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ORIGINAL PAPER

Gasterophilosis in horses in Sardinia (Italy): effect of meteorological variables on adult egg-laying activity and presence of larvae in the digestive tract, and update of species

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Abstract *Gasterophilus* larvae are common obligate parasites of the digestive tract of the equids. Horses become infected with this parasite by ingesting the larvae hatched from eggs laid by the female flies. In this study carried out monthly, we (i) counted the *Gasterophilus* eggs deposited by female flies on the coat of 30 grazing horses, (ii) counted and identified the *Gasterophilus* larvae retrieved from the digestive tract of 128 slaughtered horses, and (iii) compared these results to meteorological data. Eggs were deposited on all monitored horses, and were present from October to January and from May to September, whereas they were absent from February to April. The number of laid eggs was significantly different between the months, body regions, genders, and age classes ($p<0.05$). Larvae were recovered in 112 (87.5 %) horses, and 6 species of *Gasterophilus* were identified. The prevailing species were *Gasterophilus intestinalis* (recovered in 110 horses; 85.9 %) and *Gasterophilus nasalis* (69 horses; 53.9 %), recovered in all months. *Gasterophilus inermis* (5 horses; 3.9 %), *Gasterophilus pecorum* (3 horses; 2.3 %), *Gasterophilus haemorrhoidalis* (3 horses; 2.3 %), and *Gasterophilus meridionalis* (2 horses; 1.6 %) larvae were also found. Significant differences were found among monthly larval burdens for both *Gasterophilus* spp. and *G. intestinalis* ($p<0.05$), but not for *G. nasalis* ($p>0.05$). Larval burdens and prevalences did not differed significantly between both genders and age classes ($p>0.05$). Monthly eggs and larvae trends were not significantly correlated ($p>0.05$). With regard to the meteorological variables, minimum air temperature was significantly correlated with the eggs trend (rho=1.000; $p<0.001$) and maximum air temperature with the *Gasterophilus* spp. (rho=0.972; $p<0.001$) and *G. intestinalis* (rho=0.972; $p<0.001$) larvae trends. In addition, the number of hours with a temperature below +10 °C was significantly correlated with *G. intestinalis* larvae trend (rho=0.602; $p<0.05$). Our findings confirmed that in Sardinia, Gasterophilosis is an important parasitosis in the horses, and it needs more attention and extensive and/or correct treatment to reduce its prevalence.

Keywords Gasterophilosis · Egg-laying activity · Horse · Sardinia · Italy

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Introduction

The larvae of *Gasterophilus* flies are common obligate parasites that infect the digestive tract of the equids. These larvae cause a myiasis known as Gasterophilosis, "horse bot" or simply "bot," of which the clinical and pathological aspects are well known (Zumpt 1965; Urquhart et al. 1998). Seven of the known eight *Gasterophilus* species infect the horse, whereas one species (*Gasterophilus ternicinctus*) has been recovered only in donkeys and zebras. Currently, Gasterophilosis has a worldwide distribution, and shows prevalences ranged from 3 to 94 %. However, *Gasterophilus* species have different distribution in the world (Bucknell et al. 1995; Gawor 1995; Höglund et al. 1997; Agneessens et al. 1998; Coles and Pearson 2000; Lyons et al. 2000; Sequeira et al. 2001; Otranto et al. 2005; Felix et al. 2007; Gökçen et al.

2008; Anazi and Alyousif 2011; Niedźwiedź et al. 2013; Rehbein et al. 2013). In fact, only *Gasterophilus intestinalis* and *Gasterophilus nasalis* are distributed worldwide, whereas the other *Gasterophilus* species have been reported in more limited areas of the world. In Sardinia (insular Italy), the most recent data showed in horses a prevalence of 85.2 % (Garippa et al. 2004). In this study, *G. intestinalis*, *G. nasalis*, and *Gasterophilus haemorrhoidalis* were the three species recovered.

Horses become infected with this parasite by ingesting the larvae hatched from eggs laid by *Gasterophilus* female flies. The sites on the horse body where female flies deposit their eggs differ among *Gasterophilus* species. *G. intestinalis* female flies deposit their eggs mainly on distal ends of the forelegs, and occasionally on the hindlegs and ventral regions of the abdomen. *G. nasalis*, *G. haemorrhoidalis*, *Gasterophilus inermis*, and *Gasterophilus nigricornis* female flies deposit their eggs on the head, near the mouth. Unlike the other species, *Gasterophilus pecorum* female flies lay their eggs on grass and hay (Zumpt 1965). After ingestion, the first instar larvae (L1) pass through the digestive tract where remain attached to the gastric mucosa up to 10–12 months and evolve up to the third instar (L3). The *Gasterophilus* species differ also for the final localization of the larvae in the digestive tract (Zumpt 1965; Urquhart et al. 1998). *G. intestinalis* third instar larvae attach themselves to the *pars oesophagea* of the stomach. *G. nasalis* and *Gasterophilus meridionalis* third instar larvae are prevalently recovered in the pyloric mucosa and in the first tract of duodenum. *G. haemorrhoidalis* larvae develop to third instar attached to the mucosa of pylorus or duodenum. Subsequently, these larvae leave stomach and duodenum, and re-attach themselves to the rectum mucosa. *G. pecorum* third instar larvae localize themselves in different sites such as pharynx, esophagus, stomach, duodenum, and rectum. *G. inermis* third instar larvae are prevalently found in the rectum (Zumpt 1965; Urquhart et al. 1998).

Gasterophilosis shows a clear seasonality, but studies dealing with the effects of meteorological variables on its biology are quite scarce in the literature (Bucknell et al. 1995; Sequeira et al. 2001; Otranto et al. 2005). In particular, studies that investigated the effects of meteorological variables on the female fly egg-laying activity are lacking.

The aim of this study was to elucidate some aspects of Gasterophilosis in the horses in Sardinia, such as (i) control of the seasonal egg-laying activity, (ii) estimation of the overall prevalence and seasonal presence of the *Gasterophilus* larvae in the digestive tract, (iii) analysis of the effect of meteorological variables on both egg-laying activity and presence of larvae in the digestive tract, (iv) assessment of correlation between the egg-laying activity and the presence of larvae in the digestive tract, and (v) update of the *Gasterophilus* species in Sardinia.

Material and methods

Study design

This study was carried out monthly, over a period of 1 year from October 2005 to September 2006. It comprised two simultaneous steps: (i) control of the eggs deposited by adult *Gasterophilus* female flies on the coat of horses and (ii) control and identification of the *Gasterophilus* larvae retrieved from the digestive tract of slaughtered horses. The data obtained from the control of eggs and larvae were compared to meteorological data.

Egg-laying activity control

The egg-laying activity control was carried out monthly on 30 grazing horses. Age and gender of each horse were recorded. The body of the horses was ideally divided in five anatomical regions: head and neck, thorax, abdomen, foreleg, and hindleg. The eggs control was performed only on the left side of the body. One month prior to starting the control, any *Gasterophilus* eggs eventually present on the coats were eliminated. Monthly, the eggs laid on each body region were counted and their number recorded. After each control, the present eggs were eliminated from the coat in order to facilitate the eggs counting in the next month.

Larvae control and identification

Control and identification of *Gasterophilus* larvae were carried out on the digestive tract of 128 slaughtered native horses. Age and gender of each horse were recorded. No information was available on the history of the horses such as pasture, management, and antiparasitic treatments. The digestive tracts, from esophagus to the rectum, were removed from the carcass, and opened longitudinally along their entire length. The larvae of *Gasterophilus* spp. were harvested, counted, and the species identified according to the morphological keys described by Zumpt (1965).

Meteorological data

The meteorological data were provided by the Regional Agrometeorological Service of Sardinia (S.A.R.). The data, recorded in six meteorological stations situated in the territory of Sardinia, comprised air temperature (minimum, maximum, and mean values), air humidity (minimum, maximum, and mean values), rainfall, global radiation (maximum and mean values), number of hours below +3, +7, and +10 °C, number of hours over +25 °C, and wind at 10 m (mean intensity and mean direction).

Statistical analysis

The data obtained from the egg-laying activity control were summarized as monthly mean±standard deviation (s.d.) by body region, and as overall mean±s.d. by both body region and month. The Friedman test was used to compare the laid eggs between both body regions and months. Post hoc comparisons were performed using the Wilcoxon test corrected by the Bonferroni method.

The data obtained from the control of the larvae in the digestive tract were summarized as both overall and monthly percentage of horses positive to the presence of larvae. Percentages were calculated for both *Gasterophilus* spp. and each *Gasterophilus* species recovered. In addition, overall and monthly larval burdens were calculated as mean±s.d. for both *Gasterophilus* spp. and each *Gasterophilus* species recovered. The Kruskal–Wallis test was used to compare the larval burdens between months for *Gasterophilus* spp., *G. intestinalis*, and *G. nasalis*. Post hoc comparisons were performed using the Mann–Whitney test corrected by the Bonferroni method. Due the insufficiency of data, any statistical comparison was not performed for the other *Gasterophilus* species. The larval burden was calculated according the definition of mean abundance by Bush et al. (1997).

The data from the eggs and larvae controls were also summarized as mean±s.d. by both gender and age class. Moreover, the data from larvae control were calculated as percentage of positive/negative horses by both gender and age class. To verify any significant difference between both gender and age class, the Mann–Whitney and Kruskal–Wallis tests were used for the data of eggs and larvae, respectively. To compare percentages, the Chi-square and Fisher's exact tests were used, as appropriate.

The Spearman's rank test was employed to assess any correlation between the meteorological data and both eggs and larvae monthly means. With regard to the data of larvae, the correlation was assessed only for *Gasterophilus* spp., *G. intestinalis*, and *G. nasalis*, since the data of the other recovered species were insufficient to perform this evaluation. The Spearman's rank test was also employed to assess any correlation between the monthly means of larvae and eggs.

The statistical analysis was performed using S.P.S.S. ver.15.0., and a significant difference was admitted when $p \leq 0.05$, except for the tests used for post hoc comparisons corrected by the Bonferroni method, for which the significance level is specified.

Results

Egg-laying activity control

Of the 30 horses included in this study, 7 were male and 23 female. The age ranged from 1 to 20 years. Eggs were deposited on the coat of all monitored horses. Eggs were found from October to January, and from May to September, whereas they were absent from February to April (Table 1). The Friedman test showed a significant difference between both the months and the body regions ($p<0.05$). Overall, 59.2 and 23.7 % of eggs were laid on the foreleg and thorax, respectively. The Wilcoxon test, used for post hoc comparison between body regions (corrected by the Bonferroni method: $p<0.005$), showed significant differences among body regions, except among the head and neck region and the thorax region. The results of the post hoc comparison between months, by using the Wilcoxon test corrected by the Bonferroni method ($p<0.0007$), are shown in Table 1. Among months, the highest means of eggs were in September, October, and November (60.35±98.3, 158.5±237.0, and 134.9±178.1, respectively). The eggs deposited during these 3 months amounted to 76.3 % of the total laid eggs. The Mann–Whitney test showed that the number of the laid eggs was significantly higher in female horses than in males ($p<0.05$) (Table 2). The Kruskal–Wallis test showed a significant difference among age classes ($p<0.05$). The Mann–Whitney test corrected by the Bonferroni method ($p<0.017$) showed that the number of the laid eggs was significantly higher in >3–≤10 years age class than in the other ones, whereas there was no significant difference between ≤3 and >10 years age classes ($p>0.017$) (Table 2).

Larvae control and identification

In this study, the digestive tracts of 66 male and 62 female horses with age ranged 1 to 29 years were examined. Of the 128 horses examined, 112 (87.5 %) were infected with *Gasterophilus* larvae. A total of 14,901 larvae were collected with an overall mean intensity of 133.04 larvae per horse. The larvae were identified as belonging to six *Gasterophilus* species: *G. intestinalis* larvae were recovered in 110 horses (85.9 %), *G. nasalis* in 69 horses (53.9 %), *G. inermis* in 5 horses (3.9 %), *G. pecorum* and *G. haemorrhoidalis* in 3 horses (2.3 %), and *G. meridionalis* in 2 horses (1.6 %). The highest number of larvae recovered from a horse was 801 (671 *G. intestinalis*, 122 *G. nasalis*, and 8 *G. inermis*) in a 5-year-old female. With regard to the monthly distribution, *Gasterophilus* larvae were recovered in all months. Prevalences and larval burdens are detailed in Table 3. However, solely larvae of *G. intestinalis* and *G. nasalis* were recovered in all months, whereas *G. pecorum* larvae were recovered only in May and June, *G. haemorrhoidalis* larvae

Table 1 Monthly means of eggs laid in each horse body region, and overall means±standard deviation (s.d.) and burden (%) for both each month and each body region

Month	Head and neck	Thorax	Abdomen	Foreleg	Hindleg	Total		
						Mean±s.d.[a]	Multiple comparison[b]	%
October	3.3±9.6	162.0±80.9	99.6±64.8	399.4±208.0	10.4±22.3	134.9±178.1		29.1
November	3.9±7.3	193.0±144.9	140.5±130.0	430.8±355.6	24.3±52.2	158.5±237.0		34.2
December	1.2±2.1	55.6±79.9	32.3±57.8	123.2±194.0	2.2±7.0	42.9±106.1	a, b	9.2
January	1.2±3.3	31.5±63.8	23.4±54.6	61.2±113.0	1.7±7.0	23.8±66.0	c, d, e, f, g	5.1
February	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	h	0.0
March	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	h	0.0
April	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	h	0.0
May	0.0±0.0	0.8±2.7	0.6±1.9	11.2±14.5	0.0±0.0	2.5±7.9	c	0.5
June	0.2±1.1	7.9±8.8	3.4±4.4	70.1±70.2	0.0±0.0	16.3±41.4	a, d	3.5
July	0.2±1.1	16.8±19.1	4.7±5.7	68.0±67.1	0.0±0.0	17.9±40.3	a, e	3.9
August	0.0±0.0	3.7±4.6	1.7±3.0	28.6±349.	0.0±0.0	6.8±18.7	f	1.5
September	0.1±0.7	78.0±72.1	42.9±47.7	180.6±138.0	0.0±0.0	60.35±98.3	b, g	13.0
Total								
Mean±s.d.[a]	0.8±3.9	45.8±87.2	29.1±65.7	114.4±202.7	3.2±17.8	39.7±111.5		
%	0.4	23.7	15.0	59.2	1.7			100.0

Months with same letter were not significantly different ($p>0.0007$)

[a] *s.d.* standard deviation

[b] Performed using Wilcoxon test corrected by the Bonferroni method

only in April and May, and *G. meridionalis* larvae only in July. The Kruskal–Wallis test showed a significant difference among monthly larval burdens for both *Gasterophilus* spp. and *G. intestinalis* ($p<0.05$), but not for *G. nasalis* ($p>0.05$). The Mann–Whitney test corrected by the Bonferroni method ($p<0.0007$) used for post hoc comparison between months showed a significant difference both for *Gasterophilus* spp. and *G. intestinalis* between March and November, and March and September. This test showed a significant difference also between March and July for *Gasterophilus* spp., and between March and October for *G. intestinalis* ($p<0.0007$). With regard to the gender, the Mann–Whitney test did not shown any significant difference among *Gasterophilus* spp. larval burdens ($p>0.05$). In addition, the Chi-square test did not shown any significant difference between prevalences (Chi-square=2.163, d.f.=1,

Table 2 *Gasterophilus* eggs (mean±s.d.), horses positive and negative (number and percentage) to presence of *Gasterophilus* larvae in the digestive tract, and larval burdens (mean±s.d.) regrouped by gender and age class

	Gasterophilus eggs		*Gasterophilus* larvae in the digestive tract			
	Controlled horses (no.)	Eggs laid in the coat (mean±s.d.)	Controlled horses			Larval burden (mean±s.d.)
			Positives [no. (%)]	Negatives [no. (%)]	Total (no.)	
Gender						
Male	7	17.5±63.2	55 (83.3)	11 (16.7)	66	116.4±161.2
Female	23	45.1±121.4	57 (91.9)	5 (8.1)	62	120.0±162.1
Age class						
≤3 years	7	23.7±84.4	50 (84.7)	9 (15.3)	59	118.2±160.8
>3–≤10 years	14	50.4±129.4	44 (89.8)	5 (10.2)	49	118.1±161.9
>10 years	9	32.0±96.0	18 (90.0)	2 (10.0)	20	124.1±170.1

No. number, *s.d.* standard deviation

Table 3 Examined and positive horses, and recovered larvae (number and mean±standard deviation) in total and by *Gasterophilus* species, per month

Month	Examined horses (No.)	*Gasterophilus* spp.			*G. intestinalis*			*G. nasalis*			*G. inermis*			G. pecorum			*G. haemorrhoidalis*			*G. meridionalis*		
		Positive horses	Larvae		Positive horses	Larvae		Positive horses	Larvae		Positive horses	Larvae		Positive horses	Larvae		Positive horses	Larvae		Positive horses	Larvae	
		[no. (%)]	No.	Mean±s.d.	[no. (%)]	No.	Mean±s.d.	[no. (%)]	No.	Mean±s.d.	[no. (%)]	No.	Mean±s.d.	[no., (%)]	No.	Mean±s.d.	[no. (%)]	No.	Mean±s.d.	[no. (%)]	No.	Mean±s.d.
October	20	19 (95.0)	1706	85.7±94.2	19 (95.5)	1115	56.1±66.9	14 (70.0)	591	29.6±38.2	–	–	–	–	–	–	–	–	–	–	–	–
November	22	16 (72.7)	1272	57.9±87.7	15 (68.2)	1107	50.4±72.0	8 (36.4)	165	7.5±18.7	–	–	–	–	–	–	–	–	–	–	–	–
December	6	5 (83.3)	429	71.5±78.6	5 (83.3)	300	50.0±49.7	3 (50.0)	129	3.4±4.0	–	–	–	–	–	–	–	–	–	–	–	–
January	5	5 (100.0)	738	147.6±175.1	5 (100.0)	721	144.2±177.3	3 (60.0)	17	21.5±40.4	–	–	–	–	–	–	–	–	–	–	–	–
February	5	5 (100.0)	1909	381.8±269.9	5 (100.0)	1717	343.4±229.1	3 (60.0)	182	36.4±49.4	2 (40.0)	10	2.0±4.5	–	–	–	–	–	–	–	–	–
March	14	13 (92.9)	4344	309.7±273.0	13 (92.9)	3667	261.4±226.6	11 (78.6)	669	47.8±63.5	1 (7.1)	8	0.6±2.1	–	–	–	–	–	–	–	–	–
April	9	8 (88.9)	570	63.3±55.0	8 (88.9)	438	48.7±45.5	5 (55.6)	130	14.4±19.1	–	–	–	–	–	–	1 (11.1)	2	0.2±0.4	–	–	–
May	13	12 (92.3)	1465	112.7±113.0	12 (92.3)	1200	92.3±110.7	6 (46.2)	258	19.8±36.1	–	–	–	2 (15.4)	4	0.3±0.9	2 (15.4)	3	0.2±0.6	–	–	–
June	8	8 (100.0)	838	104.8±100.9	8 (100.0)	624	78.0±98.3	5 (62.5)	190	23.8±38.1	2 (25.0)	20	2.5±5.3	1 (12.5)	4	0.5±1.4	–	–	–	–	–	–
July	12	9 (75.0)	945	78.8±115.4	9 (75.0)	816	68.0±116.5	3 (25.0)	55	4.6±12.6	–	–	–	–	–	–	–	–	–	2 (16.7)	74	6.2±15.3
August	5	5 (100.0)	284	56.8±49.0	5 (100.0)	279	55.8±48.5	3 (60.0)	5	1.0±1.0	–	–	–	–	–	–	–	–	–	–	–	–
September	9	7 (80.0)	452	44.6±46.0	6 (66.7)	284	31.6±37.6	5 (55.6)	117	13.0±16.4	–	–	–	–	–	–	–	–	–	–	–	–
Total	128	112 (87.5)	14901	117.3±160.5	110 (85.9)	12268	96.6±139.8	69 (53.9)	2508	19.7±35.9	5 (3.9)	38	1.4±3.7	3 (2.3)	8	0.4±1.1	3 (2.3)	5	0.2±0.5	2 (1.6)	74	6.2±15.3

No. number, *s.d.* standard deviation

$p>0.05$). With regard to the age class, the Kruskal–Wallis test did not show any significant difference among *Gasterophilus* spp. larval burdens ($p>0.05$). Consistently with this, the Fisher's exact test did not show any significant difference among prevalences of the age classes ($F=0.678$; d.f.=2; $p>0.05$).

Eleven (9.8 %) of the 128 horses harbored three different *Gasterophilus* species, 58 (51.8 %) harbored two species, and 43 harbored one species. Details of the association between the recovered species are shown in Table 4.

Correlation analysis between meteorological data and egg-laying activity

A significant correlation was found only between the monthly means of *Gasterophilus* eggs and minimum air temperature (rho=1.000; $p<0.001$). The other meteorological variables did not shown any significant correlation with the deposited eggs ($p>0.05$).

Correlation analysis between meteorological data and larval burdens

Maximum air temperature showed a significant correlation with both *Gasterophilus* spp. (rho=0.972; $p<0.001$) and *G. intestinalis* larvae (rho=0.972; $p<0.001$). In addition, the number of hours with a temperature below +10 °C was significantly correlated with *G. intestinalis* larvae (rho=0.602; $p<0.05$). The other meteorological variables did not shown any significant correlation with the larval burdens ($p>0.05$).

Table 4 Associations among the *Gasterophilus* species recovered in the digestive tracts

Species	Positive horses	
	No.	%
G. intestinalis	41	36.6
G. intestinalis+*G. nasalis*	57	50.9
G. intestinalis+*G. meridionalis*	1	0.9
G. intestinalis+*G. nasalis*+*G. haemorrhoidalis*	3	2.7
G. intestinalis+*G. nasalis*+*G. inermis*	4	3.6
G. intestinalis+*G. nasalis*+*G. pecorum*	3	2.7
G. intestinalis+*G. inermis*+*G. meridionalis*	1	0.9
G. nasalis	2	1.8
1 species	43	38.4
2 species	58	51.8
3 species	11	9.8

No. number

Correlation analysis between Gasterophilus larvae and eggs

No significant correlation was found between the monthly means of *Gasterophilus* eggs and larvae ($p>0.05$).

Discussion

Our data showed the occurrence of the egg-laying activity by female bot flies during the most part of the year. In fact, the eggs were recovered on the horses from October to January, and from May to September, and they were absent solely from February to March. The egg-laying activity showed a bimodal trend, with a higher peak from October to November, and a second lower peak from June to July.

Only few studies investigated the bot fly activity by means of a monthly control of the eggs laid on horse coats. In Swisse, Brocard and Pfister (1991) found eggs deposited on horses from July to November, with a peak from September to October. In Delaware (USA), Cope and Catts (1991) observed an increased number of female bot flies from June to September. In Chile, Sievers and Weber (2005), recorded egg-laying activity from November to May. In southern Italy, Otranto et al. (2005) deduced the occurrence of the bot fly activity in summer and autumn from the presence of larvae in the digestive tract. Compared with these previous papers, our data showed a longer period of bot fly activity in Sardinia.

With regard to the body regions interested by egg-laying activity, the forelegs account for 59.2 % of total laid eggs. This is in agreement with previous reports. Brocard and Pfister (1991) found about 70 % of eggs on the medial aspect of carpus, 15 % on the forearm and elbow. Cope and Catts (1991) found 83 % of eggs on the foreleg. Our data confirm the existence of preferred sites for egg deposition by female bot flies (Cogley and Cogley 2000). However, in our study, thorax and abdomen were somewhat involved by the egg deposition, accounting for 23.7 and 15.0 % of the total laid eggs, respectively. This could be explained by the hypothesis that, when the preferred sites have been saturated, female bot flies directed themselves to others body regions (Cope and Catts 1991).

In this study, the amount of eggs laid on the horse coat was significantly different between both genders and age classes. To the best of our knowledge, there are not other studies in the literature that investigated any relationship between the amount of laid eggs and the horse gender, to which compare our results. Conversely, the differences found between age classes are in disagreement with the findings of Brocard and Pfister (1991) and Cogley and Cogley (2000), according to which the female bot flies do not discriminate between horses with different age. However, the differences found in this study may be attributable to different management conditions for both genders and age. In fact, some male horses were more frequently kept indoors. Instead, the females were housed only few days after foaling.

The high prevalence (87.5 %) of horses infected by *Gasterophilus* larvae found in this study confirms the importance of this parasitosis in the horses in Sardinia. Our findings are similar to what was previously reported in Sardinia by Garippa et al. (2004), and in southern Italy by Otranto et al. (2005), which found prevalences of 85.2 and 82.2 %, respectively. In agreement with our findings, high prevalences were found in England (63–100 %; Coles and Pearson 2000), Australia (81 %; Bucknell et al. 1995), and Saudi Arabia (86.6 %; Anazi and Alyousif 2011). Conversely, lower prevalences were found in the most part of European Countries, such as Swisse (65 %; Brocard and Pfister 1991), France (2–34 %; Bernard et al. 1994), Poland (40–47 %; Gawor 1995; Niedźwiedź et al. 2013), Sweden (9.9 %; Höglund et al. 1997), Belgium (58 %; Agneessens et al. 1998), and Germany (2.8 %; Rehbein et al. 2013), and in Countries outside Europe, such as the USA (14 %; Lyons et al. 2000), Brazil (17–32 %; Sequeira et al. 2001; Felix et al. 2007), and Turkey (9.8 %; Gökçen et al. 2008). Low prevalences of infestation by *Gasterophilus* spp. have been attributed mainly to the current extensive use of antiparasitic drugs, which are highly effective against all *Gasterophilus* instars (Sweeney 1990; Lyons et al. 2000; Sequeira et al. 2001; Gökçen et al. 2008; Niedźwiedź et al. 2013; Rehbein et al. 2013).

In agreement with all previous studies, *G. intestinalis* confirms to be the predominant species, and *G. nasalis* the second prevailing species, both with a worldwide distribution. In addition, we identified the highest number of species found in a study, compared to previous studies including the most recent one carried out in Sardinia. In fact, in this latter, only *G. intestinalis*, *G. nasalis*, and *G. haemorrhoidalis* were found (Garippa et al. 2004). Our findings are similar only to those of Otranto et al. (2005), which found five species of *Gasterophilus* in horses in southern Italy. In particular, it is noteworthy that we found *G. meridionalis*, a species described by Zumpt (1965) but unreported in the subsequent studies. Thus, *G. meridionalis* appear to be present from several decades only in Sardinia. The low prevalence of the species other than *G. intestinalis* and *G. nasalis* can be explained on the one hand by a likely tendency toward an extinction of these species of *Gasterophilus*, in agreement with Otranto et al. (2005), and on the other hand by their persistence in sites where the pressure of competition is lower. This latter is suggested by the fact that *G. meridionalis* larvae were found attached to the pyloric mucosa, which is the same site of localization of *G. nasalis* larvae, in a horse where these latter were not present. Thus, we could speculate that *G. meridionalis* larvae can survive in horses when *G. nasalis* larvae are absent. This is also corroborated by the fact that, although more species were found in the same horse, larvae of different species were never found mixed together. In addition, also an insufficient use of antiparasitic drugs in grazing horses could have promoted the persistence of these species.

In the present study, only *G. intestinalis* and *G. nasalis* larvae were found throughout the observation period. *G. intestinalis* larvae showed a bimodal trend, with the higher peak from February to March, and the lower peak in May. *G. nasalis* larvae showed a trend with three distinguishable peaks: the highest peak from February to March, the lowest one in June, and a third peak of intermediate size in October (Fig. 1). For the other species, it was not possible to draw a trend. However, the findings of previous studies showed differences depending on different climatic conditions. Our findings are similar to those of Otranto et al. (2005) in southern Italy, which found *G. intestinalis* and *G. nasalis* third instars larvae throughout the observation period. In addition, in this latter study, both *G. intestinalis* and *G. nasalis* larvae showed a bimodal trend, with the highest peak from January to August, and the lowest one from September to November. In Victoria (Australia), Bucknell et al. (1995) found larval burdens higher in winter than in summer and autumn. In Sweden, Höglund et al. (1997) found the highest prevalence and intensity of larvae from October to November, whereas larvae were absent between early June and late September. In Kentucky (USA), Lyons et al. (2000) found higher burdens of *G. intestinalis* second instars larvae from November to December, and of third instars larvae from May to June and in December. In Brazil, Sequeira et al. (2001) found the major occurrence of *G. nasalis* larvae in winter. Our findings suggest that *G. intestinalis* and *G. nasalis* larvae leave the digestive tract prevalently in April.

The mean burden found in this study was similar to that found by Otranto et al. (2005) in southern Italy, and by Bucknell et al. (1995) in Victoria (Australia), whereas it was higher than that found by Höglund et al. (1997) in Sweden, Agneessens et al. (1998) in Belgium, and Felix et al. (2007) in Brazil.

In this study, we found no significant differences between both prevalences and larval burdens among genders and age classes. This is consistent with the most previous studies (Bucknell et al. 1995; Höglund et al. 1997; Agneessens et al. 1998; Otranto et al. 2005; Gökçen et al. 2008; Anazi and Alyousif 2011). Conversely, some authors found differences between genders and/or age groups. Felix et al. (2007) found that males were more parasitized than females, whereas Agneessens et al. (1998) and Niedźwiedź et al. (2013) found that mares were more frequently infected than stallions and geldings. However, according to Agneessens et al. (1998), the difference between genders is not likely to be genetically determined, but it may be a reflection of different management conditions. Bucknell et al. (1995) found that *G. nasalis* was less prevalent in horses under 2 years of age than in older ones,

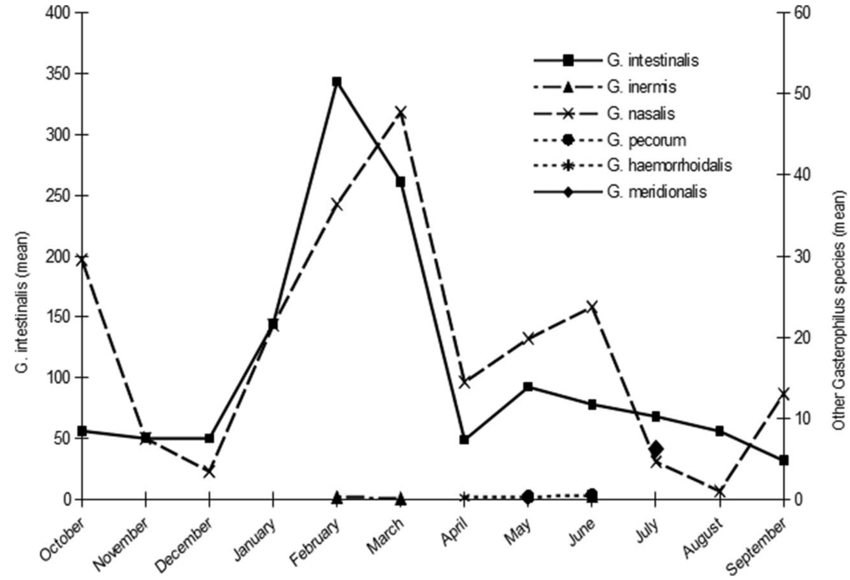

Fig. 1 Monthly trends (means) of the larvae of *Gasterophilus* species

whereas they did not found a difference for *G. intestinalis* prevalence.

Our results showed that the egg-laying activity was significantly correlated only with minimum air temperature. However, the egg-laying activity appeared the highest when mean air temperature ranged from 13.7 to 17.5. This reduced drastically until to cease when minimum air temperature fell below 10 °C, and reappeared when minimum air temperature went up to above this value.

The mean larval burdens of both *Gasterophilus* spp. and *G. intestinalis* were strongly correlated with maximum air temperature. They showed the highest value during the colder months (January to March), when mean air temperature ranged from 8 to 12 °C. Our findings suggest that annual climatic changes affect the biological cycle of *Gasterophilus* spp. This is consistent with results from other studies. Sweeney (1990) reported that a minimum temperature of 12.8 °C is apparently required before *G. intestinalis* is capable of flying, and temperatures greater than 15.5 °C are required before ovisposition can occur. Sievers and Weber (2005) found that egg-laying activity coincided with a mean temperature higher than 15 °C. Cope and Catts (1991) observed the egg-laying activity mainly during warm and sunny days, when temperature was of 24–27 °C, whereas it was absent during

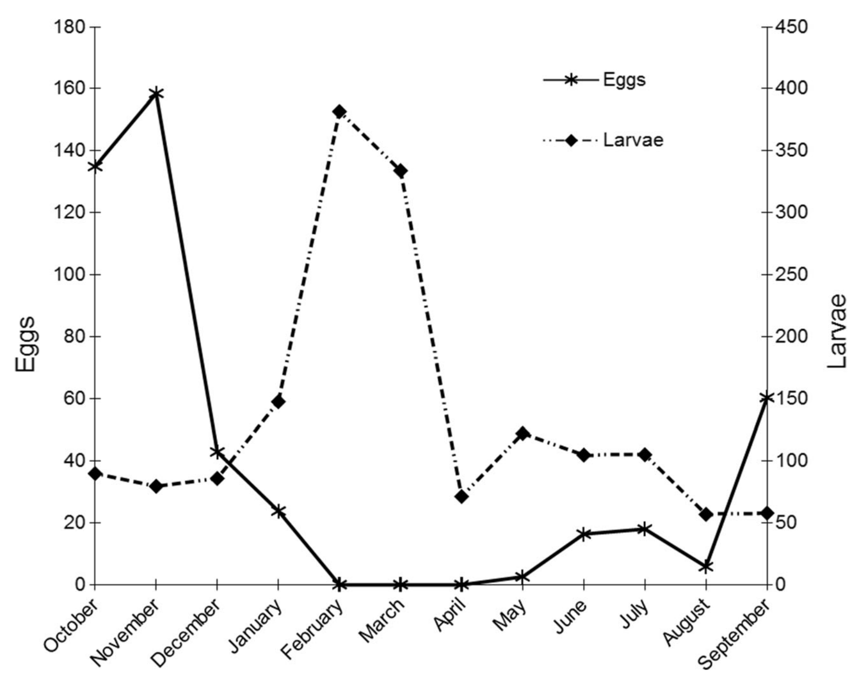

Fig. 2 Monthly trends (means) of *Gasterophilus* eggs and larvae

colder days (<21 °C). Gökçen et al. (2008) supposed a fly activity throughout all seasons in Turkey, where winter temperatures average 10–15 °C in January, and reach above 35 °C from May to September. Sequeira et al. (2001) in Brazil and Anazi and Alyousif (2011) in Saudi Arabia found that *G. nasalis* larvae were more prevalent during the cool and dry months, but this was not found for *G. intestinalis* larvae.

In Victoria (Australia), Bucknell et al. (1995) found a significant effect of seasonal rainfall on the infection prevalence. In Chile, Sievers and Weber (2005) showed that the egg-laying activity was reduced by rainfall in November and December. These findings were not confirmed in our study.

Although the monthly means of *Gasterophilus* eggs and larvae were not significantly correlated, overlapping the eggs and larvae trends the highest peak of the larval burden coincides with the period of absence of eggs on horse coats, and, in turn, the highest peak of eggs coincides with the period of the lowest larval burdens (Fig. 2). The highest peak of eggs anticipates of 4 months the highest peak of larvae. The dramatic decrease of the larvae in April anticipates of 1 month (corresponding to the mean duration of the pupal period), the restarting of the egg-laying activity.

The eggs and larvae trends would suggest the presence of two cohorts of flies also in Sardinia, in agreement with the findings of Otranto et al. (2005). However, in the literature, the presence of more than one cohort of flies per year is controversial. According to some authors, *G. intestinalis* and *G. nasalis* flies have only one generation per year (Sweeney 1990; Brocard and Pfister 1991). Alternatively, or in addition, to the presence of two cohorts of adult flies, others authors suppose the occurrence of an overwintering phase of dormant pupae in the soil (Otranto et al. 2005). According to Sweeney (1990), instead, there is no evidence of the overwintering of pupae. In agreement with this, our results did not support the occurrence of an overwintering phase of dormant pupae, since the highest larval burdens were recovered in winter. In fact, our results suggest that *G. intestinalis* and *G. nasalis* overwinter as third instars larvae in the digestive tract.

As mentioned above, several authors attributed the reduction of the prevalence of Gasterophilosis to the extensive use of antiparasitic drugs with larvicidal activity, such as ivermectin and moxidectin. Therefore, the high prevalence of Gasterophilosis found in our study would suggest that in Sardinia the treatment of this parasitosis is not extensive, or not correctly applied. This needs to be further elucidated. The frequency per year of the treatments against *Gasterophilus* spp. depends on the biologic cycle. Our results suggest that in Sardinia the treatment should be administered in the period from December (when the egg-laying activity begins to decrease) to March (corresponding to the highest peak of larvae in the digestive tract). A second treatment may be administered in the period between May and July, corresponding to the second peak of the larvae in the digestive tract. In this second period, the egg-laying activity is low.

Since first instars larvae are able to survive up to several months inside the eggs, depending on the environmental conditions (Zumpt 1965), the eggs represent a source of infection for the horses, even when the egg-laying activity is over. Therefore, it is advisable to eliminate the eggs from horse coats in order to prevent any re-infestation. We easily eliminated the eggs by use of a knife with smoothed edges. Urquhart et al. (1998) suggest sponging down the coat with hot water (about 40 °C).

Conclusions

In Sardinia, Gasterophilosis is confirmed to be an important parasitosis in the horses. With six species found in this study in Sardinia, and five species found in the Apulia region, Italy prove to have the highest biodiversity of this genus. Furthermore, *G. meridionalis* appears to be currently present only in Sardinia. The data arising from the control of both the eggs in the horse coat and the larvae in the digestive tract suggest the occurrence of two cohorts of adult flies during the year. In addition, these data suggest that *Gasterophilus* spp. overwinter as larvae in the digestive tract. Among the meteorological variables, only air temperature showed to affect both the egg-laying activity and the presence of larvae in the digestive tract. However, this would need further investigations in order to better understand it. Finally, since the high prevalence of Gasterophilosis found in this study suggests that anthelmintic drugs are not extensively or correctly used, it is advisable to enhance the awareness of the horse farmer about the importance of this parasitosis.

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Ethical statement This study complies with the current laws of both the Italian Republic and the European Union, and it was performed in accordance with the national guidelines for animal welfare.

Conflict of interest The authors declare that they have no conflict of interest.

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