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Exposure to *Gasterophilus* spp. in horses in NW Spain by ELISA

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Abstract
Gasterophilosis is a myiasis related with gastric ulcers in equids and even colics. A serological survey to assess the presence of IgG antibodies against *Gasterophilus* spp. in horses from an oceanic climate area was conducted. A total of 672 blood samples were analyzed by an enzyme-linked immunosorbent assay (ELISA) and excretory/secretory antigens from *G. intestinalis* second-instar larvae. Results were analyzed according to intrinsic and extrinsic factors. Sixty-seven percent of the horses were seropositive. The Arabian Pure Blood and the autochthonous Pura Raza Galega showed the highest and the lowest exposure to horseflies, respectively. Geldings showed the maximum values of seroprevalence (96%), while mares achieved the minimum. The highest percentage of seropositivity was reported in the oldest ones (72%). Significant differences were recorded ($P<0.05$). Our findings underline a high risk of sensitization among horses to bot fly in regions with an oceanic climate.

Keywords: *Gasterophilus*, horse, ELISA, IgG, serological survey

1. Introduction
Infestation by *Gasterophilus* spp. is one of the main myiasis in horses. Although it is known that equid family may be affected by eight *Gasterophilus* species [1]; only *G. intestinalis* (De Geer, 1776) and *G. nasalis* (Clark, 1795) have a worldwide distribution.
Egg laying activity occurs in spring and summer warm days [2], being the highest risk period from the mid-morning to the evening [3]. In previous studies carried out under oceanic climate conditions, it has been concluded that the adult bot fly emerges in the spring and the egg-laying period occurs from late spring [4, 5]. After the ingestion of eggs deposited by the adult flies on the hair, first instars hatch and penetrate into the mouth. Second instars (L2) appear five weeks after infection and go to the stomach and intestine, where L2 molt into third instars (L3) and remain until the end of winter (during 8–10 months) [6]. Finally, L3 go out with the feces and pupation takes place. The presence of hooked mouthparts and spines in the third stage *Gasterophilus* larvae can provoke hemorrhages, chronic gastritis, ulcers or stomach rupture [4], and even colic and fever [7].
Routinely diagnosis of gasterophilosis is performed by visual inspection of larvae at slaughter, the observation of eggs on the hair of the horses or third instars in their rectum or feces. The highest prevalence was obtained in countries with mild climate such as Italy (82.2% *Gasterophilus* spp., 92.5% *G. intestinalis* and 44.8% *G. nasalis*) [8] and Portugal (65.7% *Gasterophilus* spp.) [9]. However the lowest values were obtained in cold countries such as Sweden [10]. Detection of horses naturally infested with gastric bots in slaughterhouse surveys is often expensive and difficult, and usually a large number of animals are necessary before significant data can be achieved [11]. Therefore it is essential to seek other diagnostic methods, as those based in enzyme-linked immunosorbent assays (ELISAs) to estimate the presence of IgG antibodies in serum samples. This non-invasive technique allows the *in vivo* detection of *Gasterophilus* infection.
The usefulness of excretory/secretory antigens from *G. intestinalis* L2 for the detection of infestation by *G. intestinalis* or *G. nasalis* has been formerly reported [4]. Therefore, in the current study a serological survey was conducted to assess the seroprevalence and the risk factor for the presence of IgG antibodies against *Gasterophilus* spp. in horses from an oceanic climate area.

2. Material and methods

2.1 Animals and study area

Blood samples were obtained from 672 horses in Northwest Spain (42°20'–43°45' N, 6°49'–8°00' W). This area has an oceanic climate characterized by mild temperatures and elevated precipitation [12]. According to Kottek *et al*. [13] it could be classified as a type C, subtype Cs and variety Csb climate, characterized by a rainy winter and a dry and slightly warm summer.

Several intrinsic factors of animals were taken into account (breed, age and gender). According to the breed, five groups were established (Table 1): Spanish Sport Horse (SSH), Spanish Pure Breed (SPB), English Pure Breed (EPB), Arabian Pure Breed (APB), autochthonous Pura Raza Galega (PRG) and Crossbreed. By considering the age, horses were divided into three groups; G-1 (< 3 yr), G-2 (3–10 yr) and G-3 (> 10 yr). With regard to the gender, horses were divided into geldings, males and mares. Also, four groups according to housing as extrinsic factor were established, silvopasturing (horses maintained in forests and natural pastures), pasturing (horses focused to the maintenance of small pastures; they always remain in the natural meadows), box + pasturing (horses go to pasture if weather conditions are good) and box (equids stabled and that go out during certain times of day).

Other interesting aspects as treatment history and information on the hygienic conditions of animals were seldom provided by the owners.

2.2 Larvae collection, identification and antigen preparation

Larvae of *Gasterophilus* spp. were recovered from the stomach and intestine of horses slaughtered at a local abattoir located in A Estrada (Pontevedra, Spain) and maintained in phosphate-buffered saline (PBS, pH 7.4). Once in the laboratory, the larvae collected were identified to species-level following morphological keys [1, 14]. In addition, L2 of *G. intestinalis* were selected regarding the size and the shape of the larvae, in particular the shape of the body-spines and posterior respiratory spiracles [1, 15] and washed in PBS prior to the preparation of antigens. With this purpose, L2 were incubated in RPMI (Roswell Park Memorial Institute) medium at 37 °C and 5% CO_2 atmosphere for 3 days [4]. Prior to use, protein concentration of the excretory/secretory antigens (GphiL2ES) was estimated using the bicinchoninic acid method (BCA Protein Assay Reagent; Pierce Biotechnology, Inc., Rockford, IL, U.S.A.).

2.3 ELISA protocol

Detection of IgG antibodies against *Gasterophilus* was done according to Sánchez-Andrade *et al*. [4] The color reaction was stopped by the addition of 100 µL of 3N sulfuric acid and absorbance was recorded using a spectrophotometer (680XR; Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 450 nm. GphiL2ES-ELISA reported 89% sensitivity and 78% specificity [4].

Pooled sera from 12 infested and 8 uninfested horses were used as positive and negative controls, respectively. Positive control sera were obtained from horses harbouring *G. intestinalis* and *G. nasalis* L2 in their digestive tracts at abattoir. Negative controls sera were collected from foals aged 2–3 months old which did not go outside and therefore had a low probability of previous exposure to horse bot fly. The IgG values of negative controls were markedly lower than those of positive sera by ELISA. The cut-off value of absorbance was calculated with the negative sera [4], and positive horses were considered when OD > 0.3262.

2.4 Statistical analysis

The differences in the seroprevalence values were analysed using the chi-squared test and expressed as percentages with the 95% Confidence Interval (CI) [16]. Differences were considered as significant if $P<0.05$. Statistical analysis was performed by using the non-parametric Kruskal-Wallis test. The existence of correlation among the different parameters was assessed by using the non-parametric Spearman's rank correlation test. The relationship between the seroprevalence and the intrinsic and extrinsic risk factors was established by calculating the odds ratio (OR) values [16]. Statistical analyses were performed using IBM SPSS Statistics for Windows Version 20.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1 Overall seroprevalence

The percentage of horses with IgG positive values against GphiL2ES was 67% (95% CI: 63 to 71), in agreement with previous studies performed in the same area [4]. Similar results were obtained in several investigations conducted on slaughtered horses from countries with similar climatic pattern [6, 8, 17, 18]. On the contrary, the presence of *Gasterophilus* spp. was lower in horses slaughtered in Sweden [19].

Serological diagnosis helps to identify animals exposed to myiasis agents, thus avoiding much invasive parasitological diagnosis. Information on equine gasterophilosis by ELISA is sparse, which makes difficult the discussion of the results. It is important to point out that procedures based in the detection of antibodies lead us to establish if an animal has been exposed to a parasite, and often the results cannot be related with the presence of the active infestation at the moment of the collection of the samples [4]. In the current study clinical signs of gasterophilosis were not observed in the horses. Although clear clinical signs are rarely seen during the course of the disease, the parasites may directly or indirectly influence the health and performance of horses [20].

It should be emphasized that *G. intestinalis* and *G. nasalis* are the main species observed in horses [6, 8, 17], so the possibility of mixed infestations in the same animal could contribute to the presence of cross immunity. The use of GphiL2ES was shown to be suitable for detecting infestation by both species, thus avoiding the need for different species-specific antigens [4].

3.2 Intrinsic factors

3.2.1 Breed

The highest percentage of horses with antibodies against *Gasterophilus* spp. was found among the Arabian Pure Breed (APB) individuals, whereas autochthonous Pura Raza Galega (PRG) horses achieved the lowest values (Table 1). Statistical analysis showed significant differences concerning the breed of horses ($\chi^2 = 30.144$; P = 0.001 and $\chi^2 = 40.758$; P = 0.001, respectively). PRG horses are often maintained under areas at high altitude, cold and windy, which seem inappropriate for the presence of the fly. It is known that *Gasterophilus* flies are considered as relatively stenothermal parasites, and matured females need warm, sunny, and windless weather. Moreover, cold climates do not favor the reproduction of the adult flies [21]. Also, other possible explanation to these results could rely on autochthonous horses are agility gregarious specimens, which could avoid oviposition and therefore their infestation by botfly.

A significant highest risk of developing IgG against gastric bots was detected in Arabian Pure Breed (APB) and in Spanish Sport Horses (SSH) (Table 1), i.e. horses dedicated

to sport or leisure. This could be linked to their participation in sportive events held outdoors, where a large number of horses are involved. It has been demonstrated that the *Gasterophilus* flies are guided by smell and different substances in the respiration, sweat or smell could serve as attractants for the adult flies [2].

Table 1: Distribution of samples according to the breed, age, gender and housing of equids from Spain and their effect on the risk of exposure to *Gasterophilus* spp.

		N	Seroprevalence % (95% CI)	OR
Breed	SSH	76	80 (71–89)	2.2
	SPB	56	77 (66–88)	1.72
	EPB	88	74 (65–83)	1.48
	APB	31	97 (91–100)	16
	PRG	247	54 (48–60)	0.42
	Crossbreed	174	66 (59–73)	0.97
	Statistics	$X^2 = 40.758$		$P = 0.001$
Age (yr)	G1 (<3)	105	55 (45–65)	0.56
	G2 (3-10)	422	68 (63–72)	1.12
	G3 (>10)	145	72 (64–79)	1.33
	Statistics	$X^2 = 7.552$		$P = 0.023$
Gender	Geldings	28	96 (90–100)	14.3
	Males	138	69 (61–77)	1.13
	Mares	506	64 (60–69)	0.65
	Statistics	$X^2 = 12.596$		$P = 0.002$
Housing	Box	81	91 (85–97)	5.26
	Box + pasturing	203	58 (51–64)	0.9
	Pasturing	161	87 (82–92)	4.39
	Silvopasturing	227	52 (45–58)	0.64
	Statistics	$X^2 = 82.867$		$P = 0.001$

Abbreviations: SSH, Spanish Sport Horse; SPB, Spanish Pure Breed; EPB, English Pure Breed; APB, Arabian Pure Breed; PRG, autochthonous Pura Raza Galega and Crossbreed.

3.2.2 Age

A significant increment in the seroprevalence with the age of the animals has been recorded (Table 1), with the highest risk of exposure to botflies in the animals of G3 (Table 1). This seems to support the hypothesis that sensitization to horse bot fly increases with the age of the equines and suggests thus a cumulative effect of infestation throughout their life.

Previous investigations were carried out on slaughtered horses from other areas with a similar climatic pattern (Switzerland [3], Belgium [22] and Southern Italy [8]).

Furthermore, several studies showed that younger animals achieved the highest values of prevalence [23, 24] and seroprevalence [5]. This fact may show a low level of protective immunity caused by repeated infections in older horses [25].

3.2.3 Gender

As shown in Table 1, a significantly highest seroprevalence was observed in geldings. These horses are free from tension and anxiety, being more relaxed than stallions and mares and therefore female bot fly can oviposit more easily. Geldings exhibited the highest OR value (Table 1). These results are in agreement with a previous work carried out in Brazil [26] which found higher prevalence in males, as reported Ibrayeva *et al*. [25] in Kazakhstan. By opposite, Agneessens *et al*. [22] and Duque de Araújo [5] pointed that mares reached higher prevalence than stallions. Three investigations conducted in Switzerland, Southern Italy and Turkey showed the absence of relation between the gender and the presence of *Gasterophilus* species [3, 8, 19].

3.3 Extrinsic factors

3.3.1 Housing

The highest percentages of seropositivity were observed in horses maintained in box as well as in pasturing horses, while the lowest values were observed in silvopasturing animals (Table 1). These differences were statistically significant. The OR values was reflected in Table 1.

One surprising result was the finding of highest percentages of stabled horses exposed to botfly. It should be taken into consideration that *Gasterophilus* flies are difficult to see because they live abroad and never come indoors, thus infestation of horses occurs when they are spending time outdoors [2, 4, 22]. It seems more plausible to consider that these horses are spending time outdoors. The possible explanation to these results could be the owners maintain horses in grasslands if weather conditions are favorable, especially in hot seasons as summer and remaining in boxes only for the night or when the weather conditions are unfavorable (rain, cold, etc). In addition, another interpretation could be associated with the aptitude of these animals, because these equids often are dedicated to sport outdoors and they are immobilized with bridles and horsemen, a fact that favors the infestation [27]. The preference of botflies by shady areas has been pointed [2].

Conversely, it is also interesting to underline that silvopasturing horses were less sensitized, in agreement with Francisco [28]. These results confirm those obtained regarding the effect of the breed. In our study zone, a broad majority of silvopasturing horses belong to PRG breed, and they are managed in forests and natural pastures thus receiving an improper handling for the control for parasites [29].

4. Conclusions

In conclusion, a remarkable level of exposure to *Gasterophilus* spp. in horses belonging to a mild climate area has been shown in the present investigation. In view of this, the seroprevalence and the risk of sensitization are influenced by the breed, age, gender and housing of examined horses. It may be suggested that different management procedures regarding the maintenance of horses is one of the main factors influencing the seroprevalence of gasterophilosis in NW Spain. Serological detection of antibodies provides a very helpful and non-invasive

procedure for gaining information on the distribution and importance of horse exposure to *Gasterophilus*. Further studies are in progress to ascertain the relationship between seropositivity and pathological damage.

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