Chronobiology of *Gasterophilus* infestations in silvopasturing horses from NW Spain


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ABSTRACT

The humoral immune response in horses from NW Spain was evaluated to establish the chronobiology of gasterophilosis. From January 2007 to January 2008 one herd of 13 horses was monthly bled and sera analyzed by an enzyme-linked immunosorbent assay (ELISA) and *Gasterophilus intestinalis* second-stage larvae excretory/secretory antigens. Another group of 12 foals born in April 2007 was also sampled until slaughtered in October. Climatic data were collected from automatic weather stations to establish the pattern in the area of study. The examination of *Gasterophilus* eggs on the hair of the equines and 3rd instars passed by faeces were also monthly done. The kinetics of IgG response decreased from January to July, a slow increment was noted from August to November, and then antibodies increased again to January. Third-instar larvae were observed in the faeces in March-May, and *Gasterophilus*-eggs from June to September. It is concluded that in oceanic climate areas, the egg-laying period occurs from late spring. First-instar larvae are in the mouth in early summer. During summer second-instar larvae move into the stomach and intestine, where third-instar larvae remain until the end of winter, then pupation takes place and the adult horse bots emerge in the spring.

Key words: Silvopasturing horses, *Gasterophilus intestinalis*, oceanic climate, IgG, excretory/secretory antigens, chronobiology.

RESUMEN

En caballos del NO de España se evaluó la respuesta inmune humoral con objeto de establecer la cronobiología de la gasterofilosis. Desde enero de 2007 a enero de 2008, en un grupo de 13 caballos, se tomaron muestras mensuales de sangre, y los sueros se analizaron mediante un inmunoensayo (ELISA) con antígenos de secreción/excreción de larvas de segundo estadio de *G. intestinalis*. También se investigó otro...
INTRODUCTION

Gasterophilosis is a myiasis affecting equid hosts caused by Gasterophilus spp. larvae (Diptera: Oestridae) mainly in the Palaearctic and Afrotropical regions (Colwell et al., 2006). Adult bot flies deposit their eggs on the host’s hair except G. pecorum, as females leave them on the grass (Cogley & Cogley, 2000). When the eggs are introduced into the mouth, the first-instar larvae hatch and moult to L2, which can be present in different regions of the gastrointestinal tract where the L3 remains attached to the mucosa for 8-10 months (Zumpt, 1965).

The clinical signs showed by horses infested by Gasterophilus may include swallowing, gastrointestinal ulcerations, gut obstructions or volvulus, rectal prolapses, anaemia, diarrhoea or digestive disorders (Gökçen et al., 2008). Although only few, there are reports of human myiasis associated with Gasterophilus larvae, causing subcutaneous tinitus or ophtalmomyiasis (Royce et al., 1999; Chen, 2001; Anderson, 2006). Infections by Gasterophilus larvae have also been described in dogs (Taylor et al., 2002).

Gasterophilosis is a myiasis with a wide geographical distribution, as shown by investigations in different countries as Belgium, Turkey, Ireland, France, Germany or Sweden (Colebrook & Wall, 2004; Gökçen et al., 2008), but the highest prevalences were found in warm areas from Italy, Mexico or Brazil (Escartín-Peña & Bautista-Garfias, 1993; Otranto et al., 2005; Rodrigues-Félix et al., 2007).

In previous investigations G. intestinalis and G. nasalis have been the species most frequently reported (Lyons et al., 1994; Sequeira et al., 2001), and the occurrence of infestations by different species of Gasterophilus have also been usually mentioned.

The presence of this myiasis is commonly detected at the slaughter of the horses, or even when L3 instars are observed in the rectum or they are passed by faeces (Gökçen et al., 2008). Another possibility consists of the examination of eggs laid on the hair of the horses (Dawson, 2003). Few studies on the suitability of immunoenzymatic probes for the diagnosis of gasterophilosis have been developed (Escartín-Peña & Basutista-Garfias, 1993; Boulard et al., 1996).

It is noteworthy the difficulty to associate the migration and maturation of stages of the larvae with the clinical signs (Cogley & Cogley, 1999). The absence of information on the humoral immunological response to Gasterophilus complicates the development of serological studies, although the characterization of L2 and L3 proteins of G. intestinalis have been recently reported (Roelfstra et al., 2009).

In the current study, the presence of IgG antibodies against L2 excretory/secretory antigens in horses from NW Spain was analyzed. The climatic pattern was established by collecting different parameters from automated meteorological stations in the study area. The results were discussed together with data from the examination of Gasterophilus.
eggs on the hair of the equines, and 3rd instars passed by faeces.

MATERIALS AND METHODS

Variations on climatic parameters: The current investigation was conducted in Lugo (NW Spain, 42°20'–43°45'N, 6°49'–8°00'W). To establish the climatic pattern in the area where the study was conducted, the values of maximum temperature, minimum temperature, rainfall, and relative humidity were obtained monthly from 32 automatic weather stations.

Horses: Between January 2007 and January 2008, blood samples from two groups of silvopasturing equines in NW Spain were collected. Group 1 was formed by 13 adult horses belonging to the autochthonous Pura Raza Galega (PRG). Group 2 was constituted by 12 PRG foals born in April. These equines were only sampled from April to September, because they were slaughtered in October.

All the animals in each group had the same management conditions. The autochthonous PRGs under silvopasture are grazing on forest areas with natural pastures characterized by annual grass species, and food supplementation is never provided by their owners (Francisco et al., 2009). No veterinary attention is provided. The main benefit afforded is reduction of uncontrolled vegetation, and very few economical benefits can be achieved by meat-production. Most mares foal between April–June.

Direct detection of infestation: The examination of the equines’ hair was monthly done for the visual appraisal of bot eggs attached on the lower legs, lips or jaw. Faeces were also macroscopically examined for the presence of 3rd instars.

Indirect diagnosis of gasterophilosis: The humoral immune response of equines against Gasterophilus was analyzed by an ELISA and G. intestinalis excretory/secretory antigens.

a) G. intestinalis excretory/secretory antigen preparation: The use of excretory/secretory antigens was based on prior reports of successful use of these antigens regarding their immunogenicity (Sánchez-Andrade et al., 2005: Roelfstra et al., 2009).

Briefly, L2 larvae obtained from naturally infected horses at a local slaughterhouse were washed in PBS (phosphate buffered saline, pH 7.2). In the lab, the 2nd instars were incubated in RPMI (Roswell Park Memorial Institute) culture medium at 37°C and 5% CO2, atmosphere for 3 days, with changes every 8-10 hours. Then the medium was collected, dialyzed exhaustively against water, and lyophilized. The protein concentration was estimated by using the BCA® kit (Pierce, Mo, USA).

b) ELISA protocol: ELISAs using excretory/secretory products from L2 G. intestinalis were performed on serum samples (Suárez et al., 2005). The antigen was diluted in phosphate buffered saline (PBS, pH 7.4) to a concentration of 2.5 mg mL⁻¹ to coat the wells of the ELISA plates (Costar, Corning Inc.). Serum samples were diluted 1:250 in PBS containing 0.05% Tween and 1% skim milk, and horseradish peroxidase-conjugated (HRP-conjugated) goat anti-horse IgG (Sigma-Aldrich Co., Madrid, Spain) was used at 1:2500 dilutions. Absorbances were read using a spectrophotometer (Titertek Multiskan, Hæmmelina) at 492 nm.

Pooled sera from 15 uninfested and 27 infested horses were used as negative and positive controls, respectively. Positive control sera belonged to horses with many G. intestinalis larvae when slaughtered. Negative control sera were obtained from yearlings, which had been kept housed since birth to avoid many G. intestinalis infestation.

Statistical analysis: Statistical analysis was conducted using analysis of variance (ANOVA), and differences were considered significant when $P < 0.05$. The Pearson’s correlation test was applied to evaluate the existence of correlation among the different variables considered. All tests were performed by the statistical package SPSS, version 15 (SPSS Inc.).

RESULTS & DISCUSSION

Climatic pattern: The annual average values of maximum and minimum temperature and rainfall
are represented in Figure 1. The values of rainfall ranged from 0.6 (July) to 16 cm (May).

An average value of temperature from 18ºC in August to 5.3ºC in December was observed. The minimum and maximum temperatures exhibited the same pattern. This is typical of an oceanic climate area, and is usually found along the west coasts at the middle latitudes (40°-60°N) of all the world continents and in southeastern Australia; similar climates are also found at coastal tropical highlands and tropical coasts on the leeward sides of mountain ranges (Kottek et al., 2006).

Direct diagnosis: *Gasterophilus intestinalis* third-instar larvae were observed in the faeces of G-1 in March-May, and *Gasterophilus*-eggs from June to September (Figure 1). Sievers & Weber (2005) indicated that the egg laying period occurred when the mean temperatures were over 15ºC, and that it is negatively influenced by rainfall. In the current investigation, these conditions were present from June to August.

**Kinetics of the humoral IgG immune response:** The variations in the IgG kinetics in the equines are represented in Figure 1. The IgG antibodies decreased from January to July, when the lowest values were reached. A slow increment was noted from August, and a plateau level until November was noted in the horses of G-1. The antibodies increased again in January. The IgG response in the foals was detected in September. Significant differences regarding the month were observed in G-1 (F= 13.459, P= 0.001).

Relationship among the IgG humoral immune response, the presence of L3 *Gasterophilus* in faeces and eggs attached to the horse’s hairs: The existence of 3rd instar larvae of *Gasterophilus* in the faeces was matched in March-May, whereas *Gasterophilus*-eggs were observed from June to September (Figure 2). Both *G. intestinalis* and *G. nasalis* 3rd stages were identified and classified according to Zumpt (1965).

After the 3rd instars being matured, they leave the gastrointestinal tract and are voided in the faeces, and then pupate into the soil or dried manure (Cogley & Cogley 2000). Pupation is temperature-dependent, taking 22-28 days at 22-25ºC and 32 days at 18ºC (Edwards, 1982). By considering that adult activity needs for temperatures in excess of 15ºC to fly (Sievers & Weber, 2005), and that rainfall has a negative influence, it seems very reasonable to assume that under the climatic
conditions reflected in Figure 2, pupation ends on June and adults fly from June to August. The finding of *Gasterophilus*-eggs on the horses’ hair between June and September (Figure 1) supports this hypothesis, corroborated by the finding of L3 larvae 8 months later (since March) (Zumpt, 1965). The observation of the lowest IgG values in July reflects that the antigenic stimulus reduced or ended at that period, which is in coincidence with the presence of *Gasterophilus*-eggs fixed to the hairs. It has been described that eggs hatch and the larvae either crawl to the mouth or are ingested and remain in the mouth for approximately 1 month (DuPonte & Larish, 2003).

The first instars molt to 2nd instars and move into the stomach, where they become into third-instar larvae and remain immobile for the following 8-10 months. Roelfstra *et al.*, (2009) reported that the larval stage L2 possesses more antigenic properties and induces a stronger immune reaction in the host than the larval stage L3, probably helpful for larval migration. In the present work, a significant increment in the antibody response was noted from August to reach a plateau level until November. This hypothesis might be supported by the observation of IgG antibodies in September in the foals born in April.

Nevertheless, although L3 stay attached for 8-10 months to the stomach wall, and an hypometabolic status with reduced immunogenic properties has been suggested (Roelfstra *et al.*, 2009), it appears very conceivable that the rising of the IgG response in January herein could be related to the presence of hooked mouthparts and spines in the *Gasterophilus* larvae, responsible for the occurrence of hemorrhages, chronic gastritis, ulcerated stomach or even stomach rupture, which enhance the antigen presentation to the immune system in the horse.

Our results lead us to define the chronobiology of *G. intestinalis* in horses from NW Spain (Figure 2). The cycle begins with the egg-laying period (late spring and summer). The eggs are introduced into the horse mouth, and the L1 burrow into the tongue, and feed there for about 1 month (summer). After wandering in the mucosa of the mouth, these larvae molt to 2nd instars and move into the stomach and intestine (summer-autumn). The second and later third stage larvae attach to the lining of the stomach in the non-glandular portion near the junction of the esophageal and cardiac regions (summer-winter). After the third instars have matured, they detach from the gastrointestinal tract and exit in the feces, where they pupate (spring). The adult horse bot fly emerges after a 1-3 month period (summer).

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