROVAL NUMBER 035/2014 FROM THE ETHICS COMMITTEE OF THE SECTOR OF AGRICULTURAL SCIENCES OF THE FEDERAL UNIVERSITY OF PARANÁ

Universidade Federal do Paraná
Setor de Ciências Agrárias
Comissão de Ética no Uso de Animais – CEUA SCA

CERTIFICADO

Certificamos que o protocolo no. 035/2014, referente ao projeto "Correlação do diagnóstico parasitário e desempenho corporal com parâmetros de comportamento em equinos", sob a responsabilidade de Marcelo Beltrão Molento, na forma em que foi apresentado (uso de 90 cavalos), foi aprovado pela Comissão de Ética no Uso de Animais do Setor de Ciências Agrárias, em reunião realizada dia 31 de julho de 2014.

CERTIFICATE

We certify that the protocol number 035/2014, regarding the project "Correlation od=f parasite diagnostic and body performance with behavior parameters of equine", under Marcelo Beltrão Molento's supervision, in the terms it was presented (use of 90 horses), was approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Universidade Federal do Paraná (Federal University of Paraná, Brazil) during session on July 31st, 2014.

Curitiba, 31 de Julho de 2014.

Ricardo Guilherme D'Otaviano de Castro Vilani
Presidente

Ananda Portella Félix
Vice-Presidente

Comissão de Ética no Uso de Animais
Setor de Ciências Agrárias
Universidade Federal do Paraná.

APPENDIX D – CERTIFICATE OF APPROVAL NUMBER 026/2014 FROM THE ETHICS COMMITTEE OF THE SECTOR OF AGRICULTURAL SCIENCES OF THE FEDERAL UNIVERSITY OF PARANÁ

Universidade Federal do Paraná
Setor de Ciências Agrárias
Comissão de Ética no Uso de Animais – CEUA SCA

CERTIFICADO

Certificamos que o protocolo no. 026/2014, referente ao projeto "Análise de marcadores moleculares associados a resistência de parasitores gastrintestinais de equinos", sob a responsabilidade de Marcelo Beltrão Molento, na forma em que foi apresentado (uso de 120 equinos), foi aprovado pela Comissão de Ética no Uso de Animais do Setor de Ciências Agrárias, em reunião realizada dia 31 de julho de 2014.

CERTIFICATE

We certify that the protocol number 026/2014, regarding the project "Analysis of molecular markers associated with resistance to gastrointestinal parasites in horses", under Marcelo Beltrão Molento's supervision, in the terms it was presented (use of 120 horses), was approved by the Animal Use Ethics Committee of the Agricultural Sciences Campus of the Universidade Federal do Paraná (Federal University of Paraná, Brazil) during session on July 31st, 2014.

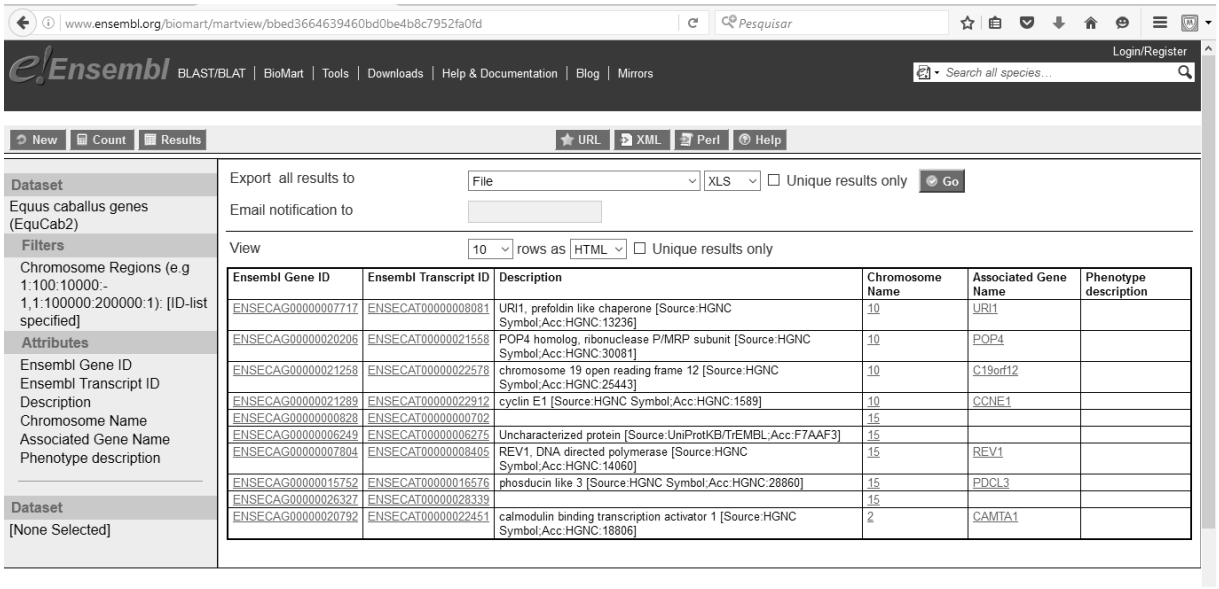
Curitiba, 31 de Julho de 2014.

Ricardo Guilherme D'Otaviano de Castro Vilani
Presidente

Ananda Portella Félix
Vice-Presidente

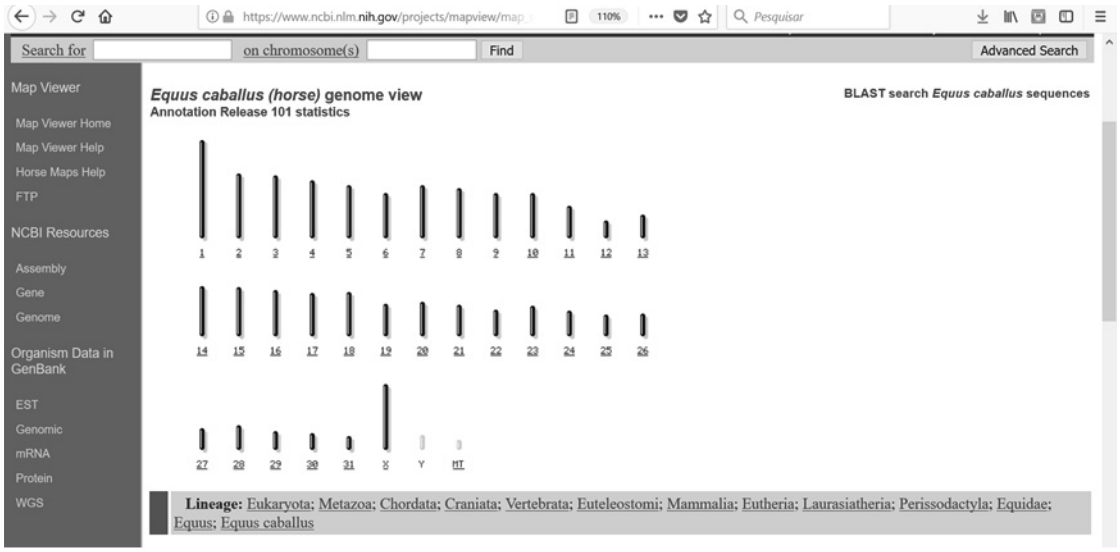
Comissão de Ética no Uso de Animais
Setor de Ciências Agrárias
Universidade Federal do Paraná.

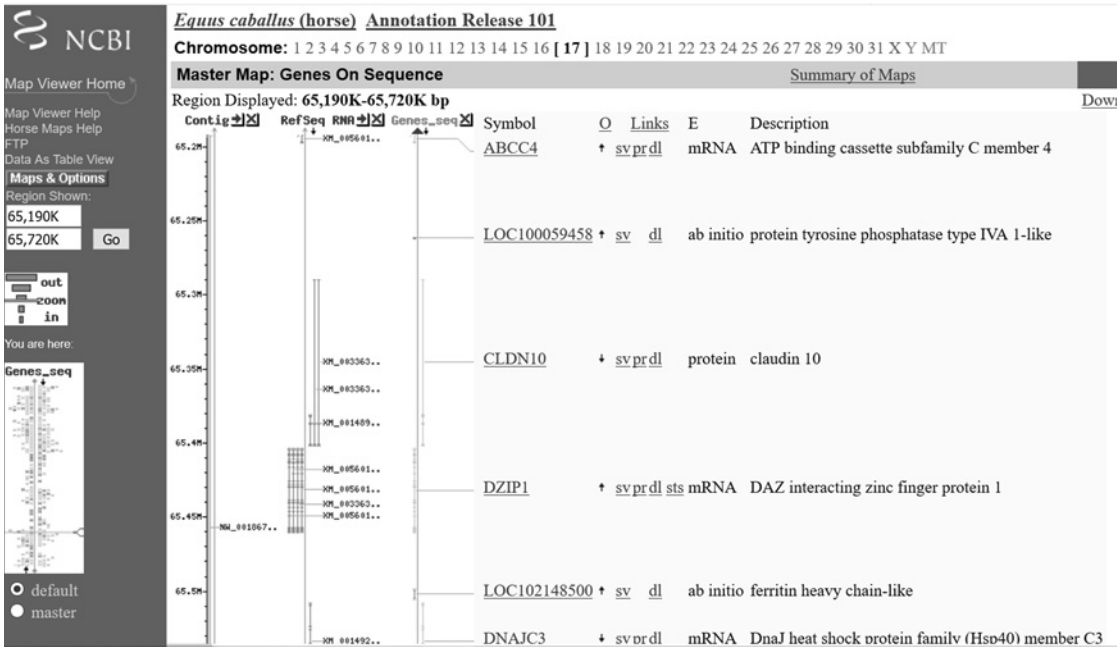
APPENDIX E – GENE SEARCH ON ENSEMBL GENOME BROWSER DATABASE, BASED ON THE EQUINE GENOME REFERENCE EQUCAB2, WITH *BIOMART* TOOL



Ensembl Gene ID	Ensembl Transcript ID	Description	Chromosome Name	Associated Gene Name	Phenotype description
ENSECAG00000007717	ENSECAT00000008081	URI1, prefoldin like chaperone [Source:HGNC Symbol;Acc:HGNC:13236]	10	URI1	
ENSECAG00000020206	ENSECAT00000021558	POP4 homolog, ribonuclease P/MRP subunit [Source:HGNC Symbol;Acc:HGNC:30081]	10	POP4	
ENSECAG00000021258	ENSECAT00000022578	chromosome 19 open reading frame 12 [Source:HGNC Symbol;Acc:HGNC:25443]	10	C19orf12	
ENSECAG00000021289	ENSECAT00000022912	cyclin E1 [Source:HGNC Symbol;Acc:HGNC:1589]	10	CCNE1	
ENSECAG00000000828	ENSECAT00000000702		15		
ENSECAG00000006249	ENSECAT00000006275	Uncharacterized protein [Source:UniProtKB/TrEMBL;Acc:F7AAF3]	15		
ENSECAG00000007804	ENSECAT00000008405	REV1, DNA directed polymerase [Source:HGNC Symbol;Acc:HGNC:14060]	15	REV1	
ENSECAG00000015752	ENSECAT00000016576	phosducin like 3 [Source:HGNC Symbol;Acc:HGNC:28860]	15	PDCL3	
ENSECAG00000026327	ENSECAT00000028339		15		
ENSECAG00000020792	ENSECAT00000022451	calmodulin binding transcription activator 1 [Source:HGNC Symbol;Acc:HGNC:18806]	2	CAMTA1	

APPENDIX F - GENE SEARCH ON NATIONAL CENTER FOR BIOTECHNOLOGY INFORMATION (NCBI) DATABASE, WITH *MAP VIEWER* TOOL





APPENDIX G - MANUSCRIPT "BODY DEVELOPMENT OF THOROUGHBRED FOALS FROM BIRTH TO 18 MONTHS OF AGE" SUBMITTED TO THE ARCHIVES OF VETERINARY SCIENCE

BODY DEVELOPMENT OF THOROUGHBRED FOALS FROM BIRTH TO 18 MONTHS OF AGE

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[†] These authors contributed equally to the development of this manuscript.

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ABSTRACT

Although the body development of foals is a predetermined genetic factor, the growth rate may be influenced by environmental and nutritional factors. The objective of this work was to determine the body development (weight and withers height) of 119 Thoroughbred foals from Paraná, Brazil. The data was gathered from the 2008 to 2012 seasons and each year corresponded to a new generation of foals. The body development data were obtained monthly, from the animals' first 24h until they reached 18 months. The variables were analyzed by ANOVA and the Tukey test. Fillies were taller and heavier than colts, with statistically differences only at birth (weight, $p=0.0091$; height, $p=0.0065$). The colts showed 9.8% and the fillies had 11.3% of their adult weight at birth and 44.7 and 49.6%, respectively at 6 months; 61.8% and 68.7% at 12 months; and 65 and 89.1% when they reached 18 months. The animals were born from July to November, and the animals born in November, both males that reached 6 months of age and females that reached 12 months, showed the lowest weight ($p \leq 0.03$). Our research can be used as a reference to the national Thoroughbred databank, demonstrating that the body development of foals in Brazil is similar to those of other countries.

Key-words: Brazil. Growth. Horse. Performance. Race-horse.

DESENVOLVIMENTO CORPORAL DE POTROS PURO SANGUE INGLÊS DESDE O NASCIMENTO ATÉ 18 MESES DE IDADE

RESUMO

Embora o desenvolvimento corporal de potros seja um fator genético predeterminado, a taxa de crescimento pode ser influenciada por fatores ambientais e nutricionais. O objetivo desse trabalho foi determinar o desenvolvimento corporal (peso e altura da cernelha) de 119 potros Puro Sangue Inglês no Paraná, Brasil. Os dados foram coletados nos anos de 2008 a 2012 e cada ano correspondeu a uma nova geração de potros. As avaliações de desenvolvimento corporal foram obtidas mensalmente, desde as primeiras 24h até os 18 meses de vida. As variáveis foram analisadas por ANOVA e teste de Tukey. As potras foram mais altas e pesadas do que os potros, estatisticamente diferentes apenas ao nascer (peso, p=0,0091; altura, p=0,0065). Os potros atingiram 9,8% e as potras 11,3% do seu peso adulto no momento do nascimento; 44,7 e 49,6%, respectivamente, aos 6 meses; 61,8% e 68,7% aos 12 meses; e 65 e 89,1% quando atingiram 18 meses. O período de nascimento dos animais foi de julho a novembro, e os animais nascidos em novembro, tanto machos aos 6 meses, como as fêmeas aos 12 meses, apresentaram menor peso (p≤0,03). Os resultados obtidos neste trabalho podem ser utilizados como referência ao banco de dados nacional de cavalos PSI, demonstrando que o desenvolvimento corporal de potros no Brasil é semelhante ao de outros países.

Palavras-chave: Brasil. Crescimento. Cavalo. Performance. Cavalo de corrida.

1. INTRODUCTION

Horses usually reach physical maturity at four to five years of age (PAGAN and NASH, 2009) and the maximum body size is mostly genetically predetermined. The growth rate may be influenced by factors such as environmental, nutrition, health and local management (HINTZ et al., 1978; HINTZ et al., 1979; BROWN-DOUGLAS et al., 2005). In the case of animals for horse racing, such as Thoroughbreds, the breeders select these animals to become athletes, as early as possible, with training and competitions starting at approximately two years of age. For this, all nutritional and health management is aimed to accelerate the foals' growth rate (PAGAN and NASH, 2009). The development of young animals is mainly evaluated by traits such as age, body weight and height at the withers or garrote (PAGAN et al., 1996).

Previous studies on the growth rate of Thoroughbred horses have been limited to populations mainly located in the Northern hemisphere (GREEN, 1969; HINTZ et al., 1978; HINTZ et al., 1979; PAGAN et al., 1996). There is less data available for the Southern hemisphere populations; i.e. New Zealand (BROWN-DOUGLAS et al., 2005) and Brazil (GARCIA et al., 2011). However, this information is extremely important for Brazilian breeders, since in Brazil the financial movement around horse racing generates approximately US$ 164 million/year, representing 5% of the horse agribusiness (LIMA et al., 2006).

The aim of this study was to determine the body development (weight and withers height) of young Thoroughbred horses, correlating the gender and month of birth of the animals, between 2008 to 2012 from São José dos Pinhais, state of Paraná, Brazil.

2. MATERIAL AND METHODS

For this study, we followed five year of birth of horses in a breeding farm located in São José dos Pinhais, state of Paraná, South of Brazil. The data from 119 foals was gathered from the birth season of 2008 to 2012. The sample compromise a total of 60 males and 59 females, which were born in the specific year and remained in the farm until the age of 18 months (Table 1). The births were from July to November of each corresponded year.

TABLE 1 – DESCRIPTION OF MALE AND FEMALE THOROUGHBRED FOALS BORN BETWEEN 2008 AND 2012 FROM SAO JOSE DOS PINHAIS, PR, BRAZIL.

Year of Birth	Male	Female	Total
2008	9	12	21
2009	12	11	23
2010	16	11	27
2011	10	16	26
2012	13	9	22
Total	60	59	119

SOURCE: the author.

All animals were born and kept in the same farm with similar management and nutritional status, differing in year and month of birth, mare and stallion. All animals were kept on ryegrass (*Lolium multiflorum*) with white clover (*Trifolium repens*) pasture, during winter and 'bahia grass' (*Paspalum notatum*), dallis grass (*Paspalum dilatatum*) pastures during summer. In addition, the horses received oat grain (*Avena sativa*), protein and minerals from a premix and alfalfa hay (*Medicago sativa*) twice a day, equivalent to 2.5 to 3% of body weight and water ad libitum. All foals were weaned at 6 months of age when they were separated into lots of males and females.

The body weight was measured using a mechanical weight scale and the withers height was measured with the aid of a hypsometer. Both measurements were performed monthly, from within the first 24 hours (day 1) until the last month of the animal at the horse farm. The data of weight and withers height of the animals were provided by the veterinarian responsible for the farm and checked by our team. The protocol for this study was approved by the Ethical Committee for Animal Experimentation of the Federal University of Parana, Brazil (number 035/2014), appendix C.

Data were analyzed in four periods: birth (24h), 6, 12 and 18 months of age. For the 'period', the data relating to animals of all generations were grouped. For the statistical evaluation of the paternal effect, gender, month and year of birth, the data was analyzed according to the analysis of variance (ANOVA) in a completely randomized design with 5% significance level, followed by the Tukey test, using Statistix 10.0.1 software.

3. RESULTS AND DISCUSSION

The body weight and the withers height of all animals are described on Table 2. During all periods of assessment, it was observed that females were taller and heavier than males, with a significant difference only in weight (p = 0.0091) and height (p = 0.0065) at birth. Garcia et al. (2011) evaluated the body development of Thoroughbred foals at birth, 6, 12 and 18 months of age and reported, unlike the present study, that males were taller and heavier than females in Bagé, state of Rio Grande do Sul. Pagan et al. (2009) have also determined that males were taller and heavier than females, but the evaluations were performed at 7 days, 1, 2, 3, 4 and 5 months old, in Kentucky, USA.

TABLE 2 – MEAN AND STANDARD DEVIATION OF THE WEIGHT (KG) AND WITHERS HEIGHT (CM) OF THOROUGHBRED FOALS ACCORDING TO GENDER, FROM 2008 TO 2012 FROM A HORSE FARM LOCATED IN SAO JOSE DOS PINHAIS, PR, BRAZIL.

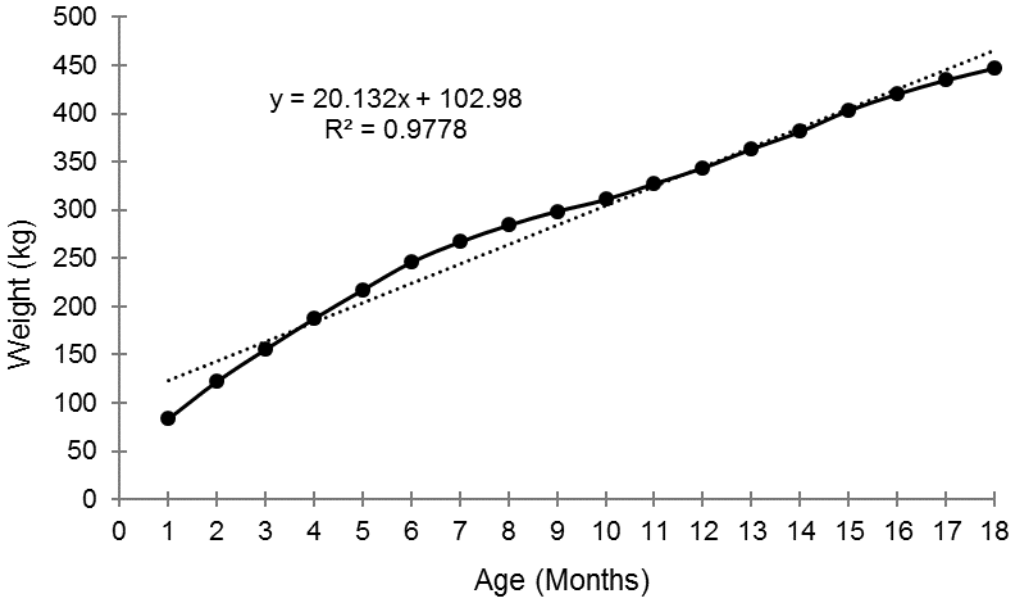
	Weight (kg)			Withers height (cm)		
	Male	Female	p	Male	Female	p
Birth	53.82^{B} ± 5.88	56.63^{A} ± 5.63	0.0091	102.15^{B} ± 3.39	103.64^{A} ± 2.35	0.0065
6 Months	243.83 ± 20.71	248.21 ± 18.25	0.2253	135.79 ± 3.55	136.44 ± 2.70	0.2694
12 Months	337.24 ± 19.15	343.62 ± 19.77	0.8961	146.46 ± 2.80	147.60 ± 2.79	0.5867
18 Months	432.72 ± 22.22	445.45 ± 30.82	0.1746	154.18 ± 3.18	155.51 ± 3.09	0.9430

Different letters in the same line indicate statistical difference ($p<0.05$).

SOURCE: the author.

Considering that the average weight and withers height of adult animals is 550 kg and 1.62 m for the males and 500 kg and 1.60 m for females (NRC, 1989), at birth, male foals in this study had 9.8% and females had 11.3% of their adult weight; when they reached 6 months, males and females achieved 44.7 and 49.6%, respectively; at 12 months, they had 61.8% (colts) and 68.7% (fillies); and 65.6 and 89.1% at 18 months of age (Figure 1). The growth of the animals showed a linear increase along the months (R: 0.9778). The results are similar to those observed in Brazil (GARCIA et al., 2011) and the USA (PAGAN et al., 1996). Regarding withers height, at birth males and females had on average 63.1 and 64.8% of the adult height; at 6 months 94.9 and 85.3% for colts and fillies, respectively; at 12 months, 90.4 and 92.3%; and 95.2 and 97.2% at 18 months (Figure 2), showing an optimal linear regression of 0.9119.

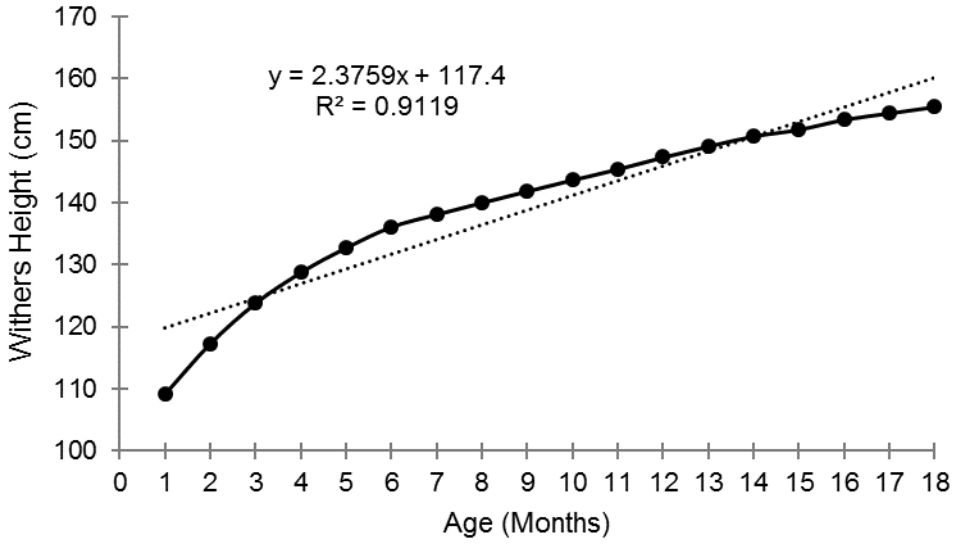
FIGURE 1 - LINEAR REGRESSION OF MEAN BODY WEIGHT (KG) ACCORDING TO AGE (MONTHS) AND TREND LINE (DASHED) OF THOROUGHBRED FOALS FROM SÃO JOSÉ DOS PINHAIS, BRAZIL.



SOURCE: the author.

Thoroughbred horses raised in India were significantly smaller than those from Australia (BROWN-DOUGLAS et al., 2009), such differences may be imposed by nutritional regimen (JELAN et al., 1996), as well as genetic and environmental factors. Another evidence that foals from our experiment can be compared to other countries, is that the average weight of the horses from our study at 18 months of age were 7.9 times higher than their birth weight. In Australia and India, foals were 6.6 and 6.5 times heavier for this age, respectively (BROWN-DOUGLAS et al., 2009).

FIGURE 2 - LINEAR REGRESSION OF MEAN WITHERS HEIGHT (CM) ACCORDING TO AGE (MONTHS) AND TREND LINE (DASHED) OF THOROUGHBRED FOALS FROM SÃO JOSÉ DOS PINHAIS, BRAZIL.



SOURCE: the author.

At six months of age, when the animals were weaned, only males born in November were lighter than the animals that were born in the other months (p = 0.0002) (Table 3). At 12 months of age, females born in September and October were significantly heavier than animals born in the other months, and those born in November were the lightest (p = 0.03). When evaluating the withers height, we observed that there were no significant differences for any of the birth months' for both genders. Huntington et al. (2007) evaluated the effect of the month of birth on the growth of Thoroughbred foals in Australia, from neonate animals to up to 18 months. The foals born in November were significantly lighter than all the others at six months of age. This finding is similar to our study, since animals born in November, both males that reached 6 months of age and females that reached 12 months, showed the lowest weight.

Santos et al. (2007) evaluated the effect of month of birth on body development of 110 colts from the Pantaneiro breed, born in areas of native grasslands of Pantanal, Brazil and observed that animals born in September were taller and heavier at weaning. This was related to higher pasture offer in the region and better quality in September. Furthermore, the foals born in December, a period

of low supply of pasture for the mares, had a reduction of their body development. This was similar to what happened to the animals born in November in our study.

The difference in the weight of the animals born in November, observed in the present study, may be related to a reduction of milk production by the mares due to a lower protein and energy intake. During milking period, the mares that gave birth from July to October had more contact with temperate pastures (C3 plants), which have higher digestibility (BASSO and BARBERO, 2015) and more access time to legumes (White clover), which have a lower content of neutral detergent insoluble fiber and a higher crude protein content (RIBEIRO FILHO, 2003). These nutrient supplies could positively influence the milk production of the mares and consequently have enhanced the development of their foals until weaning.

TABLE 3 – AVERAGE WEIGHT (KG) AND STANDARD DEVIATION OF THOROUGHBRED FOALS ACCORDING TO GENDER, EVALUATED BY CATEGORY MONTH OF BIRTH FROM A HORSE FARM LOCATED IN SÃO JOSÉ DOS PINHAIS, BRAZIL.

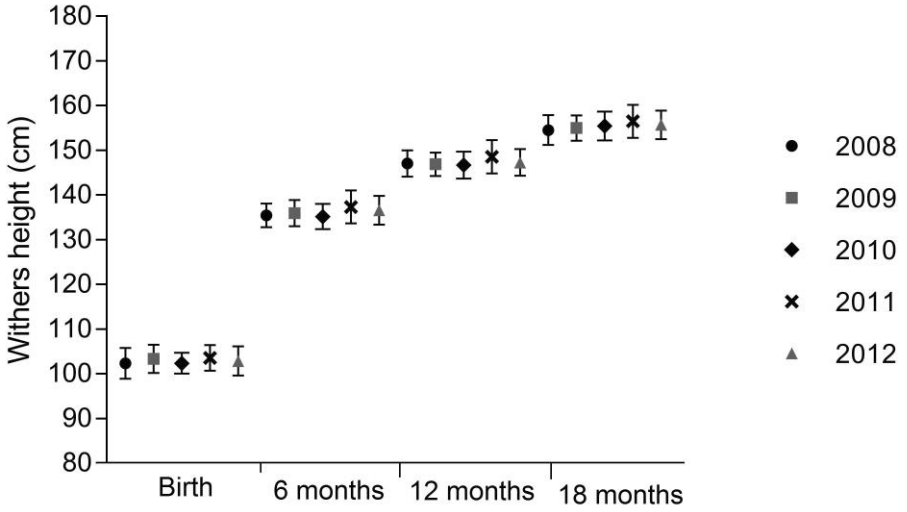
		Jul.	Aug.	Sep.	Oct.	Nov.
Birth	Male p=0.88	54.0 ± 6.3	52.2 ± 5.5	54.0 ± 5.6	54.1 ± 6.7	56.0 ± 1.0
	Female p=0.11	54.8 ± 5.2	55.5 ± 5.6	59.4 ± 5.7	56.5 +- 5.2	64.0 ± 1.5
6 months	Male p=0.0002	259.4^{A} ± 14.9	241.0^{A} ± 14.6	234.0^{A} ± 19.3	241.1^{A} ± 20.8	215.9^{B} ± 15.4
	Female p=0.54	247.6 ± 16.9	248.2 ± 18.3	251.8 ± 17.4	246.8 ± 21.3	219.0 ± 18.6
12 months	Male p=0.43	350.6 ± 19.6	341.8 ± 19.0	338.8 ± 20.4	342.2 ± 24.1	330.9 ± 9.8
	Female p=0.03	336.0^{B} ± 19.5	336.4^{B} ± 21.5	355.4^{A} ± 18.5	348.4^{A} ± 14.1	326.0^{C} ± 12.4
18 months	Male p=0.63	448.7 ± 28.5	437.2 ± 14.4	436.7 ± 23.3	441.2 ± 25.7	429.50 ± 0.7
	Female p=0.06	445.3 ± 26.3	436.4 ± 29.1	464.2 ± 26.1	452.3 ± 21.4	450.00 ± 20.1

Different letters in the same line indicate statistical difference ($p<0.05$).

SOURCE: the author.

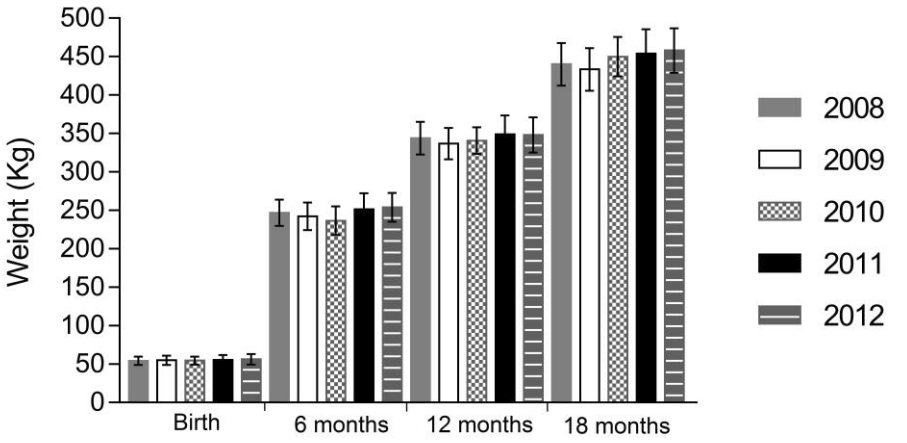
The average values of the withers height and body weight of the horses according to the year of birth, from birth to 18 months of age are described in Figure 3 and 4, respectively. The year of birth (year effect) did not influence the body development of the animals for the five years ($p > 0.05$).

FIGURE 3 - AVERAGE WITHERS HEIGHT (CM) AND STANDARD DEVIATION OF THOROUGHBRED FOALS ACCORDING TO THE YEAR OF BIRTH, FROM SÃO JOSÉ DOS PINHAIS, PR.



SOURCE: the author.

FIGURE 4 - AVERAGE WEIGHT (KG) AND STANDARD DEVIATION OF THOROUGHBRED FOALS ACCORDING TO THE YEAR OF BIRTH, FROM SÃO JOSÉ DOS PINHAIS, PR.

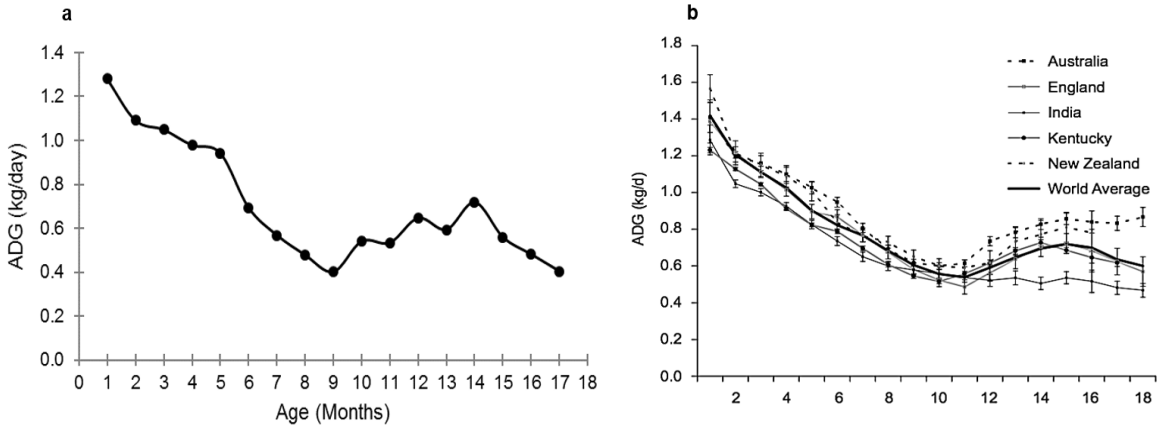


SOURCE: the author.

In Figure 5a, it is shown the average daily weight gain of all Thoroughbred animals from the first measurement up to 18 months. The average daily weight gain (ADG) showed different stages during the development of the animals, being more pronounced in the first month, gaining on average 1.28 kg/day and at 14 months with a gain of 0.72 kg/day. Brown-Douglas et al. (2009) reported that the world average

for the weight gain in Thoroughbred foals was 1.5 kg/day in the first month and 0.7 kg/day in the 13th month (Figure 5b). In India, the average weight gain was 1.3 kg/day in the first month and 0.58 kg/day in the 13th month. Our results suggest that the animals from Brazil showed a weight gain comparable to foals raised in the USA, England, Australia, and New Zealand.

FIGURE 5 - IN (**a**) ARE EXPRESSED THE AVERAGE DAILY WEIGHT GAIN (ADG) OF THOROUGHBRED ANIMALS REARED IN SÃO JOSÉ DOS PINHAIS, BRAZIL, FROM THE FIRST MEASUREMENT UP TO 18 MONTHS. IN (**b**) IT IS SHOWN THE ADG (KG/DAY) ACCORDING TO THE AGE IN MONTHS OF THOROUGHBRED FOALS REARED IN AUSTRALIA, ENGLAND, INDIA, KENTUCKY, AND NEW ZEALAND, INCLUDING WORLDWIDE AVERAGE. SOURCE: BROWN-DOUGLAS ET AL. (2009).



SOURCE: the author (2018) and Douglas et al. (2009).

The height data of the present study shows that the animals in Brazil, in the first month of life, had a mean height of 109.1 cm, similar to foals from Australia (110.9 cm), and superior to horses of other studies (106.1 cm) as measured by Brown-Douglas et al. (2009), which evaluated approximately 13.5 thousand animals from 10 generations. In our study, when foals reached 18 months of life, the animals measured 155.5 cm, similar to Australian animals (156 cm).

4. CONCLUSION

From the results of the present study, it can be verified that females were taller and heavier than males during the whole evaluation period. The month of birth influenced the development of the animals, probably due to a better forage quality during winter for the mares. This study may contribute to establish a body development reference data to the national Thoroughbred Association of Brazil due

to the long experimental period of measurements. In addition, the results indicate that the development of foals in the experimente were similar to those in other countries in different continents.

Acknowledgment

The authors wish to thank the Stud farm team for their technical support and data supply; to Dr. Paulo Guimarães of the Statistics Department of UFPR for their assistance in the statistical analysis; and the support of CAPES for the Dr. of L.L. Dias de Castro and M.Sc. of C.L.H. Abrahão.

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