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Stabling is associated with airway inflammation in young Arabian horses

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Summary

We examined the effect of stabling on upper and lower airway inflammation in 14 yearling Arabian horses that had been at pasture since birth. Horses were divided into 2 groups of 7. One group was stabled for 3 months and the other remained at pasture. The groups were then switched over for another 3 months. The nasopharynx, guttural pouches and trachea were examined endoscopically and bronchoalveolar lavage performed every month. An upper airway inflammation score was devised based on the magnitude of pharyngeal lymphoid hyperplasia and guttural pouch inflammation. During stabling this score remained constant, whereas it decreased during the 3 months at pasture. Stabling was also associated with a higher number and percentage of neutrophils in bronchoalveolar lavage fluid and with a smaller percentage of lymphocytes. There was no correlation between upper airway inflammation score and bronchoalveolar lavage cytology. During a nasal occlusion test, dorsal displacement of the soft palate occurred more times in stabled than in pastured horses, but this was heavily biased by the results from one animal. We conclude that stabling is associated with inflammation of both the upper and lower airway of young horses.

Introduction

Stabled horses can be exposed to very high levels of organic dusts that contain a variety of moulds and other components capable of inducing airway inflammation (Woods *et al.* 1993; McGorum *et al.* 1998). The relationship between stabling, hay feeding and respiratory disease has been recognised for at least 300 years. Markham (1656) wrote “the best cure [for broken wind] is grasse in summer and hay sprinkled with water in winter.” It is now very clear that acute exacerbations of airway obstruction can be induced in heaves-susceptible horses and ponies by stabling and exposure to hay dust, and airway function improves rapidly when dust levels are reduced (Lowell 1964; Derksen *et al.* 1985a; Tesarowski *et al.* 1996; Vandenput *et al.* 1998; Jackson *et al.* 2000). In these heaves-susceptible animals, neutrophils begin to accumulate in the lung within 6 h of stabling (Fairbairn *et al.* 1993) and the bronchoalveolar lavage fluid can contain more than 50% neutrophils (Derksen *et al.* 1985b; McGorum *et al.* 1993).

A less severe form of airway disease occurs in young racehorses in training. This inflammatory airway disease (IAD) occurs in 25–30% of horses in training (Sweeney *et al.* 1992; Moore *et al.* 1995) and bouts can last for 3 or 4 months (Moore *et al.* 1995; Burrell *et al.* 1996). Little is known about the pathogenesis of IAD. While an association between bacterial infection and IAD has been described (Burrell *et al.* 1996), environmental exposure may also play a major role in this syndrome. Horses kept on straw are twice as likely to suffer from IAD compared to horses that are kept on shredded paper, and recover more slowly from airway inflammation (Burrell *et al.* 1996).

Young horses in training frequently have upper airway inflammation (UAI) that is characterised by pharyngeal lymphoid hyperplasia, pharyngeal oedema and erythema. The aetiology and pathogenesis of this condition is unknown. For example, the association between IAD and UAI has not been investigated. The role of environmental factors in UAI is also unknown.

Our group has suggested recently that inflammation of the upper airway may predispose horses to developing dorsal displacement of the soft palate (DDSP) (Holcombe *et al.* 1998). The nerves that control the muscles of the soft palate travel through the guttural pouch and are intimately associated with the retropharyngeal lymph nodes (Hare 1975). These nerves eventually ramify in the pharyngeal plexus of nerves in the dorsal wall of the nasopharynx. Inflammation in these structures could affect neural function.

In this study we addressed the following questions: 1) Is stabling associated with inflammation of the upper and lower airway? 2) Do upper and lower airway inflammation occur concurrently? 3) Is a clinical mucopus score correlated with the severity of neutrophilic inflammation in the lower airway? 4) Is inflammation of the upper airway associated with DDSP?

Materials and methods

Horses

Fourteen 18-month-old Arabian horses (7 mares and 7 geldings) were used in the study. The study extended from October 1998 to April 1999. The All-University Committee for Animal Use and Care approved the experimental protocol. All horses had been kept on a pasture at the Michigan State University horse farm since weaning. All were vaccinated against tetanus, equine influenza, EHV-1 and -4 and eastern and western equine encephalomyelitis in March 1998. The horses were treated with either ivermectin,

TABLE 1: Total and differential cell counts, mean (± s.e.) in bronchoalveolar lavage fluid of 14 yearling Arabian horses during 3 months at pasture and in a stable

	Pasture			Stable		
	Month 1	Month 2	Month 3	Month 1	Month 2	Month 3
Total cells/µl	85.2 (10.2)	93.6 (12.8)	103.2 (16.0)	93.4 (13.5)	98.8 (9.7)	74.5 (7.8)
Total neutrophils/µl	2.5 (0.4)	3.2 (0.7)	4.9 (1.7)	10.2 (1.8)	5.7 (1.8)	9.1 (2.3)
Total lymphocytes/µl	36.1 (4.3)	38.4 (5.0)	48.8 (9.9)	32.3 (6.2)	36.1 (4.0)	28.8 (3.5)
Total macrophages/µl	35.3 (5.7)	40.8 (7.4)	38.9 (4.8)	37.5 (5.0)	44.3 (6.6)	31.6 (5.0)
Total mast cells/µl	6.2 (1.3)	4.7 (1.3)	5.8 (1.5)	3.7 (0.8)	4.1 (0.8)	2.9 (0.9)
Total eosinophils/µl	0.7 (0.3)	0.7 (0.7)	0.5 (0.3)	2.1 (1.2)	0.5 (0.3)	0.4 (0.2)
Total epithelium/µl	6.0 (1.2)	10.0 (2.7)	5.9 (1.6)	10.0 (3.7)	10.6 (2.4)	3.6 (1.2)
Percent neutrophils	3.6 (0.8)	3.6 (0.9)	3.7 (1.1)	12.1 (2.1)	5.7 (1.4)	13.2 (3.0)
Percent lymphocytes	42.8 (2.4)	42.4 (3.4)	42.6 (3.4)	35.0 (3.1)	36.9 (2.5)	39.1 (2.3)
Percent macrophages	39.5 (2.6)	41.9 (2.6)	41.6 (3.5)	41.1 (2.7)	42.9 (2.4)	40.1 (2.7)
Percent mast cells	8.3 (1.7)	5.5 (1.4)	5.9 (1.0)	4.7 (1.0)	4.3 (0.8)	4.1 (1.3)
Percent eosinophils	0.8 (0.4)	0.8 (0.8)	0.4 (0.2)	2.5 (1.4)	0.4 (0.2)	0.6 (0.2)
Percent epithelial cells	7.2 (1.0)	10.4 (2.0)	6.8 (2.2)	8.1 (2.5)	11.9 (3.0)	5.1 (1.7)

fenbendazole or pyrantel every 8 weeks. At the start of the study, none of the horses had overt signs of respiratory or other diseases.

For the study, we compared maintaining animals at pasture to stabling. The pasture was 4 hectares with a 3-sided shed to provide shelter from the weather. The shed had a gravel floor. Horses were fed mixed grass and alfalfa hay in a rack that was out in the open. Horses were fed approximately 3 kg hay/horse daily. A mixture of oats, corn and protein supplement was fed in a trough. Heated automatic vessels provided water *ad libitum*.

The stable was in the lower level of a barn with hay storage above. The ceiling of the stable was closed to ensure that hay dust did not come through from above. The outer walls were built of fieldstones (cobbles). Each stall was 3.7 x 4.6 m and was separated from its neighbour and the aisle by partitions that were solid wood to 1.1 m high and continued as bars to the ceiling. Mixed grass and alfalfa hay was fed in racks 1.2 m from the ground. The same hay was fed to the horses at pasture and to the stabled horses. A mixture of oats, corn and protein supplement was fed in a feeder 0.9 m from the ground. The feeder was directly below the hayrack. Heated vessels provided water *ad libitum*. Horses were bedded on straw. The aisle was 4.6 m wide. The sliding door between the aisle and the outdoors was kept closed except during morning cleaning. Horses were fed twice daily in the stable and on the pasture. No analysis of feed or bedding was performed. Neither the stabled nor the pastured horses came into contact with any other horses during the course of this study.

Experimental design

Horses were divided into 2 groups of 7. One of the groups was stabled and the other kept on pasture. At the end of 3 months, the groups were switched between pasture and stable and were followed for another 3 months.

Upper and lower airway examinations were performed at the end of each month on every horse on the same day. Horses were restrained in a set of stocks and a lip twitch applied. Endoscopic examination of the nasopharynx and larynx was performed without tranquilisation. As part of the endoscopic examination, the nares were occluded for as long as the horse would tolerate. During this nasal occlusion test we watched for dorsal displacement of the soft palate. Horses then received xylazine (0.3 mg/kg bwt i.v.) and butorphanol (0.01 mg/kg bwt i.v.) and endoscopic examination of the right and left guttural pouches and trachea was performed. All examinations were videotaped.

A bronchoalveolar lavage was performed to assess lower airway inflammation, using a 300 cm, 10 mm diameter BAL catheter with an inflatable cuff passed via the nose and wedged in a peripheral bronchus. Three hundred ml phosphate buffered saline was infused in 100 ml aliquots and recovered manually.

Scoring upper airway inflammation and function

Two independent observers (S.J.H. and N.E.R.) examined all videotapes of upper airway examinations and assigned each horse an upper airway inflammation score for each month. One of the observers was blinded as to the housing status of the animal and one was not. Horses received 1–4 points for the degree of pharyngeal lymphoid hyperplasia (Baker 1987). Inflammation of the guttural pouch was scored as follows: multiple lymph follicles on the floor of the medial compartment, 1 point (Fig 1); enlarged retropharyngeal lymph nodes, 1 point (Fig 2); hyperaemia of the guttural pouch membrane, 1 point and oedema of the guttural pouch membrane or exudate within the pouch, 1 point. A horse could earn up to 4 points for the degree of pharyngeal lymphoid hyperplasia, and 4 points for each guttural pouch. Therefore, the highest potential upper airway inflammation score was 12 points. The presence of food material in the nasopharynx was also noted.

Scoring tracheal mucus

The amount of mucus visible in the trachea was graded on a scale of 0 (no visible mucus), 1 (singular small blobs), 2 (multiple blobs only partly confluent), 3 (mucus ventrally confluent), 4 (large ventral pool) and 5 (profuse amounts of mucus occupying more than 25% of tracheal lumen), similar to that used by Dieckmann (1987) and Dixon *et al.* (1995). One observer (V.G.) graded all mucus scores based on ‘blind’ examination of the videotapes.

Fig 1: Endoscopic image of the floor of the medial compartment of the right guttural pouch. Note multiple enlarged lymph follicles.

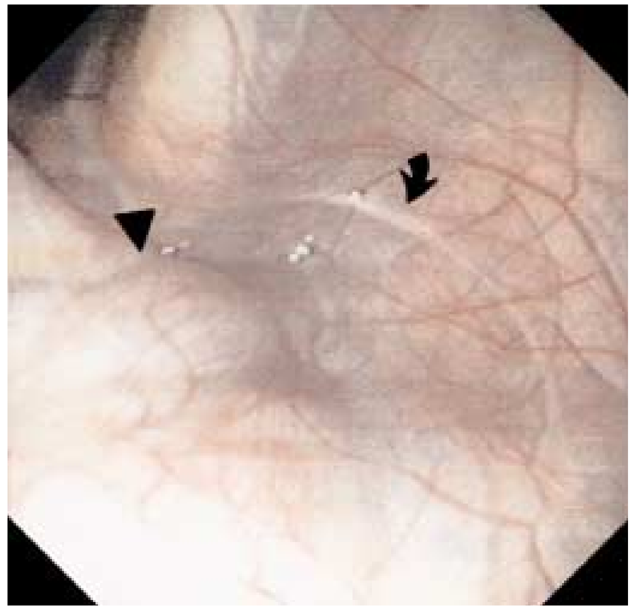


Fig 2: Endoscopic image of the floor of the medial compartment of the right guttural pouch. Note the enlarged retropharyngeal lymph nodes (arrow) and the pharyngeal branch of the vagus nerve (curved arrow).

Examination of bronchoalveolar lavage fluid

The volume of BAL fluid (BALF) recovered was recorded. Total nucleated cell counts were determined by use of a haemocytometer. Differential cell counts were determined by examination of 200 consecutive leucocytes on a cytological preparation made on a cytocentrifuge and stained with Diff-Quick.

Statistical analysis

Results of BALF cytology were examined by repeated measures ANOVA with time and environment (pasture or stable) as the main effects. The interobserver agreement in scoring upper airway inflammation was tested using the Spearman rank order correlation. For upper airway inflammation, the scores of the 2 observers were averaged and the effect of environment and time analysed using Friedman's repeated measures ANOVA on ranks. The latter test was also used for tracheal mucopus scores. Pairwise multiple comparisons were made using the Student-Newman-Keuls method, where appropriate. The number of episodes of DDSP that occurred while horses were at pasture and in the stable was compared by Fisher's exact test. The correlation between upper airway scores, BALF cytology and mucopus scores was evaluated by the Spearman rank order correlation. A significance level of $P<0.05$ was chosen. Statistical analysis was conducted with Sigma Stat for Windows, version 1.03[1].

Results

Lower airway inflammation

Results of BALF analysis are shown in Table 1. Repeated measures ANOVA demonstrated a significant ($P<0.05$) effect of environment on total and percent neutrophils and on percent lymphocytes (Fig 3a,b). There was no effect of month of sampling and no interaction between month and environment. Main effects of pasture and stable are shown in Figure 4. Stabling was associated with a higher total neutrophil count in BALF, a greater percentage of neutrophils and a lower percentage of lymphocytes. No other cells were affected significantly by stabling, but there was a strong trend toward a decrease in total mast cells ($P = 0.08$), mast cell percent ($P = 0.06$) and total lymphocytes ($P = 0.06$). We also determined the highest percentage of neutrophils for each horse during its time at pasture and in the stable. In all horses, the highest percentage occurred during stabling. The rank sum test demonstrated a significantly higher percentage (mean ± s.d. 18.6 ± 9.5%) when horses were stabled than when they were at pasture (5.9 ± 3.9%).

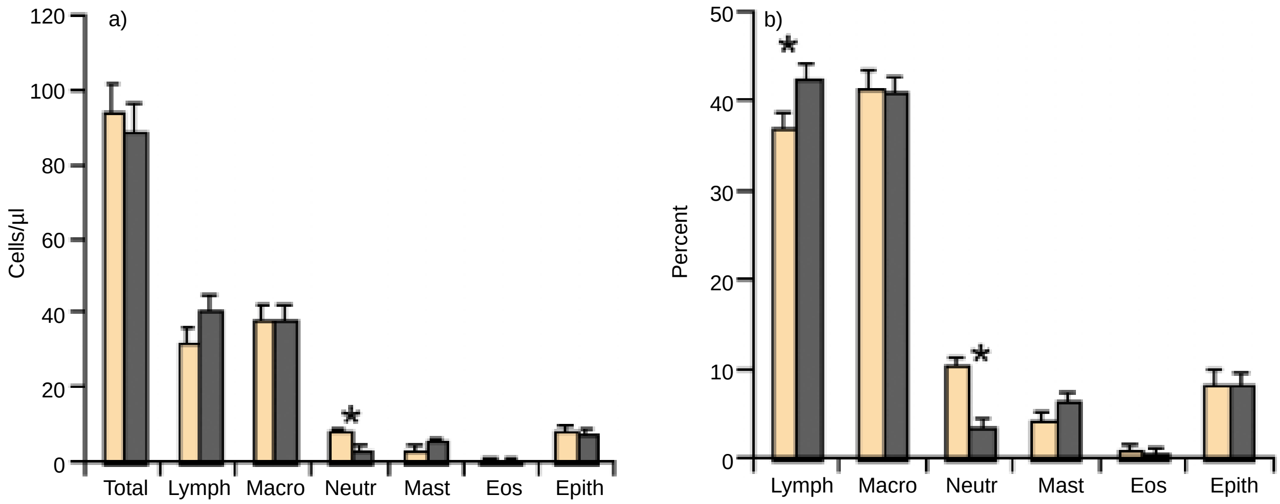
Mucus score

Mucus scores were generally low. Of 84 total scores assigned over the 6 months of the study, 73 scores were ≤2. Both time and environment had a significant effect on tracheal mucus score. However, contrary to expectations based on the neutrophils in BALF, the mucus scores were higher in horses at pasture than when they were stabled ($P<0.05$). Mean ± s.e. scores in months 1, 2 and 3 were 1.9 ± 0.3, 1.1 ± 0.2 and 1.8 ± 0.3, respectively, in horses at pasture and 0.6 ± 0.2, 0.6 ± 0.2 and 1.0 ± 0.3, respectively, in the stable. There was no correlation between mucus score and either percent or total neutrophils in BALF.

Upper airway inflammation and function

All the young Arabian horses, except 2, tolerated the monthly upper airway examinations very well without sedation. In the 2 horses that had to be sedated, we did not perform the nasal occlusion test. The nasal occlusion test was of variable duration (10–60 s) depending on the horse's temperament and the month of the examination. Specifically, the horses became more compliant with the nasal occlusion test as the investigation progressed.

There was a high correlation between the 2 observers scoring



*Figs 3a and b: Effect of stabling and pasture on bronchoalveolar lavage cytology. a) Absolute cell numbers. b) Differential cell count. Light bar = stable; dark bar = pasture. Mean ± s.e.m. * = Significant difference between stable and pasture (P<0.05).*

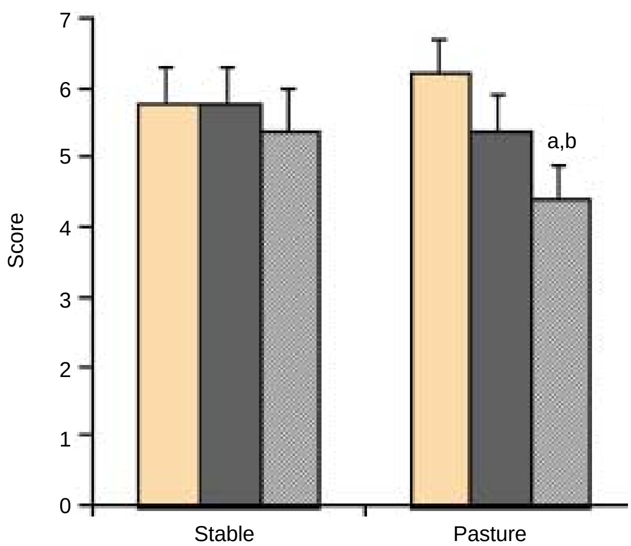


Fig 4: Effect of stabling and pasture on upper airway inflammation score. Data are shown for months 1, 2 and 3. Mean ± s.e. a = different from all stable periods. P<0.05. b = different from pasture, months 1 and 2.

the upper airway inflammation (r_s = 0.88). Upper airway inflammation scores during stabled months 1–3 were all significantly higher (P<0.05) than scores during the 3rd month at pasture (5.8 ± 0.5, 5.8 ± 0.5, 5.4 ± 0.6 vs. 4.4 ± 0.5). Upper airway inflammation scores also were significantly higher during pasture months 1 and 2 compared to pasture month 3 (6.2 ± 0.5, 5.4 ± 0.5 vs. 4.4 ± 0.5). Therefore, upper airway inflammation scores decreased while horses were at pasture. Because there was no washout period between treatments, it is possible that stabling affected the inflammation scores of the pastured horses and that 3 months at pasture influenced the upper airway inflammation scores of the stabled horses. We were not able to prove this effect of time statistically, although there was a trend (P = 0.06). There was no correlation between upper airway inflammation score and BALF cytology or mucopus score.

During nasal occlusion, the soft palate displaced in 6 of 36 trials (17%) while horses were stabled compared to 1 of 36 trials (3%) at pasture. During stabling, 3 of the episodes of displacement occurred in one horse. This horse had a small chondroid in the left guttural pouch for the first 2 months of the study, and also had the highest upper airway inflammation scores of all the horses throughout the trial. Although there was a trend (P = 0.059) to suggest that horses displaced their soft palate more frequently while in the stable compared to the pasture, this result was heavily influenced by the data from the horse that displaced for 3 consecutive months in the stable. Horses in the stable were more likely to have food particles within the nares and nasopharynx. None of the pastured horses had food material in their upper airways. This was probably because horses in the stable ate their hay from racks elevated 1.2 m from the ground, whereas pastured horses ate their hay from a rack positioned on the ground, so that their head was not elevated during eating.

Discussion

When horses are stabled and fed hay, they are in an environment in which human subjects would not be allowed to work (Schenker *et al.* 1998). Dust levels in the breathing zone around the muzzle vary with the type of feed, from less than 1 to as high as 25 mg/m^3 of air (Woods *et al.* 1993; McGorum *et al.* 1998; Vandenput *et al.* 1998). Dust levels of 10 mg/m^3 are known to be associated with a high incidence of bronchitis in man (Morgan 1982). Organic dust contains antigens, endotoxin and a variety of particulates. These components are capable of initiating airway inflammation and together may work synergistically to induce airway inflammation in the horse.

The results of the present study add to the accumulating data that demonstrate an association between stabling and airway inflammation, even in horses that have no previous history of heaves (also known as recurrent airway obstruction [RAO] or chronic obstructive pulmonary disease [COPD]). When our group of young horses were stabled, there was an increase in the number of neutrophils in the BALF and this led to an increase in the neutrophil percentage. Furthermore, the highest neutrophil percentage in BALF was associated with a period of stabling in every horse. While the percentage of neutrophils in our stabled horses was much less than that seen in heaves-affected horses, the neutrophil percentage was frequently greater than 10%. We are not the first to observe a higher level of neutrophils in stabled healthy horses. Other investigators studying heaves have noted an increase in BALF neutrophils when their control populations were stabled, even though the change was not statistically significant (Derksen *et al.* 1985b; Tremblay *et al.* 1993). The greatest percentage (mean ± s.e. 27.6 ± 7.8) was noted by Tremblay *et al.* (1993). Neutrophil percentages such as we observed have been associated with a diagnosis of COPD (Dixon *et al.* 1995). However, none of our horses showed any overt signs of respiratory disease during the examinations and the clinical significance of these elevated levels of neutrophils is unclear. Our results suggest that influx of neutrophils into airways occurs in healthy animals as a defence reaction to dust exposure in stabling.

An increased number of neutrophils in BALF coupled with other signs of respiratory disease, such as poor performance, is the basis for a diagnosis of IAD in young horses in training. The aetiology of this syndrome remains in dispute, but environmental factors are thought to play a role because the syndrome is more frequent and of longer duration when horses are kept in a high-dust environment (Burrell *et al.* 1996). Our data certainly support the idea that stabling can contribute to airway inflammation. It is perhaps interesting to note that the mean percentage of neutrophils for all the stabled and pastured horses (10.8 and 3.6%, respectively) is identical to that reported by Moore *et al.* (1995) in Standardbreds with and without IAD. A relatively mild degree of inflammation, such as we have noted, may go undetected clinically in a pleasure horse but may be sufficient to impair performance in a racing animal and in that situation is identified as IAD.

Even though stabling was associated with a higher number of neutrophils in BALF, this was not reflected in the amount of mucus observed in the trachea; quite the reverse, stabled horses had a lower mucus score than did pastured horses. The lack of association between mucus and inflammation is probably because the level of inflammation was so slight and the majority of mucus scores were in a range that would be considered clinically unimportant. We know of no reason why the mucus score should be higher in pastured horses, other than that these animals were occasionally chased around before being caught. However, not all horses at pasture were chased and this did not occur every month. If exercise altered tracheal mucus scores, it may also have influenced the BALF cytology. In a study conducted by Wong *et al.* (1990) BAL samples were collected at specified times before and after single bouts of exercise. The cellular composition of the lavage fluid was not altered by the exercise (Wong *et al.* 1990; Martin *et al.* 1999).

Our data allow us to state that upper airway inflammation is not always associated with inflammation in the tracheobronchial tree. Even though the highest scores for both upper airway inflammation and BALF neutrophils occurred in the stable, there was not a direct correlation between the two in each horse.

We developed a unique system to grade UAI that combined an established grading system (1–4) for pharyngeal lymphoid hyperplasia (Baker 1987) with a score for guttural pouch inflammation. The ventral floor of the medial compartment of the guttural pouch contains multiple lymph follicles that enlarge and proliferate. Lymphadenopathy can also be observed within the guttural pouch because the retropharyngeal lymph node chain lies beneath the membrane on the floor of the pouch. The enlarged nodes bulge into the ventral portion of the medial compartment of the guttural pouch. The epithelial lining of the guttural pouch can become erythematous, oedematous and hypersecretory. Our score accounted for each of these changes.

Follicular pharyngitis has been speculated to be a result of viral and/or bacterial infections or of environmental insult. Because lymphoid hyperplasia and other signs of airway inflammation were present in all horses at the start of the study, our data add no new information about the aetiology of the inflammation. However, our results do suggest that upper airway inflammation persists in stabled horses and may decrease at pasture. However, the study design was flawed because there was no washout period between the treatments. It would have been more appropriate for horses to have been stabled for 3 months followed by a washout period on pasture and then a second 3 month period either stabled or pastured. A washout period between treatment groups could potentially have identified the effect of stabling on pasture and pasture on stabling. It is possible that the decreased inflammation occurred over time and not because of the pasture environment. However, because we were only able to study this unique group of horses for 6 months, a washout period was not possible.

One of our reasons for conducting the present study was to test the hypothesis that DDSP is associated with upper airway inflammation in horses. This hypothesis is derived from the results of a study that showed that persistent DDSP could be induced by bilateral neuronal blockade of the pharyngeal branch of the vagus nerve as it courses through the medial compartment of the guttural pouch (Holcombe *et al.* 1998). The motor and most of the sensory supply to the nasopharynx is derived from the pharyngeal plexus of nerves on the surface of the dorsal nasopharynx. This plexus is composed of the pharyngeal branch of the vagus nerve, the pharyngeal branch of the glossopharyngeal nerve and by sympathetic branches from the superior cervical ganglion (Moore 1992). All of these structures course rostroventrally through the medial compartment of the guttural pouch to ramify in the pharyngeal plexus of nerves. These nerves are involved in swallowing. Guttural pouch inflammation, caused by aspergillus and streptococcal infections, can be associated with dysphagia (Mayhew 1989; de Lahunta 1983). We hypothesise that DDSP may also result from neuromuscular dysfunction. Specifically, inflammation of the pharyngeal branch of the vagus nerve may cause neuropraxia, resulting in inappropriate motor function of the *palatinus* and *palatopharyngeus* muscles, which control the position of the caudal portion of the soft palate. Inflammation in the guttural pouch, retropharyngeal lymph nodes and the nasopharynx may be responsible for this dysfunction. Inflammation of the upper airway was most severe in horses when they were stabled and it was at this time that we observed the most instances of DDSP. Furthermore, the horse with the most severely inflamed guttural pouch and a chondroid was responsible for 3 of the episodes of DDSP. However, the lack of standardisation of the nasal occlusion test (i.e. 10–60 s) and the biased results (i.e. one horse displaced 3 times during stabling) makes it difficult to draw any conclusions about the effect of stabling on the occurrence of DDSP.

In conclusion, stabling horses was associated with lower airway inflammation and persistence of upper airway inflammation. Many, if not all, young racing and performance horses are stabled. This is precisely the population of horses most frequently affected with IAD and UAI. In addition, upper airway dysfunction such as DDSP is the most prevalent in these horses. Minimising dust exposure should be the aim of management for all horses, but especially those, such as racehorses, that place high demands on their respiratory systems. Keeping young racing horses and performance horses at pasture may be impractical for reasons relating to time management, personnel and injury.

However, the cumulative effects of repeated stable-induced airway inflammation may predispose horses to the development of more severe lower airway disease such as heaves or performance-limiting upper airway dysfunction.

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