THE DIAGNOSIS AND TREATMENT

OF *CULICOIDES* HYPERSENSITIVITY IN HORSES

IN BRITISH COLUMBIA

by

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The diagnosis and treatment of Culicoides Hypersensitivity in horses in British Columbia

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ABSTRACT

Culicoides Hypersensitivity (CH) is a chronic, recurrent, seasonal dermatitis of horses first reported in British Columbia in 1984. *Culicoides obsoletus* (Meigen) was the major causal agent in southwestern British Columbia as shown by skin testing affected and non-affected horses with extracts of *C. obsoletus*. Light-trap captures determined that the seasonal distribution of *C. obsoletus* corresponded well with the presence of lesions. Trapping inside and outside stables indicated that significantly more *C. obsoletus* were trapped outside, but some still entered stables, indicating that the previously recommended treatment of stabling may not be completely effective. Affected horses in B.C. sensitized to *C. obsoletus* also reacted to other horse-biting species of *Culicoides* despite the fact that these are rare or absent in southwestern B.C. This indicates that the allergen is common to many species, including a strain of *C. variipennis* (Coquillett) reared in a laboratory for over 30 years. Clenbuterol hydrochloride is thought to be an effective mast cell stabilizer, preventing the release of the pharmacological mediators of allergy. However, it was ineffective in preventing the clinical signs of CH, despite proven efficacy in respiratory allergies in horses. This indicates an inherent difference between cutaneous and other hypersensitivities. No effective drug therapy is available, so a two-year immunotherapy trial was conducted on affected horses, using increasing doses of a crude *C. variipennis* extract. Horses were injected weekly, and local reaction to the injections decreased over time, despite the increasing dosage, indicating the development of hyposensitization. A noticeable improvement in clinical signs was observed at a dose extracted from 1680 insects. During the first year, 9 of the 10 horses improved, with 3 recovering completely. 8 horses were injected weekly with a maintenance dose during the second year, and 2 horses recovered completely, 3 improved greatly, 2 improved slightly, although 1 was unchanged. Of the 2 horses that received immunotherapy for only 1 year, one recovered completely and one was unchanged. One year after therapy, the clinical signs appeared unchanged or only slightly worse in most cases, indicating that the treatment had lasting effect. Western blotting techniques were used to identify a single causal allergen.

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1. GENERAL INTRODUCTION

1.1 HISTORY, HISTOPATHOLOGY AND ECONOMIC IMPORTANCE OF THE DISEASE

A serious, chronic, recurrent, seasonal dermatitis of horses, *Equus cabalus* L. was first described in 1829 in the East Indies (Freeborn *et al.* 1927). It has since been reported from France (Henry and Bory 1937), India (Datta 1939), Algeria (Le Seac'h 1946), the United States of America (Dikmans 1948), Australia (Riek 1953a), Israel (Ralbag 1954), Japan (Nakamura *et al.* 1954), Germany (Becker 1964), Britain (McCaig 1973), Ireland (Baker 1973), Denmark (Hesselholt and Agger 1977), Hong Kong (Baker and Collins 1984), Canada (Kleider and Lees 1984) and Sweden (Troedsson and Brostrom 1986). In many countries, this disease has been colloquially called "sweet itch".

The etiology of the disease was disputed for many years, being attributed to such diverse agents as fungi, nematodes, sunshine, adverse climate, poor diet or lack of work. However, in 1891 in Australia, it was first noted that when an affected horse was protected from insect attack, the clinical signs regressed (Bancroft 1891). This finding was not followed up until Riek (1954) determined that the disease in Australia, termed 'Queensland itch', was caused by a hypersensitive reaction to the bites of *Culicoides* spp., in particular *Culicoides robertsi* (=*brevitarsis*) Lee and Reye (Diptera:Ceratopogonidae), using intradermal skin tests of extracts of this and other biting flies. Later studies in Ireland (McCaig 1975; Baker and Quinn 1978; Quinn *et al.* 1983), Israel (Braverman *et al.* 1983) and Hong Kong (Baker and Collins 1984) have indicated a similar etiology.

The disease was first recognized in British Columbia in 1984 (Kleider and Lees 1984) and is thought to have a similar etiology to Queensland itch and sweet itch because the epidemiology and histopathology of the disease are identical. Hence, it has been called *Culicoides* Hypersensitivity or CH in Canada.

The prevalence of the disease varies worldwide, depending on the region and the animals surveyed. In British Columbia 26% of horses examined were affected (Anderson *et al.* 1988). Prevalence was similar in Germany at 29% (Becker 1964), in Israel at 22% (Braverman *et al.* 1983) and in Queensland, Australia at 32%, although in some areas of Queensland over 60% of horses were affected (Riek 1953a).

In Britain, the prevalence is lower, ranging from 0.3% (British Equine Veterinary Association 1965) to 3% (McCaig 1973; Pelham 1984), depending on the animals surveyed. The prevalence in Japan was reported as 4.5% (Nakamura *et al.* 1956).

This variation in numbers of horses affected in different geographical regions may be related to the abundance of *Culicoides*, their biting habits, or the housing, maintenance and treatment of the horses in that particular country or region, but is probably more closely related to recognition of the disease. CH is frequently misidentified as mange, lice-infestation, onchocerciasis or unspecified dermatitis by both owners and veterinarians who are unfamiliar with the true cause of the problem. Also, mild cases may pass unnoticed, particularly when the lesions are in the ventral midline, as the owner must stoop or lie beneath the animal to see the clinical signs. If the disease is not recognized for what it is, the prevalence recorded for that area will be low.

In most cases, the disease appears during the warmer months of the year, affecting only certain animals in a herd, and these animals are affected year after year. In particularly severe cases, clinical signs may be apparent all year (Kleider and Lees 1984, Anderson *et al.* 1988), probably due to a scratch reflex similar to that seen in flea-sensitive dogs. Scratching renews the pruritis, even though fleas are no longer present, and this phenomenon can also occur in humans (Baker and O'Flanagan 1975). The disease usually occurs in horses that are pastured, rather than stabled, but some horses in open or badly screened stables are also affected. The clinical signs and histopathology of the disease are similar worldwide, varying only in the location of the lesions.

Initially the disease is characterized by numerous papules, tufted hair and skin sensitization. This is followed by intense irritation, scratching and rubbing, which leads to serous effusion, localized hair loss and the development of secondary lesions (Riek 1953a, Robinson 1983, Kleider and Lees 1984). Affected horses may cause severe injury to themselves, or their riders in an effort to alleviate the irritation. Recurrent attacks lead to thickening of the skin, scaling and the formation of transverse ridges or ruggae in the skin. These are a result of extensive fibrosis, hypertrophy of epidermal tissues and marked hyperkeratosis (Riek 1953a).

The location of the lesions on the horse varies from region to region, geographically. In British Columbia, they are found most commonly in the ventral midline (83%), mane (50%) and tail (46%), with the withers, head, chest, hindquarters, neck, hocks and inguinal groove being affected less commonly (Anderson *et al.* 1988). However, in almost all other parts of the world, the lesions are found almost exclusively on the dorsal midline, affecting, in particular, the mane and tail (Riek 1953a; Ralbag 1954; Nakamura *et al.* 1956; McMullan 1971; Mellor and McCaig 1974; Baker and Quinn 1978; Fadok 1984). In France, the entire dorsal midline, from poll to tail is affected (Henry and Bory 1937), but in Germany, although the dorsal midline is most commonly affected, the shoulders, chest, head and ventral midline sometimes show clinical signs (Becker 1964).

The histopathology of a typical lesion shows an eosinophilic perivascular dermatitis (Fadok and Mullowney 1983). The eosinophils are found at all levels of the dermis. Subepidermal edema occurs, with the collagen bundles appearing separated, and in some areas an amorphous eosinophilic deposit may be present (Baker and Quinn 1978). Daily fluctuations in blood histamine levels and eosinophil counts were positively correlated with the diurnal activity of *Culicoides* spp. (McMullan 1971). The epidermis exhibits hyperkeratosis, usually associated with spongiosis (Kleider and Lees 1984) and mast cells show evidence of degranulation (Baker and Quinn 1978).

The horse's appetite is unaffected and its overall body temperature remains normal except when secondary infection occurs (Riek 1953a). However, local skin temperatures do increase at the site of lesions (personal observations), and this may persist throughout the off-season (Braverman 1989a). Local skin temperatures were also found to increase in cattle hypersensitive to *Culicoides* (Akey *et al.* 1989).

The disease is primarily a problem in horses, but it has also been reported as a serious problem in mules in the State of Georgia where a prevalence of 10% was reported (King 1947), and in Hong Kong, with a prevalence of 20% (Baker and Collins 1984). It is not surprising that mules are affected by CH as they are hybrids between horses and donkeys. However, the disease has also been reported in more distantly related animals. Over 30% of sheep in a flock in England exhibited ventral lesions which were demonstrated to be the result of hypersensitivity to *Culicoides obsoletus* (Meigen) using skin tests (Connan and Lloyd 1988). A hypersensitivity to *Culicoides* bites was induced in cattle (Akey *et al.* 1989), although no natural cases have been reported. The rarity of reported cases in domestic farm animals may be due to the fact that, unless the disease is severe enough to result in a reduction in milk, meat or wool production, it is unlikely to be noticed. The disease in sheep was only noticed during a routine inspection for other problems, and since becoming aware of the disease, Connan and Lloyd (1988) have noticed many other cases and anticipate that it is a common problem. Severe hypersensitive reactions to *Culicoides* bites have also been reported in humans (Areán and Fox 1955).

It is very difficult to determine the economic loss caused by a chronic disease such as CH in an animal species which varies so much in value from region to region and owner to owner, depending on its use. The disease severely debilitates an affected horse for at least eight months out of every year in climates where *Culicoides* are seasonal, and year round in milder climates. The severity of the disease varies from horse to horse. In a survey in British Columbia, 72% of horses with CH were affected severely enough to warrant veterinary attention and 6% were destroyed due to the disease (Anderson *et al.* 1988).

The lesions may be primarily in the ventral or the dorsal region of the body, depending on country, but in either case, the position of CH lesions makes it extremely difficult to use a saddle or harness on an affected horse. Even when the saddle does not directly interfere with active lesions, exercise will result in sweating which in turn results in greatly increased pruritis (Riek 1953a), making the horse almost impossible to control. In fact, it is thought that the original derivation of the term "sweet itch" used for the disease in Britain, Ireland, Israel and Hong Kong is from "sweat itch", as an affected animal was far more noticeably pruritic when it was sweating (Anon. 1984). Consequently, an affected horse is frequently unridable and cannot even be worked in a harness when it is showing clinical signs. Therefore, an animal which is used as a working horse, such as a stock horse, is virtually worthless during the *Culicoides* season. This must have resulted in much greater economic loss in the past when horses were used on ranches and farms much more than today. In Australia, the Report of the Parliamentary Commission of 1888 indicated that allergic dermatitis in horses was of considerable economic significance (Riek 1953a). Today horses are rarely used on farms, but are commonly used on ranches in Canada, Australia and America, and their inability to be worked for several months of the year would result in severe loss to the owner. Such animals would probably be destroyed, resulting in the loss of the price of the animals, and the inconvenience and cost of replacing them and training others.

Many horses in British Columbia are owned for pleasure, primarily recreational riding. Summer is the most popular time for such recreation, due to favourable weather and extended daylight hours. However, as the disease occurs from March to October, a susceptible horse bought for such purposes would be unridable during this period, so would have little or no value.

An animal which has rubbed out its mane and tail and has visible facial lesions is unsightly and could not be exhibited or entered in horse shows and would be extremely difficult to sell. Mares that are rendered unworkable as a result of other debilitating problems, such as lameness, are commonly used for breeding, to recompense the owner for loss of work.

This is inadvisable in the case of horses affected with CH. A mare with lesions in the ventral midline will frequently kick or bite at a foal as it tries to suckle the severely irritated teat area and the foal may be killed or injured. There is also strong evidence that susceptibility to CH is inherited (Riek 1953a; Ishihara and Ueno 1957; McCaig 1975; Unkel *et al.*1987; Anderson *et al.* 1988), making it even less desirable to breed from an affected horse.

Also, affected horses rub and scratch on any available obstacle in an effort to relieve the irritation, and this frequently results in damage to themselves, or their enclosure. In Scotland, horses suffered greatly from *Culicoides* attacks, to the point of exhaustion, and similar attacks on cows resulted in a lowered milk yield (MacGregor *et al.* 1948). Therefore, although it is difficult to put a monetary value on it, there is a severe economic loss as a result of CH.

At the present time there is no successful treatment for CH. Corticosteroid treatment is commonly prescribed, but it is expensive, rarely completely effective and may cause adverse side effects, such as adrenal suppression (Addison's syndrome) (Stannard 1980) and laminitis (Eyre *et al.* 1982). Insect repellents have limited use as they must be applied very frequently, and are not very effective when sprayed on hair rather than on skin, although some contact insecticides, such as permethrin, last for several weeks because they are lipophilic and bind to the hair. Treatment of CH affected horses in Britain and Belgium with permethrin resulted in some improvement in 55% of horses, but only 23% healed (Stevens *et al.* 1988). However, a pour-on treatment was used, which is feasible in Britain and Belgium where the lesions are found in the dorsal region, but would be of little practical use in British Columbia, where the lesions are primarily ventral. Also, no repellent or insectide can be used when the lesions are open.

Stabling the horse when the insects are active has been recommended, but is often inconvenient and rarely adhered to.

Eradicating the insect is not feasible as no adulticide or larvicide program will kill all the insects, and only a few bites can result in lesions (Riek 1955; Sippel 1979). Also, even if

generalized larviciding is carried out, *Culicoides* can breed in any small body of water, such as a horse trough or a rain filled hoof-print. *Culicoides* can also travel up to 1 km from their breeding sites (Brenner *et al.* 1984), so even local eradication would be impossible.

1.2 OBJECTIVES OF THESIS

My objectives were as follows :

1. to determine whether the dermatitis observed in British Columbia is a hypersensitivity to *Culicoides* bites by identifying the causal insect species;
2. to determine the seasonal occurrence and peak periods of the causal species, in relation to appearance and regression of clinical signs;
3. to determine whether the behaviour of the causal insect species, for example, in entering stables, can be exploited to prevent the disease;
4. to determine if the allergen is specific to the causal insect species in British Columbia, or is common to other species from different geographical regions to evaluate whether a treatment based on the allergen itself, such as hyposensitization, would be effective in regions other than British Columbia, and if so, to determine whether mass-reared, laboratory raised *Culicoides* contain the allergen, to assess the feasibility of a hyposensitization trial;
5. to treat affected horses with a mast cell stabilizer, to determine the efficacy of a possible new drug therapy;
6. to hyposensitize affected horses using a crude extract of *Culicoides* species and to evaluate its potential in providing long term relief from the clinical signs; and
7. to identify and characterize the protein in the insect responsible for the hypersensitivity.

1.3 GENERAL METHODS AND MATERIALS

The horses used in this study were located in most cases through veterinarians, in particular, Dr. N. Kleider. In this way, I was confident that each affected horse exhibited clinical signs that were considered serious enough to warrant the services of a veterinarian, and the diagnosis of each case was confirmed by a veterinarian familiar with the disease. Some cases were recruited by word of mouth from one owner to another, and the diagnosis was again confirmed by a veterinarian. The objectives of the study both short and long term, the consequences, the amount of involvement with horse and owner, the amount of co-operation required and any risks involved were clearly explained to each owner. Each owner then signed a consent form. In all cases, before I was involved, the owners had tried for some time to treat their horses, using recognized proprietary forms of treatment, and also more esoteric methods. In every case, these had failed to bring relief, and the owners volunteered their horses for these experiments in the hope that a long term cure could be found.

For the purposes of the thesis, each horse has been designated with a letter A for Affected, and N for Normal, followed by a number. These numbers are used consistently throughout this work, but in some tests these letters are suffixed by another number to indicate that a particular animal belonged to a specific experimental group.

I have shown previously that no breed, sex, age or height of horse is particularly likely to develop the disease (Anderson *et al.* 1988). Consequently, affected horses were chosen based on the severity of their clinical signs, their geographic location and the willingness of their owners to co-operate in the experiments. Normal horses pastured with affected horses were selected as controls. Both the affected and the normal horses consisted of a variety of breeds and cross-breeds, ages, colours and heights. Both mares and geldings were used, but no stallions were involved due to their scarcity and unpredictable nature. The horses ranged in age from 2 to 23 yrs at the beginning of the study. Many owners did not know when their animals first showed signs of the disease as they had unknowingly purchased diseased horses and, therefore, did not know when the clinical signs first appeared. However, in most cases, the age

of onset was between 2 and 7 yrs. The percentage of affected horses with lesions in particular areas were : ventral midline (89.5%), mane (84.2%), tail (100%), chest (26.3%), face (15.8%), withers (31.6%), rump (5.3%) and other regions (5.3%). The individual details of the affected and normal horses are summarized in Appendix I.

Most of the horses were used for recreational riding, or kept purely as pets. In some cases, the animals were valuable show, cattle and racehorses or brood mares.

Many of the tests in this work involved injecting foreign proteins. This always entails a slight risk of anaphylactic shock. Therefore, all injections were performed by a veterinarian unless otherwise stated. An emergency medical kit containing adrenalin, cortisone and other resuscitating drugs, together with the means to administer them intravenously, was carried at all times as a precaution against anaphylactic shock. Each horse was observed for at least 10 min after each injection. No horse in the program reacted adversely to any of the injections or treatments at any time.

2. DETERMINING THE CAUSAL AGENT OF *CULICOIDES* HYPERSENSITIVITY IN BRITISH COLUMBIA

2.1 INTRODUCTION

Kleider and Lees (1984) proposed that sweet itch in British Columbia was caused by a hypersensitive reaction to *Culicoides* bites, due to the similarities in histopathology and epidemiology to Queensland itch in Australia, and to sweet itch in England, Ireland, Israel, and Hong Kong.

The objective of this section was to determine whether *Culicoides obsoletus* can cause CH disease in horses in British Columbia. *Culicoides* seemed the most likely suspect as their seasonal occurrence and geographical distribution correspond with the appearance of clinical signs in British Columbia (Costello 1982; Chapter 3). Hornflies, *Haematobia irritans* (L.) cause a ventral midline dermatitis, but the lesions are usually only found in the umbilical region (Fadok and Mullowney 1983). Also, hornflies are obligate parasites of cattle, and the adult fly usually remains on the host. Therefore, only horses pastured with cattle are likely to be attacked (Foil *et al.* 1990). No animals used in this study were pastured with cattle. Other insects which bite horses include tabanids, blackflies and mosquitoes. Tabanids bite most commonly in the chest, flanks and limbs (Foil and Foil 1986), where CH signs rarely occur. Blackflies feed most commonly in the ears of horses (Foil and Foil 1990) and are not common in Southwestern British Columbia. Although mosquitoes are known to bite horses, no specific skin diseases have been attributed to their feeding (Foil and Foil 1986). Riek (1954) compared skin tests using extracts of *Culicoides*, mosquitoes and stable flies in horses affected by Queensland itch and obtained consistently positive responses only with the *Culicoides* extract. This was later confirmed by similar tests in Ireland (Baker and Quinn 1978). *Culicoides* were also the most common biting species to land on a horse in Israel (Braverman 1988), and *C. variipennis* (Coquillett) was the most common of 34 species of biting flies attacking horses in three southwestern States (Jones *et al.* 1977).

The species responsible for the disease worldwide varies with the geographical region. *C. obsoletus* seemed the most likely candidate in Southwestern British Columbia as it made up 99.3% of this genus caught in light traps in this area (Costello 1982). *C. obsoletus* was one of the three most common *Culicoides* captured on a horse (Schmidtmann *et al.* 1980) in New York and the most common species captured on horses in Ireland (Townley *et al.* 1984) and England (Mellor and McCaig 1974). In England and Israel, lesions were found dorsally, where the *Culicoides* spp. most commonly feed (Mellor and McCaig 1974; Braverman 1988). However, in British Columbia, the most commonly affected area was the ventral midline, although the mane and tail were also affected (Anderson *et al.* 1988). *C. obsoletus* bites horses most frequently along the ventral midline in North America (Schmidtmann *et al.* 1980) and England (Mellor and McCaig 1974), and bites cattle most frequently in this region in Denmark (Nielson 1971) and Australia (Kettle 1977). In England, *C. obsoletus* is not considered to be a causal agent (Mellor and McCaig 1974). However, *C. obsoletus* is a species complex (*avaritia* group) and may be genetically different in British Columbia and England.

Sensitivity of skin to insect bites in certain animal species is known to go through five stages (Larrivee *et al.* 1964; Feingold 1973a). The first is induction, during which there is no change in response to bites. In the second stage, delayed skin reactions appear. In the third, both immediate and delayed reactions are present, and by stage four, only immediate reactions occur. At stage five, no skin reaction is observed and the animal is considered desensitized. Such a sequence occurs in guinea pigs (Benjamini *et al.* 1961) and could also occur in horses. If so, affected animals should naturally desensitize after many years of exposure. Therefore, it should also be possible to induce such a state by regularly exposing or injecting an affected animal with the allergen. Determining the causal insect is the first step in such a procedure.

2.2 METHODS AND MATERIALS

Six clinically normal horses (N1 through N6) and six affected with severe and typical lesions (A1 through A6) were evaluated. The affected horses had had the disease for several

years severely enough to warrant veterinary attention. Each normal horse was kept in the same pasture, under the same conditions as an affected horse both before and during the tests. The horses were exposed equally to *C. obsoletus* under natural conditions. Both affected and normal horses included geldings and mares, ranged from 7 to 22 yrs old, were grey to bay in colour, and belonged to a variety of breeds and cross-breeds (Appendix I). All affected horses had severe clinical signs in the ventral midline region and tail. Most also had lesions in the mane, withers, face, rump, chest and genital region. All of the affected horses were known to have developed lesions yearly for a minimum of five years before this study.

Culicoides obsoletus were collected from the sites of five of the affected horses using New Jersey light-traps equipped with 100 watt light bulbs. Captured *C. obsoletus* were freeze-dried and stored at -18^{o}C until used. Approximately 3,500 whole freeze-dried insects, weighing 0.17 g were crushed in 0.5 mL of physiological saline (0.9% NaCl). The extract was diluted 100-fold with saline and then sterilized by filtering through a series of sterile Millipore filters, with pore sizes of 0.8μm, 0.45μm and 0.22μm respectively. The extract was measured into aliquots and kept frozen at -18^{o}C until just before use. An aliquot of the extract was incubated in nutrient L-broth (bactotryptone, bactoyeast extract and sodium chloride) at 37^{o}C for 48 h to test for sterility. Aliquots of physiological saline were also autoclaved and frozen until used.

Each horse was clipped along the side of the neck, using size 40 clipper blades. The skinfold thickness in the approximately 25cm^2 clipped area was measured with vernier calipers and the area was cleaned and sterilized with 70% ethanol immediately before injection.

All twelve horses were challenged, or inoculated, with the *Culicoides* extract. Each affected horse received four intradermal injections approximately 5 cm apart, in two rows. Three were of 0.1 mL of 1% *C. obsoletus* extract (*i.e.* 168 insect equivalents) and one was 0.1 mL physiological saline, which acted as a control. Each normal horse received two injections: one of 0.1 mL of 1% *C. obsoletus* extract, and one of 0.1 mL saline. These were injected side

by side, approximately 5 cm apart. Tuberculin syringes with 26 gauge needles were used to minimize skin trauma.

The increase in skinfold thickness (difference between pre- and post-inoculation measurements) and width of the welt at its widest point were measured with vernier calipers at 20 min, 1, 2, 3, 4, 24, 48, and 72 h after injection. In cases where the reaction had not subsided completely after 72 h, readings were also made up to three weeks later. In horse A3 both increase in skinfold thickness and welt width measurements were repeated three times at each of the first five reading times to estimate the error associated with each measurement technique.

The results were analyzed by comparing relative areas under the curve, for both welt width and skinfold thickness increase from 0-72 h after inoculation and by comparing the reactions 24 h after inoculation. The reactions of the six affected horses to the extract at each assessment time were also compared with those in the normal horses to determine how soon after inoculation the affected horses could be differentiated from normal ones. The mean of measurements made after the three inoculations in the affected horses was used at each assessment time. The reactions of the affected horses were compared with those of the normal horses with a two sample t-test using Minitab (Ryan *et al.* 1985).

Three of the affected horses, A1, A3 and A5, were tested again approximately two years after the first challenge. Measurements were made as before, and the housing and management of these horses had not changed. Each horse was clipped and skin thickness was measured using vernier calipers, as before. Each animal was then given one 0.1 mL intradermal injection of freshly made 1% *C. obsoletus* extract. The subsequent welts were measured for both welt width and skin thickness increase at 20 min, 1, 2, 3, 4, 24, 48 and 72 h after inoculation as before. The results of these tests were compared with those of the earlier tests and with the progress of clinical signs over this time, but statistical comparisons were not made because only three horses were involved.

2.3 RESULTS

Immediately after inoculation with *C. obsoletus* extract or 0.9% saline, the affected horses developed a raised swelling or welt at the site of each injection. Within 20 min, the welts at the extract injection sites had swollen much more than those at the saline sites, which indicated the amount of swelling to be expected from the injection of 0.1 mL of liquid intradermally. In affected horses, the reaction to *Culicoides* extract did not peak, in most cases for at least 24 h (Table 2.3-1). Horse A6 peaked 4 h after challenge, but the welt width decreased only slightly by 24 h, while skinfold thickness had decreased greatly, indicating a large shallow welt. Horse A1 reached peak welt width at 24 h, but skinfold thickness peaked at 4 h, again indicating a change in the shape of the welt. The duration of a measurable welt in affected horses ranged from 72 h to $>$ 3 weeks. During the reaction the welts on the affected horses changed appearance. For the first 4 h they were firm, hard to the touch and well defined, while after 24 h, they became less indurated, larger and poorly defined. The welts were round or oval in shape after 4 h, but were usually well extended down the neck, beyond the border of the clipped area, within 24 h. The reaction always extended down from the injection site further than it extended above the site.

In contrast, the reaction in the six unaffected horses peaked 2 to 4 h after challenge. Welt width returned to zero between 4 and 48 h, and skinfold thickness to normal after 72 h, except in horse N4 which retained a very minor increase in skinfold thickness for up to 120 h after inoculation. All reactions were greatly reduced after 4 h.

Affected horses developed a significantly larger welt and had a significantly greater skinfold thickness increase than did normal horses 24 h after inoculation (Table 2.3-2). Total area under the curve of a graph showing the reaction from time of inoculation to 72 h after inoculation was measured for each affected and normal horse and these data were also compared. Affected horses showed a greater welt width ($P \leq 0.03$) and skinfold thickness increase ($P \leq 0.02$) over time than normal. The mean reactions of affected and normal horses over time are shown in Fig. 2.3-1.

Table 2.3-1. The peak and duration of the reaction of affected (A) and normal (N) horses after challenge with 0.1 mL of 1.0% *Culicoides* extract or a control of 0.9% saline.

	PEAK REACTION								DURATION OF REACTION (h)			
	Welt width (mm)		Time of occurrence (h)		Skinfold thickness increase (mm)		Time of occurrence (h)		Welt width		Skinfold thickness	
HORSE[a]	Extract	Saline	Extract	Saline	Extract	Saline	Extract	Saline	Extract	Saline	Extract	Saline
A1	49.6	0.0	24	0	9.1	2.4	4	4	>72	0.0	>72	48-72
A2	80.2	27.8	24	0.3	7.5	2.6	24	0.3	283-529	2-3	283-529	0.3-1
A3	128.8	19.5	24	1	14.6	0.8	24	0.3	114-165	24-48	114-165	1-2
A4	315.0	14.0	72	4	13.7	0.8	3	0.3	120-214	4-24	242-357	2-3
A5	235.8	0.0	24	0	28.8	0.2	24	0.3	72-169	0.0	169-266	0.3-1
A6	68.8	18.1	4	1	13.7	2.7	3	4	48-72	4-24	72-189	4-24
Mean±SE	146.4±43.4	13.2±4.6			14.6±3.1	1.6±0.5						
N1	27.8	10.4	2	0.3	4.5	2.0	4	0.3	24-48	0.3-1	48-72	1-2
N2	38.4	11.5	3	0.3	4.0	2.0	4	0.3	4-24	4-24	48-72	24-48
N3	38.8	11.6	3	1	4.4	1.0	3	0.3	24-48	1-2	48-72	2-3
N4	46.2	11.9	4	0.3	12.4	0.6	4	0.3	24-48	3-4	48-72	0.3-1
N5	36.0	11.6	4	0.3	4.0	0.1	4	0.3	4-24	1-2	48-72	1-2
N6	35.0	20.0	4	4	8.2	1.4	4	3	4-24	4-24	48-72	4-24
Mean±SE	37.0±2.4	12.8±1.4			6.3±1.4	1.2±0.3						

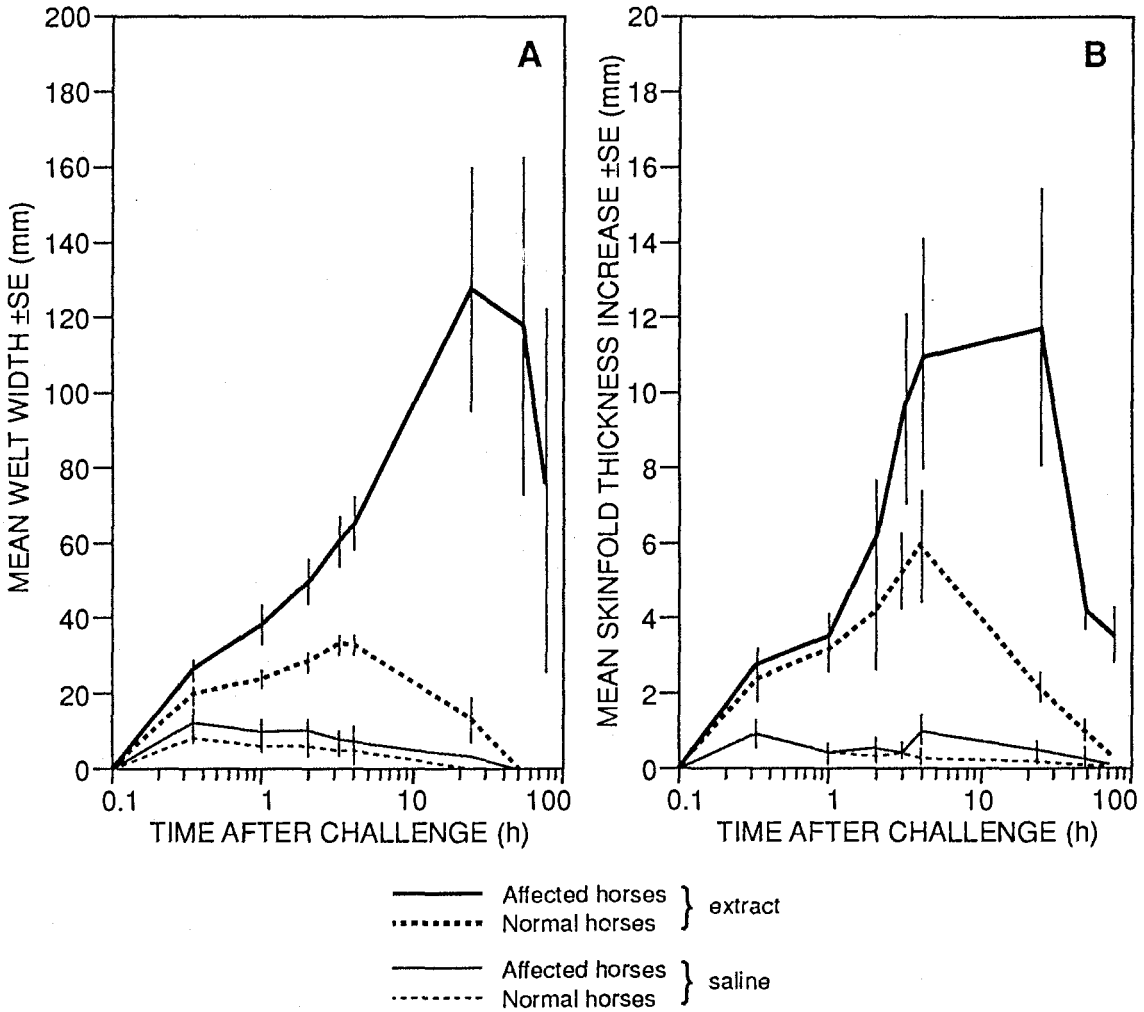
[a] Horse A1 was monitored for only 72 h; horse N4 had a very minor skinfold thickness increase of 0.6 mm up to 120 h post-inoculation, but the increase was very minor by 72 h after inoculation.

Table 2.3-2. The reaction of affected and normal horses 24 h after injection with 0.1 mL of 1.0% *Culicoides* extract or a control of 0.9% saline.

	Welt width (mm)[a]		Skinfold thickness increase (mm)[a]		Probability of significant difference between extract and saline effects (t-test)	
Horses	**extract**	**saline**	**extract**	**saline**	**Welt width**	**Skinfold thickness increase**
Affected	128.7±32.4a	2.8±2.8a	11.8±3.8a	0.5±0.3a	0.01	0.03
Normal	13.5±6.7b	0.0a	2.2±0.4b	0.2±0.2a	N.S.	0.004

[a] Means within a column followed by the same letter are not significantly different, t-test $P \leq 0.05$.

Figure 2.3-1. Welt width A) and skinfold thickness increase B) of six affected and six normal horses after intradermal challenge with *Culicoides obsoletus* extract and saline control over time.


A
B
MEAN WELT WIDTH ±SE (mm)
MEAN SKINFOLD THICKNESS INCREASE ±SE (mm)
TIME AFTER CHALLENGE (h)
TIME AFTER CHALLENGE (h)
0.1
1
10
100
Affected horses
Normal horses
extract
Affected horses
Normal horses
saline

Several days after injection, the affected horses showed small vertical ridges in the injection site similar to the ruggae seen in natural cases of the disease at the lesion sites (Fig. 2.3-2). The ridges ran vertically down the length of the neck and each was 1-2 cm wide. The ridges were not present before the test, and I have never seen the side of the neck affected by natural lesions. These ruggae disappeared within a few weeks. Normal horses never showed such ridging and did not appear at all discomforted by the injections. However, all the affected horses showed some irritation at the injection site. Most attempted to rub the area, and in horse A3 the entire injection site was extremely sensitive during the first 24 h, despite the fact that the reaction had not yet reached its peak. During this period, the horse reacted violently to being handled in the region of the injection site. The following day, however, it showed little sign of discomfort. Several of the affected horses were irritable and difficult to handle at the injection site during the first 24 h after challenge.

After 2 h, horse A4 developed a number of extraneous small welts around the injected area, and after 4 h, five extra welts appeared. These welts were separated from the injection sites by several centimetres of unaffected skin and were not a result of insect bites as the area was monitored closely. By 72 h, eight small scabs had appeared and some were pustular. Three weeks (498 h) after challenge, four of the extra welts were still present, although the original welt at the injection site had disappeared. Two of the other affected horses showed small welts near the injection site, but none was as extreme as horse A4. The normal horses did not display these extra welts, so they were not attributed to clipping. In later tests, unclipped horses have shown similar extra welts.

There was no significant difference between the reaction of affected and unaffected horses to saline (Table 2.3-2) indicating that differences were due to the solution injected rather than to the trauma of inoculation. In the affected horses peak welt width from the saline injection ranged from 0 to 27.8 mm which occurred at 20 min to 4 h after injection (Table 2.3-1). The peak width in unaffected horses ranged from 10.4 to 20.0 mm, and it also occurred 20 min to 4 h after injection. The welt following saline injection disappeared between 0 to 48 h in

Figure 2.3-2. Ruggae in neck region after challenge with *Culicoides* extract.

affected horses and 1 to 24 h in unaffected horses. The peak skinfold increase in response to saline in affected horses ranged from 0.2 to 2.7 mm between 20 min and 4 h after inoculation, and the peak in unaffected horses ranged from 0.1 to 2.0 mm after 20 min to 3 h. The skin returned to its normal thickness between 1 and 72 h in the affected horses and 2 and 72 h in the normal horses (Fig. 2.3-1).

Affected horses had a significantly greater welt width than normal horses within 1 h of inoculation (t-test, $P \leq 0.05$), although skinfold thickness increase was not significantly different until 24 h post-inoculation (t-test, $P=0.05$). After 1 h, affected horses exhibited a consistently greater welt width than did normal horses (Fig. 2.3-1), whereas skinfold thickness was only significantly different from 24 h after inoculation.

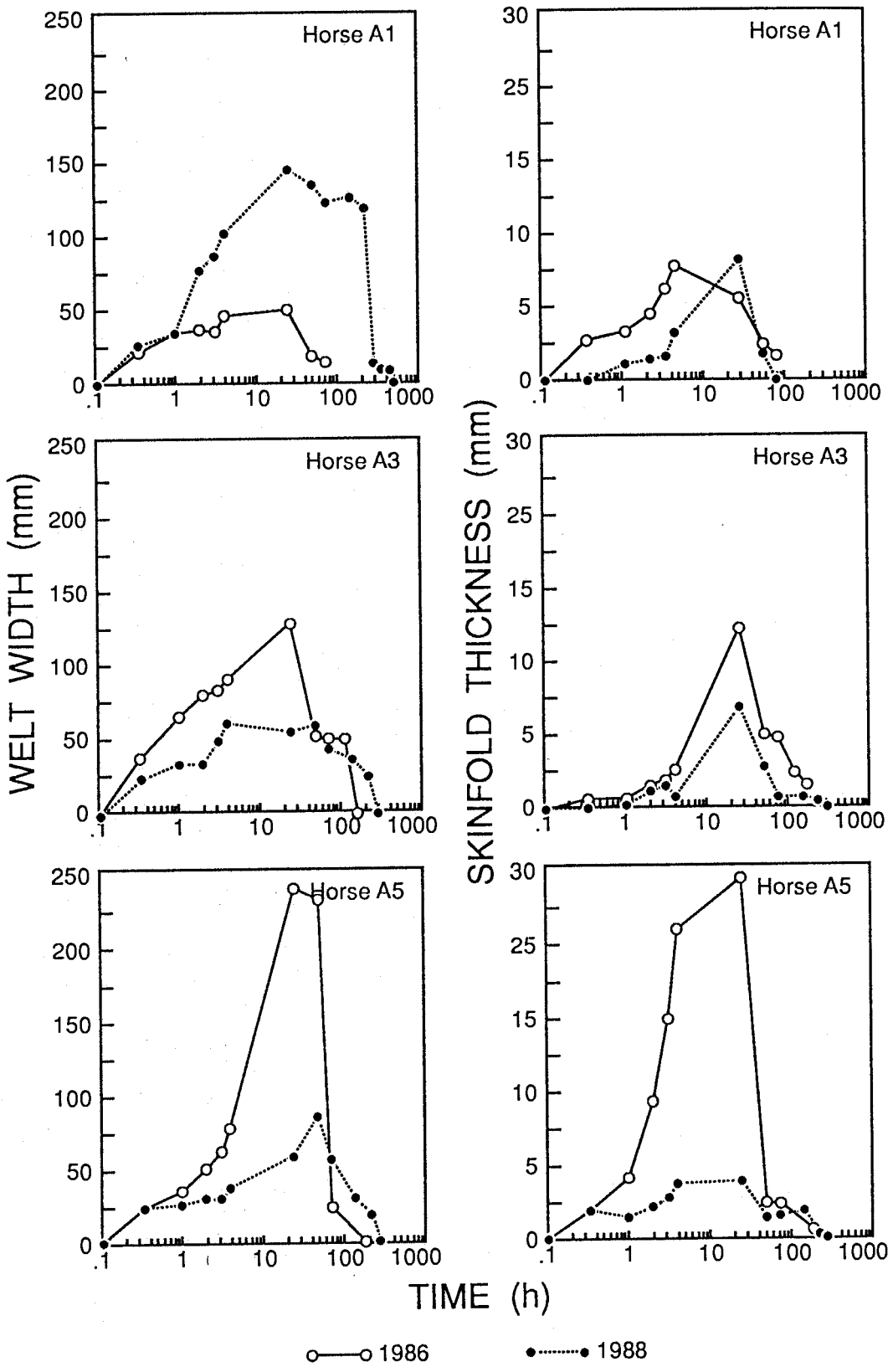
Measurements made in triplicate up to 4 h after challenge for horse A3 disclosed that the error (mean $\pm$ SE) was $\pm$0.0-0.3 mm for skinfold thickness increase and $\pm$0.1-5.5 mm for welt width. These represent 0-2% of the maximum skinfold thickness increase, and 0.1-4.3% of the maximum welt width for this horse.

When horses A1, A3 and A5 were re-tested after two years, horse A1 had a larger welt than after the first test, but the increase in skin thickness did not vary (Fig. 2.3-3). In contrast, horses A3 and A5 had smaller reactions the second time. In particular, A5 had an extremely severe reaction to the first challenge, but reacted only slightly to the second. The disease, which previously had been very severe in this horse in 1986, did not recur in 1987 and has since been very mild.

2.4 DISCUSSION

All the affected horses showed rapid reactions to challenge by *Culicoides* extract characteristic of immediate hypersensitivity or Type I reactions (Coombs and Gell 1975). A typical Type I reaction is a sharply defined, indurated welt, appearing a few minutes after the challenge, reaching peak size in a few hours and then subsiding (Pepys 1975). This accurately describes the appearance of the welt for the first few hours following extract injection. The

Figure 2.3-3. Welt width and skinfold thickness increase in three horses affected by *Culicoides* Hypersensitivity after two separate injections of *Culicoides obsoletus* extract administered in 1986 and 1988.


Horse A1
Horse A1
Horse A3
Horse A3
Horse A5
Horse A5
WELT WIDTH (mm)
SKINFOLD THICKNESS (mm)
TIME (h)
1986
1988

welt is, in most cases, followed by a reddening of the peripheral skin, the 'wheal and flare reaction'. A Type I reaction is an inflammatory hypersensitivity mediated by Immunoglobulin E (IgE) (Tizard 1977). IgE antibodies become bound through the Fc portion of the protein molecule to membrane receptors on basophils and tissue mast cells. Specific antigens crosslink the IgE receptor sites and elicit mast cell degranulation with subsequent release of pharmacologically active substances such as histamine, serotonin and bradykinin (Hanna *et al.* 1982; Jubb *et al.* 1985).

However, because the reactions in most of the affected horses did not peak until 24 h after challenge, they were more consistent with a delayed Type IV, than a Type I hypersensitivity reaction alone. A typical delayed reaction is characterized by a less defined welt which appears after 6 h, reaches a maximum 24-48 h after challenge, and thereafter subsides (Katz 1978). The reaction may take weeks to subside entirely. Type IV reactions are cellular rather than humoral and are mediated by sensitized T-lymphocytes that produce lymphokines which then attract non-sensitized lymphocytes (Tizard 1977; Hodgin *et al.* 1978). Both immediate wheal and flare reactions and delayed-type skin lesions frequently occur following the bite of one species of insect (Humphrey and White 1970; Roitt *et al.* 1985), and both reactions were observed in these experiments.

Tests in Australia showed that the intensity of the reaction of sensitized horses to *C. robertsi*, the causal agent in Queensland, did not vary between tests using extracts of entire male or female specimens, or among extracts of head, thorax and abdomen (Riek 1954). A similar response has also been shown to certain mosquito species (Rockwell and Johnson 1952; McKiel 1959); the allergen in fleas has been found throughout the insect's body (Benjamini and Feingold 1970; Pinnas *et al.* 1978). Even though the allergen in *Culicoides* may occur in the salivary glands, extraction of whole insects should elicit the same response as extraction of the salivary glands. Due to the minute size of *Culicoides*, dissecting out the salivary glands would be extremely labour intensive and somewhat impractical.

In Australia, the reactions of horses challenged with *Culicoides* extract were measured only 1 h after challenge, so delayed reactions were not observed (Riek 1954). However, when monitored for 72 h, horses in Ireland challenged with *Culicoides* extract exhibited both immediate and delayed reactions, although the duration of the delayed response was not measured (Quinn *et al.* 1983). Immediate and delayed responses were also seen in horses in Hong Kong (Baker and Collins 1984) and Florida (Fadok and Greiner 1990). In my study, a reaction was still discernible in some of the affected horses for up to three weeks. Even when a defined welt was no longer visible, an increase in skinfold thickness indicated the persistence of a slight edema.

The development of ruggae at the lesion sites in affected horses was similar to the occurrence of ruggae in natural lesions. Ruggae were also observed in horses skin tested in Israel (Braverman *et al.* 1983). As seen in cattle, (Akey *et al.* 1989), the development of ruggae at the lesion sites may be a form of host immunity wherein *Culicoides* are still able to lacerate the skin, but are not able to blood-feed due to the edema and inflammation. Ruggae formed after several seasons of attack are highly keratinized, which would also make it difficult for the insects to penetrate the skin.

Some reaction would be expected by normal horses to the injection of any foreign protein (Table 2.3-1, Fig. 2.3-1). A certain percentage of normal animals often respond to intradermal challenge with antigens (Kieffer and Kristenson 1979; Moriello and McMurchy 1989). Horse N4 showed a larger reaction than most of the normal horses, although it had no clinical signs of CH. It is possible that this horse had a sub-clinical sensitivity to *Culicoides*. All the normal horses had been exposed constantly to *Culicoides* bites so some may have been slightly sensitized. However, in most of the present cases, a clear distinction was seen between affected and unaffected horses. Therefore, the results clearly indicate that the disease in British Columbia is a hypersensitivity to *Culicoides* species, and suggest that *C. obsoletus* is the major causal or sensitizing agent. This conclusion is supported by the significantly larger and more prolonged reactions in affected horses, the irritation experienced by the affected but not the

unaffected horses after challenge, and the appearance of ruggae similar to those seen in natural lesions in the skin of the affected horses. *C. obsoletus* is probably the most important sensitizing agent because it is by far the most common species (Costello 1982), but other *Culicoides* species may also be involved.

Both welt width and skinfold thickness measurements demonstrated *Culicoides* sensitivity. However, measuring the width of the welt at its widest point was far more accurate than using skinfold thickness as it could distinguish an affected from a normal horse within 1 h post-inoculation. These skin tests indicated that all the affected horses were in stage three of the five stages of skin reactivity, showing both immediate and delayed reactions (Benjamini *et al.* 1961; Benjamini and Feingold 1970; Feingold 1973a).

The re-test on three of the affected horses indicated that the severity of the reaction may have been different after a second injection of extract (Fig. 2.3-3), decreasing in two instances and increasing in one. Quinn *et al.* (1983) reported challenging a single horse with *Culicoides* extract twice in two years and observing no difference in the reaction, although the reaction to other extracts did change. When first injected, horse A5 was already very severely affected by the disease, but two years later it did not develop clinical signs of CH. The clinical signs seen in A1 and A3 did not change and the normal horses did not develop clinical signs over this time. The present method of diagnosis is by the elimination of other potential causes, such as onchocerciasis by using a punch biopsy. It is highly desirable to determine whether a horse is susceptible to CH as there is evidence that there is a genetic predisposition for the disease (Riek 1953a; Ishihara and Ueno 1957; McCaig 1975; Unkel *et al.* 1987; Anderson *et al.* 1988). A diagnostic skin test for *Culicoides* Hypersensitivity would be useful in diagnosing the disease in affected animals, and also in those animals which are affected but do not exhibit symptoms due to the season, or to being continuously protected from bites. Such horses could then be eliminated from breeding programs.

3. BIONOMICS OF *CULICOIDES OBSOLETUS* IN BRITISH COLUMBIA

3.1. INTRODUCTION

Culicoides species have not been studied in great detail in British Columbia. *C. obsoletus* was first reported in British Columbia in 1903 at Kaslo (Hoffman 1925), and a further seven species were collected later in the vicinity of Kamloops (Curtis 1941). A survey in the lower Fraser Valley in 1976 determined the species of *Culicoides* present and their abundance, but did not determine seasonal trends in occurrence (Costello 1982). A survey in 1977 examined seasonal and geographical distribution of *Culicoides* species in relation to bluetongue disease of cattle, but was limited to the Okanagan region (McMullen 1978).

Culicoides obsoletus overwinter as larvae and spend 3.5 to 5 months in the larval stage (Hill 1947). Larvae develop in wet or moist habitats which can include marshy soil around pools and lakes, damp pasture, wet peaty areas (Downes 1958), freshwater sloughs (Costello 1982), acid grassland, standing water (Hendry 1986), decaying vegetation, including rotten banana stems (Braverman, pers. comm.), damp tree holes (Hill 1947), manure piles and decomposing cornstalks (Jones 1961). The pupal stage lasts 5 days, and a blood meal is usually taken within 2-3 days of adult eclosion (Hill 1947). *C. obsoletus* requires a blood meal for the production of each egg batch (Hendry 1986).

As *C. obsoletus* is hypothesized to be the major causal agent of CH in British Columbia, it is important to know the seasonal distribution and peaks of this and other *Culicoides* spp. in the Lower Mainland of British Columbia, where most of the cases of CH occur (Anderson *et al.* 1988). It should then be possible to determine whether the activity of *C. obsoletus* can be correlated with the yearly progress of the disease.

Most *Culicoides* spp. are crepuscular (Twinn 1931; Downes 1958; Kettle 1969). Riek (1953b) observed that *Culicoides* attacked after 1600 h and were particularly active during the early hours of the night, disappearing shortly after daylight. *C. obsoletus* was most active between 1900 and 2200 h in England (Hill 1947).

Stabling an affected horse during the peak diurnal hours of *Culicoides* activity can be an effective method of preventing CH (Kleider and Lees 1984). Even before the etiology of the disease was understood, in Australia, Bancroft (1891) reported that when affected horses were stabled at night they recovered. Riek (1953b) housed susceptible animals in rigorously insect-proofed stables from 1600 h to 0700 h daily over an entire season. The horses were allowed to graze during the day. When confined in this manner, known susceptible horses did not develop clinical signs. Horses already showing clinical signs recovered within 3 weeks, when similarly stabled at night; when re-exposed at night, they developed clinical signs within 3 days. Similar trials in England supported Riek's (1953b) conclusion that stabling affected horses during the hours of peak *Culicoides* activity protected them from CH (Mellor and McCaig 1974).

In the absence of effective treatment, stabling is now commonly recommended as the best method of preventing the development of clinical signs (Ralbag 1954; Pascoe 1973; Frost 1974; McCaig 1975; Baker 1978; Baker and Quinn 1978; McMullan 1982; Stannard 1982; Quinn *et al.* 1983; Robinson 1983; Dakin 1984; Kleider and Lees 1984; Unkel 1984). However, stabling does not necessarily achieve a total control. Several of the horses that I studied in this research were consistently stabled and still showed severe clinical signs. The premise that stabling is effective assumes that *Culicoides* do not enter buildings as stables are very rarely insect-proof. On the rare occasions when they are screened, the screen is designed to keep out insects such as mosquitoes that are larger than *Culicoides* spp. (Robinson 1983). McCaig (1975) and Unkel (1984) have stated that *Culicoides* do not enter buildings, but this apparent assumption has not been experimentally tested. Research in other countries has resulted in claims that *Culicoides* will enter buildings (Braverman and Rubina 1976; Hoshino 1985; Braverman 1988) but in most cases, the 'buildings' were sheds for domestic animals and were completely open on one or more sides (Birley *et al.* 1984). Flies in some species of Ceratopogonidae (species of *Forcipomyia* Meigen, *Atrichopogon* Kieffer and *Alluaudomyia bella* (Coquillett)) were trapped inside buildings in the West indies, but flies in the main mammal biting genus, *Culicoides*, were not (Davies and Giglioli 1979).

This study was conducted to examine the seasonal occurrence of *Culicoides* spp., and to determine whether *C. obsoletus* and other *Culicoides* spp. do enter stables and, if so, the numbers caught indoors, in comparison with those caught outside the stable.

3.2 METHODS AND MATERIALS

3.2.1. Seasonal Distribution

3.2.1.1 Preliminary trapping

A preliminary trapping survey was conducted at five sites in Surrey and Langley, in the Lower Mainland (Appendix II). At least one horse affected by CH was present at each site. Some sites also had several unaffected horses present.

Four New Jersey light-traps equipped with 100 watt light bulbs and one black light-trap were set up as close as possible to the horses' enclosure. The traps were set up between May and June 1985 and were removed after frost occurred in late October. Captured insects were collected twice weekly from small plastic bags at the base of the traps.

Several problems were encountered. Because the fine cotton mesh used to prevent larger insects entering the trap was not strong enough to prevent large moths from breaking through and destroying the sample, a much stronger nylon mesh was used to replace it. At first, the insect samples frequently became waterlogged, so great care was taken to reposition traps where they were not hit directly by rain. Traps were often disconnected and sometimes moved by the property owners. Some traps proved attractive to horses which kicked, butted or bit them and would sometimes actually eat the sample, and so the traps had to be kept out of reach of any animals. Finally, trap placement was limited by the availability of electrical outlets.

There was a great variability between the traps. Black light-traps are reportedly much more attractive to *Culicoides* than are white light-traps (Belton and Pucat 1967; Kwan and Morrison 1973). However, the one black-light trap used caught far fewer *Culicoides* than the

white-light traps, possibly because of poor trap placement and operability, as it frequently shut down, or was disconnected by the owners.

The preliminary trapping survey was of immense value in eliminating problems and selecting the two best sites for intensive study over an entire season.

3.2.1.2 Trapping study

The two trapping sites selected were those where the largest numbers of *Culicoides* were regularly caught. Each site had an electrical outlet which allowed the traps to be placed within 1 m of the horses' enclosure. Each site had a record of excellent owner participation.

Site 1 was in North Surrey in a semi-rural area, close to major highways. Two horses were present, A4 and N4, one of which was severely affected by CH. The trap was hung outside the stable, close to the horse paddock.

Site 2 was in South Langley in a rural area and was some distance from any major highways. Three affected horses were present, A3, A7 and A15. The trap was hung in a tree close to the horse paddock.

New Jersey light-traps equipped with 100 watt light bulbs were used. Each trap had a strong, fine mesh at the top to eliminate as many large insects as possible. As before, the insects were collected in small plastic bags attached by an elastic band at the base of the trap. A small fan prevented the trapped insects from escaping. The bags were collected and replaced twice weekly. The traps were hung 1.7 m above ground under a slight overhang, or under large tree branches in order to avoid waterlogging of the samples. They were set up on 17 March and trapping continued until 17 November 1987. The insect samples were frozen and then later identified and counted under a binocular microscope.

Temperature records were obtained for the trapping period from a Surrey weather station (Surrey Municipal Hall, Lat. 49^{o} 06', Long. 122^{o} 50', elevation 76 m).

3.2.2. Trapping inside and outside stables.

The study sites were the same as those used in the seasonal distribution studies. The traps were set up from 20 July to 25 September 1986. New Jersey light-traps equipped with 100 watt light bulbs were used. The inside traps were exchanged with the outside traps after 5 weeks at both sites to avoid trap bias.

The outside trap at Site 1 was hung outside the stable within 1 m of the horses' paddock. The inside trap was hung in the middle stall of a three-stall stable, approximately 1.7 m high. The stable was large and well built with three 3x3 m stalls and a corridor running the length of the stable. Two doors were left open continuously to allow the horses access to one stall. The horses were prevented from entering the trap stall by a half door.

The outside trap at Site 2 was hung in a tree as before. The inside trap hung 1.5 m high in a small shed within the horse paddock. This was an old structure approximately 2.5 m^2 with two small stalls. The horses did not have access to this shed. The single door was kept open.

The samples were frozen, then identified and counted under a binocular microscope. All the results were analyzed with a two sample t-test, using Minitab (Ryan *et al.* 1985).

3.3 RESULTS

3.3.1 Seasonal Distribution

The first *C. obsoletus* were trapped on 20 March, as soon as the traps were set up (Fig. 3.3.1-1). The numbers of *C. obsoletus* collected peaked in early May and again in late June, and began to decline in September, but small numbers of *C. obsoletus* were collected into mid November. The numbers of *C. cockerellii* (Coquillett)[1] and other *Culicoides* species captured generally followed the trends for *C. obsoletus* (Table 3.3.1-1). Other species collected were *C. tristriatulus* Hoffman and *C. hirtulus* (Coquillett).

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[1] Some of the specimens of *C. cockerellii* were larger than normal and may have fallen within the range of *C. sommermanae* Wirth and Blanton.

Figure 3.3.1-1. Distribution of 17,819 *Culicoides obsoletus* trapped over time in 1987, as well as maximum and minimum temperatures.

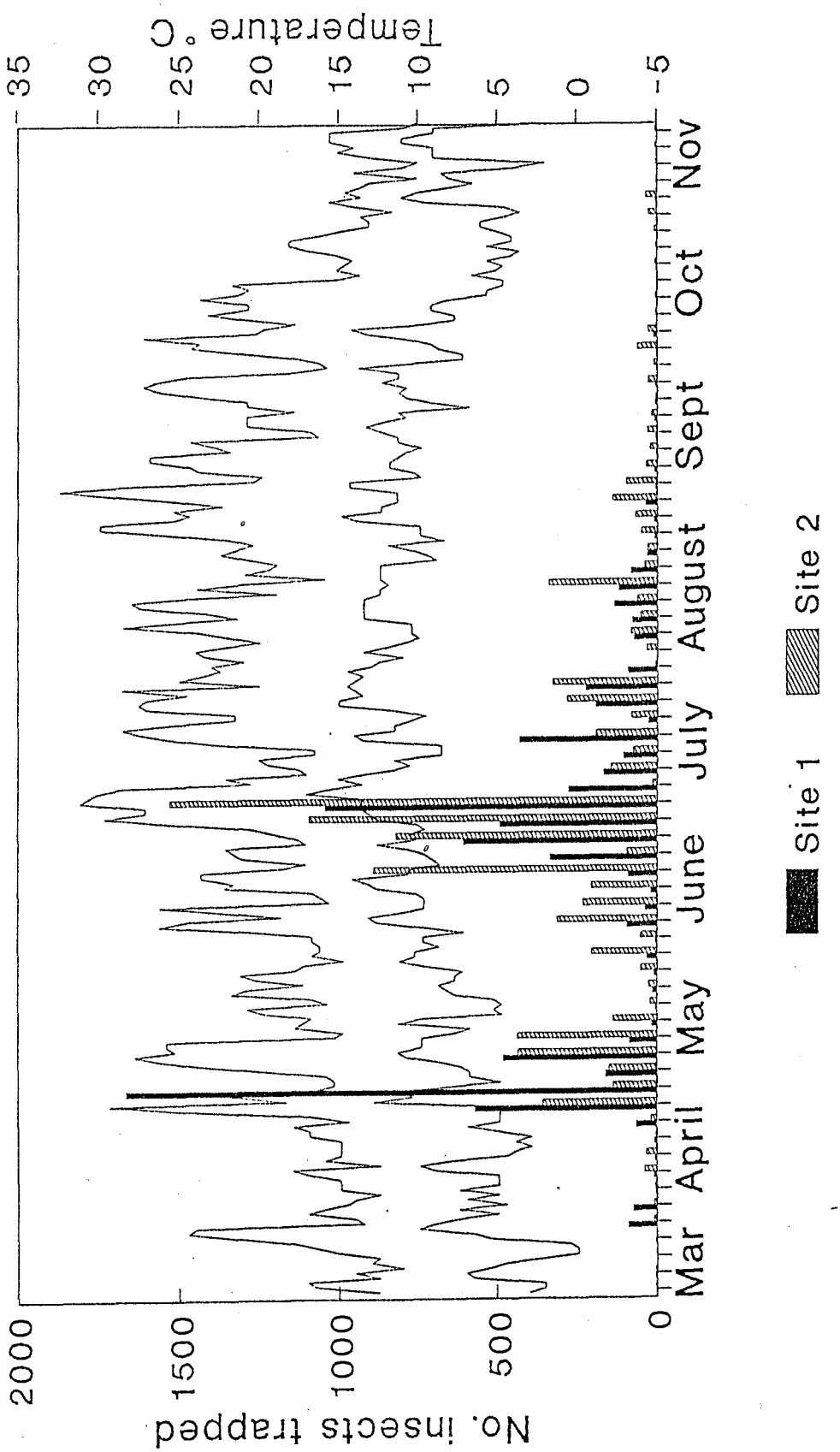

Temperature °C
35
30
25
20
15
10
5
0
-5
No. insects trapped
2000
1500
1000
500
0
Mar
April
May
June
July
August
Sept
Oct
Nov
Site 1
Site 2

Table 3.3.1-1. Numbers of *Culicoides cockerellii* and other *Culicoides* species trapped in 1987 at each site.

Date		SITE 1 *Culicoides cockerellii*	SITE 1 other species	SITE 2 *Culicoides cockerellii*	SITE 2 other species
March	17		Traps set out		
	20	0	0	0	0
	24	0	0	0	1
	27	0	0	0	0
	31	0	0	0	0
April	3	5	15	0	8
	7	5	0	0	6
	10	0	1	1	0
	14	6	0	0	1
	17	27	0	0	1
	21	18	0	0	0
	24	67	0	1	0
	28	3455	0	1	0
May	1	0	0	4	2
	5	233	0	8	6
	8	2000	0	11	5
	12	3	10	1	48
	15	79	0	0	14
	19	3	1	0	0
	22	8	0	3	0
	26	11	6	4	0
	29	2	12	71	8
June	2	2	4	2	6
	5	10	0	69	9
	9	21	5	64	0
	12	6	12	77	20
	16	17	3	57	142
	19	161	11	31	19
	23	132	0	57	44
	26	94	74	59	34
	30	351	536	43	64
July	2	41	69	0	0
	5	68	1	7	22
	9	23	1	7	20
	12	66	83	14	13
	16	17	0	4	5
	19	31	56	4	11
	23	21	29	1	3
	26	41	0	0	0
	30	15	0	0	1

Table 3.3.1-1. Contd.

Date		SITE 1 *Culicoides cockerellii*	SITE 1 other species	SITE 2 *Culicoides cockerellii*	SITE 2 other species
August	4	26	19	0	2
	7	25	1	0	1
	11	17	49	3	2
	14	15	2	0	0
	18	34	1	0	3
	21	10	0	0	0
	25	1	0	0	7
	28	5	4	4	3
Sept.	1	13	14	1	9
	4	6	1	0	0
	8	12	0	1	2
	11	1	0	0	1
	15	1	0	2	0
	18	0	0	1	1
	22	0	0	0	0
	25	0	0	0	0
	29	0	0	2	2
Oct.	2	0	1	1	3
	6	2	1	0	0
	9	0	0	0	2
	13	0	0	0	3
	16	0	0	0	0
	20	0	0	0	0
	23	0	0	0	0
	27	0	0	0	0
	30	0	0	0	0
Nov.	3	0	0	0	0
	6	0	0	0	0
	10	-	-	0	0
	13	-	-	0	0
	17	-	-	0	0
SUM		**7207**	**1037**	**616**	**556**

At Site 2, *C. obsoletus* represented 89.2% of the total *Culicoides* collected, significantly more than other *Culicoides* spp. (t-test, $P \leq 0.001$). At Site 1, it represented 49.7% of the total, but the number of other *Culicoides* species was skewed by two trapping days in which nearly 5,500 *C. cockerellii* were collected (Table 3.3.1-1), comprising 75.7% of the total *C. cockerellii* at Site 1. If these two days in late April and early May are eliminated, *C. obsoletus* represented 74.5% of the total *Culicoides* collected at Site 1 and 81.8% of the total at both sites. *C. cockerellii* were trapped almost entirely (92%) from Site 1.

The seasonal peaks of activity by *C. obsoletus* had a slight but significant relationship with maximum temperature ($r^2=0.35$, $p=0.0001$, regression line for both sites together 0.25 x maximum temperature -0.68) and minimum temperature ($r^2=0.39$, $p=0.0001$, regression line 0.37 x minimum temperature +0.88) after log transformation (Fig. 3.3.1-1). Daily peaks generally were associated with individual periods of warm temperature. Two peaks of activity, in late April and again in late June/early July were observed, suggesting two generations of flying insects (Fig. 3.3.1-1).

3.3.2 Trapping inside and outside stables

Significantly more *C .obsoletus* (Fig. 3.3.2-1), as well as other *Culicoides* spp. (Table 3.3.2-1) were trapped outside stables than inside (two sample t-test, $P \leq 0.005$), and a significantly greater total of all *Culicoides* species was caught outside the stables (t-test, $P \leq 0.005$). However, *C. obsoletus* entered stables on every trap night recorded. *C. obsoletus* represented 90.7% of the total number of *Culicoides* trapped inside stables, and 89.1% of the total *Culicoides* trapped outside stables.

C. cockerellii represented 63.8% of other *Culicoides* spp. The majority (94.4%) of *C. cockerellii* collected were trapped outside the stables (Table 3.3.2-1). As in the seasonal distribution study, most *C. cockerellii* (93.5%) were collected at Site 1. All of the *C. cockerellii* collected inside the stables were trapped at Site 1. Members of significantly more species of *Culicoides* other than *C. obsoletus* were collected at Site 1 inside the stables (t-test, $P \leq 0.001$) and outside the stables (t-test, $P \leq 0.01$) than at site 2. The difference is, again

Figure 3.3.2-1. Numbers of *Culicoides obsoletus* collected from 20 July to 25 September 1986 inside and outside stables.

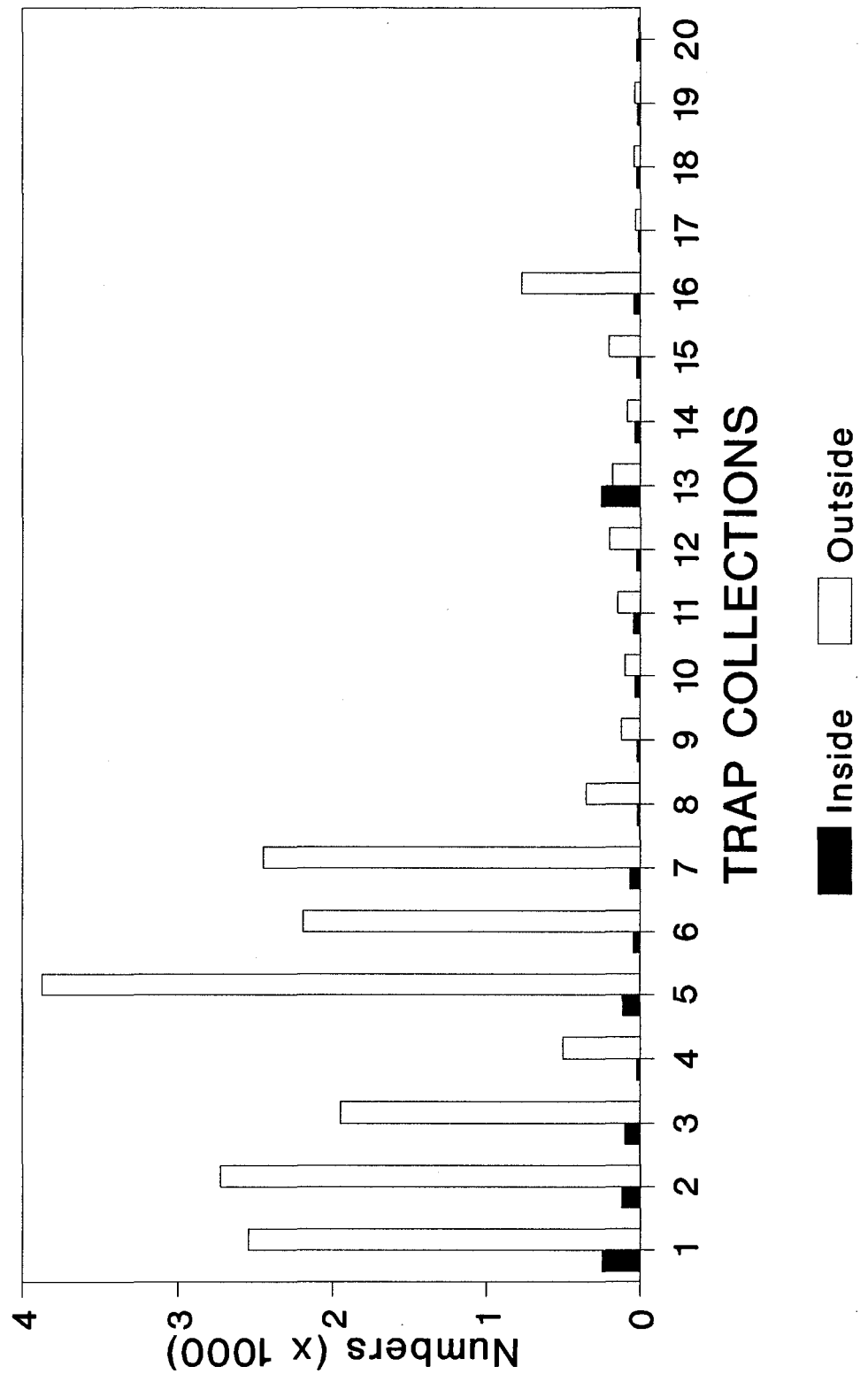

Numbers (x 1000)
0
1
2
3
4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
TRAP COLLECTIONS
Inside
Outside

Table 3.3.2-1. Numbers of *Culicoides cockerellii* and other *Culicoides* species trapped inside and outside two stables in 1986.

Date		INSIDE STABLES *Culicoides cockerellii*	INSIDE STABLES other species	OUTSIDE STABLES *Culicoides cockerellii*	OUTSIDE STABLES other species
July	20	226	10	215	133
	24	18	5	61	471
	27	4	1	137	6
	31	2	0	60	1
Aug.	4	8	3	136	2
	7	7	0	197	1
	11	5	0	160	92
	14	0	0	29	4
	18	0	8	20	1
	21	0	2	2	19
	25	4	4	46	1
	28	0	0	32	2
Sept.	1	5	8	88	2
	4	5	0	27	2
	8	4	0	33	17
	11	2	2	32	0
	15	3	0	16	0
	18	1	0	14	0
	22	2	0	10	0
	25	2	0	1	0
SUM		298	43	1,316	754

accounted for by the presence of *C. cockerellii*.

Fewer insects were trapped at both sites inside and outside stables after the traps were exchanged, indicating a seasonal rather than a trap effect.

3.4 DISCUSSION

3.4.1 Seasonal distribution

My results indicate that light-traps are an effective means of trapping and surveying *Culicoides* spp. in southwestern British Columbia. Jones *et al.* (1977) found light-traps more efficient and more attractive to biting insects than animal bait traps. New Jersey light-traps have been used extensively for collecting *Culicoides* (Jorgensen 1969). In Florida, these traps were used to survey *Culicoides* associated with cattle; they favoured species that are nocturnal, sensitive to light and active at low temperatures, but over an extended time they disclosed the most common species present and the months in which they were abundant (Kramer *et al.* 1985). Light traps are inexpensive, easy to run, need little maintenance, and collect a wide range of species (Acuff 1976). They were, therefore, considered ideal for this study. Because *C. obsoletus* was mainly confined to levels below 3 m above ground level in England (Service 1969), the placement of my traps near the ground is apparently justified.

The first appearance of *C. obsoletus* in mid March, with a peak in late April and early May (Fig. 3.3.1-1) correlates well with the first appearance of the clinical signs of CH. In British Columbia, 73% of affected horses developed the first clinical signs of the year in April and May, with 2.5% developing signs in March (Anderson *et al.* 1988). Similarly, the decline in numbers captured in late summer and early fall (Fig. 3.3.1-1) corresponds well with the regression of clinical signs which occurs in 91.7% of affected horses in British Columbia in September and October (Anderson *et al.* 1988).

However, once an affected horse has begun to show clinical signs, it will continue to worsen over the first few weeks, then will remain severely affected, with little respite, for the rest of the season. Therefore, the duration between peak activity periods (Fig. 3.3.1-1) was

apparently too short to disclose any consistent worsening or regression of lesions. A study of *C. obsoletus* in England indicated that, although density fluctuated rapidly throughout the year, survival and blood-feeding rate varied more slowly, within a seasonal time scale (Birley and Boorman 1982). It has also been shown that patterns of biting activity in *C. variipennis* do not always coincide with flight activity (Nelson and Bellamy 1971).

Culicoides spp. did not occur in the Okanagan region until late April and early May with the last specimens trapped in early October, and *C. obsoletus* was only collected occasionally (McMullen 1978). Moreover, Costello (1982) recorded peak numbers of *C. obsoletus* in early July and again in mid to late August, much later than the second peak that I observed (Fig. 3.3.1-1). In southeastern Washington, the first appearance of *C. obsoletus* did not occur until 10 May and the latest occurrence was in late October (Jorgensen 1969). Therefore, one might expect the onset and regression of CH to vary between years and regions, and possibly to vary according to the capacity of members of different species to induce CH.

The abundance of *C. obsoletus* (49.7% of the total *Culicoides* at Site 1 and 89.2% at Site 2) is somewhat lower than the 99.3% previously reported (Costello 1982). Because *C. cockerellii* was collected almost entirely from Site 1 and only three specimens were collected by Costello (1982) it seems probable that the occurrence of *C. cockerellii* is sporadic and patchy. This hypothesis is supported by the fact that *C. obsoletus* represented 91.2% of the *Culicoides* spp. collected if *C. cockerellii* is not included. McMullen (1978) collected larger numbers of *C. cockerellii* in the Okanagan, but they were found to be mostly associated with wet pastures, bordering slow, meandering streams. Site 1 was a relatively dry pasture, but the lower Mainland region is wetter than the Okanagan. However, this does not explain why so few *C. cockerellii* were collected at Site 2, which would receive equal rain.

The collection of *C. tristriatulus* and *C. hirtulus* in relatively large numbers confirms their collection for the first time in British Columbia in the Fraser Valley in 1978 (Costello 1982), when only one specimen of *C. tristriatulus* and 18 specimens of *C. hirtulus* were

captured. *C. tristriatulus* has been reported as a severe human biting pest in Alaska, where it breeds in salt marshes (Wirth 1952). There were no salt marshes within 30-40 km of my trap sites, but Costello (1982) suggested that *C. tristriatulus* may also breed in fresh water marshes.

Temperature had a slight but significant influence on the numbers of *C. obsoletus* collected; daily peaks were generally associated with periods of warm weather. In contrast with my results, temperature, within the limits of approximately 10 to 22-30^{o}C, was not reported to have an effect on the flight activity of *Culicoides obsoletus, C. pallidicornis* Kieffer, *C. heliophilus* Edwards, *C. pulicaris* L. (Parker 1949), *C. variipennis* (Nelson and Bellamy 1971; Barnard and Jones 1980), *C. impunctatus* Goetghebeur (Parker 1949; Rueben 1963), *C. grahami* (Vatter-Barnard *et al.* 1986), *C. phlebotomus* (Nathan 1981), *C. sanguisuga* (Coquillett) (Jamnback and Watthews 1963), and *C. yukonensis* Hoffman (Shemanchuck 1972), although most species are affected by wind.

3.4.2 Trapping inside and outside stables

The capture of 6% of the *C. obsoletus* inside stables and their presence inside the stables on every trap night means that although a horse is more likely to be bitten if kept outside at night, it could still be attacked in a stable. A single bite was found to cause an increase in histamine level in an affected horse (Riek 1955) and a single night's exposure to bites can result in severe clinical signs (Sippel 1979; Foil and Foil 1990). *C. imicola* Kieffer and *C. oxystoma* Kieffer are known mammal feeders, and were light-trapped inside occupied cattle sheds in Israel, presumably attracted by the presence of the cattle, whereas *C. circumscriptus* Austen, a known bird-feeder, was caught more commonly outside the cattle sheds (Braverman and Linley 1988). *C. obsoletus* was also caught both inside and outside cattle sheds in Israel, together with *C. imicola* and *C. newsteadi* Austen, but Israeli cattle sheds are open, with no wall on at least one side (Birley *et al.* 1984). Many *Culicoides* spp. including some *C. obsoletus*, were trapped inside poultry runs in Israel (Braverman and Rubina 1976; Braverman *et al.* 1977), and *Culicoides* spp. (although not *C. obsoletus*) were also trapped in poultry runs in Zimbabwe (Braverman and Phelps 1981); but again, it is probable

that these were open structures. Some *C. obsoletus* were trapped in horse stables in Israel (Braverman *et al.* 1976), but they are much more open structures than Canadian or European stables (Braverman, pers. comm.). *C. arakawae* Arakawa and *C. odibilis* Austen, which are bird feeders, were trapped in poultry houses in Japan, whereas mammal feeders were more commonly trapped in cattle sheds (Kitaoka and Morii 1964). *Culicoides kingi* Austen was trapped inside animal sheds in northern Sudan (El-Sinnary *et al.* 1985).

In Scotland, *C. impunctatus* was such a persistent nuisance to humans in cow byres that unprotected workers had to leave (Crew *et al.* 1946). *C. obsoletus* was found to pass through mosquito screens into lit, enclosed chambers in the laboratory (Jamnback 1961), and Murray (1957) found that *C. obsoletus* readily entered screened cabins in Virginia, attracted by light.

It is apparent that *Culicoides* species, including *C. obsoletus*, will enter stables in search of a host. In my study, the two stables were quite different. Site 1 involved a large, three-stall stable in which at least one horse frequently spent the night, whereas the stable at Site 2 never contained animals. However, despite these differences, similar numbers of *C. obsoletus* and total *Culicoides* spp. were trapped at both sites.

Horse A4 at Site 1 showed severe clinical signs from March through October, despite spending almost every night inside the stable, showing that it was apparently being bitten inside the stall. However, the annoyance from bites was obviously much less, and when given a free choice of staying in a pasture or entering a stable, affected horses invariably chose the stable.

Horses housed in large show barns do not appear to be affected by CH (Kleider, pers. comm.). This may be due to the large size of the buildings, as opposed to the smaller private stables studied here, or may be due to the repellent effect of increased quantities of carbon dioxide from a large number of animals housed in close quarters (Anderson, pers. comm.).

To protect stabled horses at night, stables should be kept dark and preferably screened. If light is necessary, yellow light is less attractive to *Culicoides* (Braverman 1989b). Strong

screens with a very fine mesh will help to prevent the entry of *Culicoides*, and these can be made lethal or repellent by spraying an insecticide or repellent on them (Jamnback 1961). Because wind can reduce or prevent flight activity (Parker 1949; Yamashita *et al.* 1957; Jamnback and Watthews 1963; Reuben 1963; Kettle 1969; Hendry 1986; Edwards *et al.* 1987), fans may be used to keep *Culicoides* spp. from entering stables or biting animals inside them. Fans have been used to some effect in Louisiana (Foil and Foil 1988; 1990).

Stabling a horse throughout the summer months from 1600 h onwards is usually inconvenient and the concomitant husbandry involved is time consuming. A single day's lapse can result in the development of severe clinical signs. A horse in Louisiana rubbed out its entire mane during one night of exposure (Foil and Foil 1990). Also *Culicoides* spp. will sometimes bite during the day, if it is calm and shady (Kettle 1962); therefore, stabling an affected horse, even in an insect-proof stable on a regular and consistent basis, is very rarely a completely satisfactory method of preventing CH.

4. THE SENSITIVITY OF HORSES WITH *CULICOIDES* HYPERSENSITIVITY IN BRITISH COLUMBIA TO *CULICOIDES* SPECIES FROM OTHER GEOGRAPHICAL REGIONS.

4.1 INTRODUCTION

CH is caused by different *Culicoides* spp. in different regions of the world, despite the fact that the histopathology and epidemiology are identical. *C. robertsi* was found to be the causal agent in Australia using skin tests (Riek 1954); in England the hypothesis that *C. pulicaris* was the causal agent, as its feeding sites and bimodal seasonal distribution coincided with the development of lesions (Mellor and McCaig 1974), was confirmed using skin tests (McCaig 1975). In Ireland, *C. punctatus* (Meigen) and *C. nubeculosis* (Meigen) were suspected as the major causal agents as they are the most common species landing at the lesion sites, although *C. pulicaris* may also contribute to clinical signs (Townley *et al.* 1984). It is assumed that landing sites correspond with biting sites. In Denmark, Icelandic horses frequently develop the disease, and it is usually attributed to *C. pulicaris*, as this species is known to cause the disease in England, is widespread in Denmark, but is not present in Iceland (Hesselholt and Agger 1977). However, no skin tests have been performed to test this hypothesis. In Israel, *C. imicola* was shown to be the causal agent using skin tests (Braverman *et al.* 1983), and by correlating landing sites with lesions (Braverman 1988). In Japan, *C. peregrinus* and *C. obsoletus* are considered responsible, due to their distribution and landing sites (Yamashita *et al.* 1957). In Hong Kong, *C. circumscriptus* is the most common *Culicoides* species present, but although skin tests using a mixed pool of *Culicoides* spp. were performed, individual species were not tested (Baker and Collins 1984). *C. variipennis* is frequently considered to be a causal agent in the United States, although no skin tests have been performed (Braun 1972; Jubb *et al.* 1985). In Florida, *C. insignis* Lutz, *C. stellifer* (Coquillett) and *C. venustus* Hoffman have been implicated based on their mammalophilic behaviour and their flight activity

(Greiner *et al.* 1988), although only *C. stellifer* is known to bite horses (Schmidtmann *et al.* 1980).

These reports lead to the hypothesis that although the disease-causing species itself varies between regions, the sensitizing allergen may be common to many species. My objective was to test this hypothesis by determining whether horses affected with CH in southwestern British Columbia, which were most likely naturally sensitized to *C. obsoletus*, would be equally sensitive to extracts of *Culicoides* spp. known or thought to cause CH in other regions.

This is an important hypothesis to consider when searching for an appropriate treatment for the disease, as a treatment with a worldwide application would be much more desirable than one that is only effective for a few animals. Hyposensitization is a successful therapy in human allergic diseases, and could potentially be beneficial in treating horses with CH. However, it is unlikely to be practical if the allergen is different in every region where CH is a problem.

To attempt a hyposensitization trial in British Columbia, a large number of *Culicoides* would be needed on a regular basis. However, I was unable to light-trap enough *C. obsoletus* over two years to carry out such a trial. Also hand sorting the trap collections under a binocular microscope is extremely labour intensive. Therefore, it was necessary to determine whether a laboratory raised *Culicoides* species contained the allergen.

Six different sources of *Culicoides* were selected. *C. obsoletus*, the known sensitizing agent in southwestern British Columbia, was used as a comparison for the subsequent species. *C. cockerellii,* which was caught in large numbers on two occasions in B.C. (Table 3.3.1-1) was the other local selection. *C. imicola,* the causal agent of CH in Israel (Braverman *et al.* 1983; Braverman 1988) was chosen because it is a widespread species on horses in Israel (Braverman *et al.* 1976; Braverman 1988), and has not been reported in British Columbia (McMullen 1978; Costello 1982). *C. biguttatus* (Coquillett), a horse-biting species known to cause CH in Louisiana (Foil and Foil 1990) was another exotic selection. It is extremely rare in southwestern British Columbia (Costello 1982), and was not collected in the Okanagan

region of British Columbia (McMullen 1978), but is one of the most frequent biters of horses in New York (Schmidtmann *et al.* 1980). *C. obsoletus* from Louisiana was also selected as it belongs to a species complex (*avaritia*), and the insects in Louisiana may be different from those in British Columbia. In England, *C. obsoletus* does not bite at the site of the lesions and it is not considered to cause CH (Mellor & McCaig 1974). *C. variipennis* represented a species that is mass-raised by the USDA (G. Hunt, pers. comm.). It is not present in southwestern British Columbia (Costello 1982) but was captured in small numbers in the Okanagan (McMullen 1978). It has been implicated as a causal agent of CH in the United States (Braun 1972; McMullan 1982), and was the most common biting insect found to attack a horse in three southwestern States (Jones *et al.* 1977).

4.2 METHODS AND MATERIALS

Six horses in southwestern British Columbia, A7, A8, A9, A10, A11 and A12, were selected solely on the basis of their severe and typical lesions of CH. All had the disease severely enough to warrant veterinary attention, and had been exposed to the bites of *Culicoides* spp., particularly *C. obsoletus*, under natural conditions. The horses ranged from 4 to 9 years old, palomino to bay color and were mostly pure or cross-bred Morgans, Quarterhorses, Arabians and Standardbreds (Appendix I). All had clinical signs in the ventral midline and tail, and most also had lesions in the mane, withers, face and genital region, and were known to have developed lesions for several consecutive years.

C. obsoletus and *C. cockerellii* were trapped in southwestern British Columbia, and 1% extracts in saline were prepared as in Chapter 2.2. A volume of 0.1 mL of a 1% extract resulted from 168 *C. obsoletus*. *C. biguttatus* and *C. obsoletus* were trapped in Louisiana and 1% extracts were prepared as above and shipped by Dr. L. Foil, Louisiana State University, Baton Rouge, LA. Dr. Foil also prepared and shipped a 1% extract of *C. variipennis*, which was obtained from a mass-reared colony established in 1957 from specimens collected in Texas (G. Hunt, pers. comm.). *C. imicola* were trapped in Israel by Dr. Y. Braverman, Kimron

Veterinary Institute, Beit Dagan, Israel. They were shipped freeze-dried and a 1% saline extract was prepared as above at Simon Fraser University.

Each insect extract was sterilized as before, and checked for contamination by incubating an aliquot in nutrient broth at 37°C for 48 h. Each extract was measured into 0.2 mL aliquots and kept frozen at -18°C until just before use. Aliquots of physiological saline (0.2 mL) were also autoclaved and frozen until used.

Each horse was clipped along the side of the neck, using size 40 clipper blades, and the area was cleaned and sterilized with 70% ethanol immediately before injection. The resultant cleaned area measured approximately 40 by 20 cm along the length of the near side of the neck. Each horse received seven intradermal injections approximately 5 cm apart in a row along this clipped area. The injections were 0.1 mL of each *Culicoides* extract (168 insect equivalents) and one of 0.1 mL saline as a control. The extracts were not injected in the same order in each horse. Tuberculin syringes with 26 gauge needles were used to minimize skin trauma.

The width of the welt resulting from each injection was measured at its widest point using vernier calipers at 20 min, 1, 2, 3, 4, 24, 48 and 72 h after inoculation. Skinfold thickness increase was not analyzed as it had previously been shown that the width of the welt was a far more diagnostic measurement than was skinfold thickness increase (Chapter 2.4).

The width of the horse's reactions to the individual extracts over time from 0.33 to 72 h post-inoculation were analyzed using a two-way ANOVA with repeated measurements (SAS 1988). A curve was generated for each horse's reaction to each extract over time for 0 to 72 h post-inoculation and the area under each curve was calculated using integration, then analyzed with a two-way ANOVA with repeated measurements, comparing reactions between extracts and over time.

4.3 RESULTS

The horses all developed a swelling at the site of each insect extract injection. The injection sites showed visible signs of swelling within 20 min of inoculation. The welt widths changed significantly over time from 0.33 to 72 h post-inoculation ($P=0.0026$), gradually increasing over time, but did not reach a peak for at least 24 h (Fig. 4.3-1). They then declined sharply, although some horses still exhibited a reaction for up to 288 h. For the first 4 h the welts were firm, hard to the touch and well-defined; after 24 h, they became less indurated, larger and poorly defined. As before (Chapter 2.3), the welts changed from a round to an oval shape, running down the neck, and again extended further below the injection site than they extended above it.

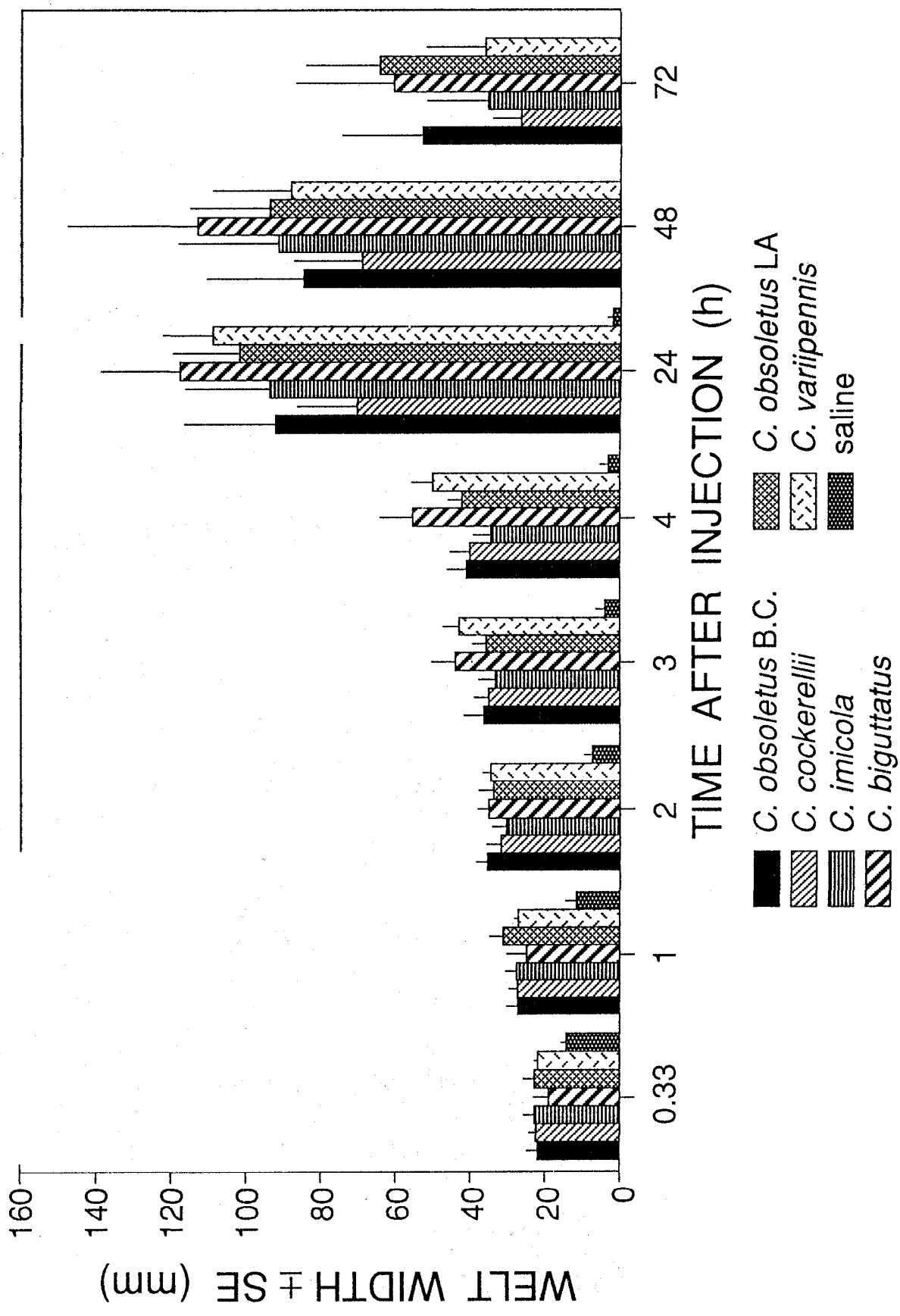
The saline injections produced a bleb in the skin, which did not increase in size, indicating that the bleb was caused by the volume of saline infiltrating the tissues, and not by an allergic reaction. The saline reaction never appeared hard, and gradually dissipated.

The horses reacted similarly to each *Culicoides* extract (Figure 4.3-1.). By 2 h after injection, welts from all extract treatments were significantly wider than those from the saline control (ANOVA, $P \leq 0.05$), and the difference between experimental and control treatments remained large and significant throughout the 72 h observation period. There was no significant difference in welt widths between extract treatments (ANOVA, $P=0.1652$), and there was no interaction between the time and treatment (ANOVA, $P=0.1463$), *i.e.* the horses reacted the same way over time, for each extract.

Also, when area under the curve plotted for each horse was compared using a one-way ANOVA with repeated measurements, there was no significant difference between treatments (ANOVA, $P=0.1522$).

After several days, all of the horses showed small vertical ridges, approximately 1-2 cm wide and running the length of the neck, at the extract inoculation sites. All the horses were extremely irritated by the inoculations and were very sensitive at the reaction sites for the first 24 h after inoculation. They were difficult to handle and attempted to rub the inoculated

Figure 4.3-1. Mean welt width over time of six horses injected with 1.0% extracts in saline of *Culicoides* spp. from six different sources.


WELT WIDTH ± SE (mm)
160
140
120
100
80
60
40
20
0
0.33
1
2
3
4
24
48
72
TIME AFTER INJECTION (h)
C. obsoletus B.C.
C. cockerellii
C. imicola
C. biguttatus
C. obsoletus LA
C. varilipennis
saline

area. Several horses had to be tethered in a way that prevented them from scratching the lesion sites to avoid self-mutilation.

4.4 DISCUSSION

The results indicate that the allergen responsible for the disease is common to all six forms of *Culicoides*. All the horses showed typical immediate (Type I) and delayed (Type IV) reactions (Coombs and Gell 1975; Pepys 1975; Katz 1978) to all the extracts, despite the fact that previously they had only been exposed to *C. obsoletus*, with perhaps some exposure to *C. cockerellii*. They did not react to the saline injection, indicating that the reactions were due to the extracts injected and not to the trauma of injection. The reactions were very similar to those recorded previously in affected horses to skin tests with locally collected *C. obsoletus*. After the six injections, all of the affected horses developed transverse ridges similar to those seen in natural cases of the disease at the lesion sites. The side of the neck is not affected by natural lesions, and the ridges were not present before inoculation, so were a direct result of the inoculations. This phenomenon was also observed in the affected horses injected with *C. obsoletus* extract, but was not seen in the normal horses (Chapter 2).

C. imicola and *C. variipennis* have not been reported from southwestern British Columbia and *C. bigutattus* is only present in extremely low numbers, so it is unlikely that the horses could have been previously sensitized by these species. The irregular and infrequent outbreaks of *C. cockerellii* may have contributed to the sensitization of susceptible horses, but it is unlikely that they play an important role.

Baker and Quinn (1978) and Quinn *et al.* (1983) skin-tested horses in Ireland with a 1% extract of a pooled mixture of Irish *Culicoides* (no species identified) caught in a light-trap, and reported positive reactions in affected horses. The test animals showed both immediate and delayed hypersensitivity. Baker and Collins (1984) found that affected horses in Hong Kong tested positively to a 1% extract of a pooled mixture of the Irish *Culicoides*. The only species of *Culicoides* they describe as common in Hong Kong is *C. circumscriptus*, which is unlikely to have been one of the species included in the pooled Irish mixture as it has not been reported

as a horse-feeder in Ireland (Townley *et al.* 1984). Thus the Hong Kong horses probably reacted to species of *Culicoides* to which they had not previously been exposed, indicating that the allergen was common to the species in Ireland and Hong Kong.

A single affected horse in Ireland was skin tested with 1% extracts of *C. circumscriptus* and *C. puncticollis* Becker from Israel, neither of which is known to bite horses in Ireland (Townley *et al.* 1984), and also with a 1% extract of pooled Irish *Culicoides* as a comparison (Braverman *et al.* 1983). The horse showed strong immediate and delayed reactions to *C. circumscriptus* and to the pooled Irish *Culicoides* and weak immediate and delayed reactions to *C. puncticollis*. This result is somewhat surprising as *C. circumscriptus* is a known bird-feeder (Kitaoka and Morii 1964), whereas *C. puncticollis* is a mammal feeder (Braverman *et al.* 1983). However, as only one horse was tested, no firm conclusions can be drawn. It is unfortunate that the suspected causal agent of CH in Israel, *C. imicola* (Braverman *et al.* 1983), was not tested.

In Australia, skin tests with extracts of three *Culicoides* spp. in three affected and three normal horses gave a consistently positive reaction only with *C. robertsi* (Riek 1954). However, the reactions were only monitored for one hour post-inoculation, and although allergic reaction can be detected after such a short time when the width of the welt is measured and compared, allergic reaction in the horses I have tested did not reach a peak until 24 h after injection. It therefore seems possible that the other species may have caused delayed skin reactions if the horses had been monitored over a 24 h period or more.

Other allergies in both man and animals have often been reported to be elicited by several species, and even genera of insects, indicating common antigens. Dogs with flea dermatitis react to dog, cat and human fleas (Baker and O'Flanagan 1975), and humans with flea allergies are sensitive to *Pulex irritans* L., *P. stimulans* Baker, both human fleas, and *Ctenocephalides felis* (Bouché), the cat flea, although they were generally most sensitive to *P. irritans* (Hudson *et al.* 1960). Michaeli and Goldfarb (1968) also reported that allergic dogs and cats reacted equally to *C. felis, C. canis* (Curtis), *P. irritans* and *Xenopsylla cheopis*

(Rothschild), indicating common allergens shared between genera as well as species. Furthermore, Johnston and Brown (1985) found that guinea pigs sensitized to *X. cheopis* reacted to the bites of a tick, *Ornithodorus parkeri* Cooley. *Aedes aegypti* (L.) and *Ae. dorsalis* (Meigen) were found to share one or more salivary antigens which elicited a response in sheep (Jones and Lloyd 1987), and guinea pigs sensitized to one species of mosquito were found to react to three other species, although their responses to the sensitizing species were strongest (French and West 1971).

Because of the virtually identical responses of extracts of *Culicoides* from widely separated geographical origins, immunotherapy based on the causal allergen might be used over a wide geographical area. Since *C. variipennis,* raised in the laboratory for over 30 years, obviously contain the allergen, it would be an ideal insect to use in a hyposensitization trial as it is easily obtained in large numbers.

5. THE EFFICACY OF A MAST CELL STABILIZER IN TREATING *CULICOIDES* HYPERSENSITIVITY

5.1 INTRODUCTION

The allergic reaction shown by horses to bites of *Culicoides* is evidently a hypersensitivity to a specific allergen or allergens, and affected horses show typical Type I and Type IV hypersensitive reactions (Chapter 2).

Type I reactions are caused by antigen-antibody interactions (Coombs and Gell 1975). Allergy can be viewed as the immune system acting incorrectly. Normally, lymphocytes are responsible for recognizing harmful foreign substances, or antigens, by means of receptors on their surface that are shaped to bind to particular conformations of molecules. When a B lymphocyte encounters an antigen, it binds to it and becomes transformed into a plasma cell, which then synthesizes antibodies against that particular antigen (Camazine 1987). When an individual is first exposed to an antigen, no allergic reaction occurs, but the plasma cells that are produced begin to manufacture millions of antibody molecules against the antigen. Plasma cells produce five classes of antibody, each with a specific immunological role. One of these is Immunoglobulin E (IgE), not usually important in normal individuals, although it can help to repel parasitic attack (Dessaint *et al.* 1979; Gamlin 1989). It normally accounts for only 0.001% of all the antibodies in human blood (Camazine 1987). However, in allergic individuals, a particular allergen may cause the plasma cells to produce large numbers of IgE antibodies that are released into the blood stream. These attach to the surfaces of mast cells and basophils (both are white cells) throughout the body's connective tissues and in the blood, through the Fc portion of the IgE molecule. The attachment of IgE molecules to mast cells and basophils sensitizes the individual to this allergen. Similar immunoglobulins have been identified in the horse (Suter & Fey 1981; 1983). When the complementary antigen (allergen), enters the system, it binds to the Fab regions of the bound IgE and cross-links two IgE molecules (Tizard 1977). This initiates rapid changes in the mast cell membrane, in particular,

increased calcium permeability, resulting in the release of granules (degranulation) containing vasoactive substances including histamine, serotonin (5-hydroxytryptamine) and kinins (Hanna *et al.* 1982). These substances pharmacologically mediate the clinical signs of allergy. Some of the mediators, such as histamine and serotonin, are liberated preformed from intracellular granules; both of these mediators have been shown to be released from lung tissue and white blood cells of horses (Burka *et al.* 1976). Other preformed mediators are eosinophil chemotactic factor of anaphylaxis (ECF-A) and neutrophil chemotactic factor of anaphylaxis (NCF-A), although they have not yet been reported in Type I reactions in horses (Hanna *et al.* 1982). Other substances, such as prostaglandins, kinins and slow reacting substance of anaphylaxis (SRS-A or leukotrienes), are synthesized *de novo* and released only after immunological triggering has occurred. A large release of anaphylactic factors may cause fatal shock.

Histamine constricts smooth muscle, increases secretions and venule permeability and stimulates sensory nerves (Hanna *et al.* 1982). Histamine is known to be involved in equine Type I hypersensitivity, with significant increases occurring during anaphylactic shock (Eyre and Lewis 1973). Serotonin also constricts smooth muscle and stimulates sensory nerves. Kinins, in particular bradykinin, constrict smooth muscle, increase secretions and venule permeability and produce pain. Prostaglandins are considered secondary mediators of hypersensitivity as they are released from tissues by histamine or by smooth muscle contractions. They regulate bronchial constriction, vascular resistance and permeability, platelet and leucocyte activity and the release of other mediators. Leukotrienes constrict smooth muscle, increase secretions and venular permeability. ECF-A and NCF-A are selectively chemotactic for eosinophil and neutrophil white blood cells, respectively (Hanna *et al.* 1982). After release, the mediators may produce their effects systemically or locally through direct actions on tissue receptors or indirectly *via* nervous reflexes. Most of the mediators are not selective for a particular target tissue or organ.

Reports of the importance of the various mediators in equine allergy vary. Histamine and serotonin were found to be less important in equine anaphylaxis than kinins, SRS-A and prostaglandins (Eyre 1976), although isolated lung fragments from horses sensitized to bovine plasma liberated histamine and serotonin when incubated with bovine plasma, but did not liberate SRS-A (Burka *et al.* 1976). Walton (1971) found that horses had a low histamine level in the blood, and Eyre (1972) found that anti-histamines were of little use in treating allergic horses. Skin tests showed that although histamine and bradykinin each caused welts in horses resembling a Type I response, serotonin alone produced a weak response (Morrow *et al.* 1986).

Anything that can inhibit or interrupt any part of the events described will prevent the release of the mediators of allergy and will thereby prevent the manifestation of the clinical signs. Drugs that act as mast cell stabilizers prevent the breakdown of the mast cell membrane, thus preventing the release of their mediators.

Ventipulmin[1] or clenbuterol hydrochloride (4-amino-α-[(tert-butyl-amino) methyl]-3,5-dichlorobenzyl-alcohol hydrochloride) is a sympathomimetic amine (Eyre *et al.* 1982) which binds selectively to $ß_2$ adrenergic receptors. Synthetic sympathomimetic amines are chemical analogues of adrenaline which cause smooth muscle dilation, vascular constriction and decreased vascular permeability, reduced glandular secretion and cardiac acceleration (Eyre *et al.* 1982). Adrenaline acts on two kinds of receptors, α and ß : α receptors are involved in blood pressure and vasoconstriction, whereas ß receptors are involved in heart and smooth muscle effects (Eyre *et al.* 1982). Adrenaline, and many synthetic sympathomimetic amines, prevent bronchoconstriction and also act by preventing the release of histamine (Assem and Schild 1969), possibly by stabilizing components of the mast cell membrane.

Clenbuterol hydrochloride was first synthesized in 1972 (Keck *et al.*) and has been used in human medicine under the name NAB 365[1] to control bronchospasm in asthmatic patients (Cummiskey *et al.* 1978; Curti and Vibelli 1979; Pasotti *et al.* 1979; Baronti *et al.*

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[1] registered trade name.

1980; Brusasco *et al.* 1980; Blom-Bülow *et al.* 1985). It has also been successfully used to control Chronic Obstructive Pulmonary Disease (COPD, "coughing horse" syndrome) in horses (Sasse and Hajer 1978).

The major action of clenbuterol hydrochloride involves its action on the β_2 receptors, resulting in bronchodilation; however, as it is also reported to have mast cell stabilizing properties (Engelhardt 1976; Del Bono *et al.* 1979; Eyre *et al.* 1982; Teitzel 1982) it should have a beneficial effect on other allergies which do not involve bronchial disease, such as CH. Stabilization of the mast cell membrane should prevent the release of allergy mediators.

As it acts selectively on the β_2 adrenergic receptors at low doses (O'Donnell 1976; Bohmer and O'Donnell 1977) and only acts on β_1 receptors at higher doses (Engelhardt 1976), clenbuterol hydrochloride has little effect on the heart, where β_1 receptors predominate (Pasotti and Vibelli 1979). A rapid increase in heart rate in horses was seen immediately after intravenous injection, but this returned to normal within 2 min (Shapland *et al.* 1981). However, no such increase was seen in horses treated orally with the drug. Also the dichlorinated substitution of the benzene ring in the structure of clenbuterol hydrochloride results in rapid oral absorption (Cummiskey *et al.* 1978; Menard 1984). This means that, in most cases, oral administration is preferable to injection, thereby further reducing the risk of side-effects.

Clenbuterol hydrochloride is also long acting, with a half-life of 35 h and a duration of action of 8-10 h, indicating that two daily doses should be effective (Pasotti *et al.* 1979).

As clenbuterol hydrochloride has shown mast cell stabilizing properties, is effective orally and has a long duration of action, it appeared to have potential in treating CH. Clenbuterol hydrochloride, under the name Ventipulmin, was used in this study.

My objectives were :

1. to determine if clenbuterol hydrochloride could partially or completely control CH in horses,

2. to determine the efficacy of clenbuterol hydrochloride in a controlled trial over an entire season, and

3. to determine whether clenbuterol hydrochloride could be effective in controlling CH after clinical signs were present, and if so, how rapidly, as this would be an important consideration for owners. When the preliminary results were analyzed, the third objective was changed. I originally planned to test this objective by giving clenbuterol hydrochloride to one of two separate groups of chronically affected horses after they had developed the clinical signs of CH. This was changed to determining whether the skin tests used during this trial to evaluate the efficacy of the drug over the season might, in themselves, have a beneficial effect on the disease.

5.2 PRELIMINARY TRIAL

A preliminary trial was conducted in 1987. The trial used two horses, A1 and A5 which were known to have been severely affected in past years. A third horse was originally included, but the owner, although at first believing that the horse's clinical signs were regressing, later decided that her horse was not benefitting from the treatment and withdrew from the preliminary trial after approximately three weeks.

Horses A1 and A5 were already showing clinical signs when clenbuterol hydrochloride[2] was prescribed. Horse A5 had developed severe open lesions in the ventral midline, and had also rubbed out its mane and tail for the past 10 years. However, it had been moved approximately 5 km from its original location in the previous winter, and had not spent an entire season at the new location before this trial began. Horse A1 had developed severe open lesions in the ventral midline, chest, face and between the hind legs for the previous five years, as well as rubbing out its mane and tail. It was kept at the same location for this time.

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[2] Clenbuterol hydrochloride was kindly donated by Boehringer Ingelheim (Canada) Ltd., 977 Century Drive, Burlington Ontario.

Treatment of horse A5 began on 17 April and of horse A1 on 3 May. Each horse was given 0.05mg/kg body weight of clenbuterol hydrochloride [0.8 μg/kg of the active ingredient, clenbuterol hydrochloride (Menard 1984)]. Both horses weighed approximately 500 kg. I examined both horses weekly; they were monitored daily by the owners.

Horse A5 began to show an improvement within two weeks of the start of treatment. The horse was still pruritic but no open lesions were present, and the mane and tail, which were previously rubbed out by the same time in previous years, were still intact. The owner considered that clenbuterol hydrochloride was at least as effective as the previous treatment, prednisolone, a corticosteriod. By 6 June, the horse was still virtually free of lesions. All previous papules and lesions had healed. Slight hair loss was noted along the ventral midline, but was very minor compared with the open lesions seen on this horse in the past. Due to this success, treatment was continued for the entire *Culicoides* season. Horse A5 did not develop clinical signs for the rest of the season. It is possible that the drastic improvement noted in this horse was due to the change of location, as some local areas may have much larger *Culicoides* populations than others. However, as the horse had already begun to develop clinical signs at this new pasture when the treatment began, it is obvious that a significant number of *Culicoides* were present.

Horse A1 also began to show an improvement two weeks after the start of the trial. Most of the open lesions healed and although the horse was still pruritic, it was not as severe as in the past. By 8 June, no open lesions were present, although some more minor lesions were still present on the inner thighs, ventral midline and chest. The horse appeared to have improved with treatment, but still showed some clinical signs. The owner considered that the treatment was 50-60% effective. Treatment of horse A1 concluded in mid July. At this time it was moved to a field approximately 2 km away and shortly after the conclusion of treatment, its condition worsened considerably. Therefore, its worsened condition may have been due to the lack of clenbuterol hydrochloride, the change of site, an increased number of *Culicoides* at this time in the season, or a combination of any of these factors.

Both horses showed clinical signs before treatment began, and there was approximately a two week lag period before any improvement could be seen. This may have been due to the presence of allergy mediators, such as histamine, in the blood, or because oral medications are much slower acting than injected medications. Also, many animals continue to scratch lesions, even when the mediator is no longer present, *e.g.* flea allergy in dogs (Chapter 1.1).

Three other affected horses, A3, A4, A15, were pastured in the same general area as A1 and A5, so acted as untreated controls. Each began to develop clinical signs in late March, and by June were severely affected. They continued to show severe clinical signs until mid October.

This preliminary trial suggested that there was potential for using clenbuterol hydrochloride to treat CH. Both horses showed an improvement, and one became completely sound. However, as only two horses were tested, it is possible that other factors may have been involved in the regression of lesions, so a larger scale trial was conducted.

5.3 METHODS AND MATERIALS

Twelve horses severely affected with CH were chosen. The horses were divided into two groups. Group 1 consisted of A1, A3, A5, A13, A14, and A15 and were identified by the number 1, followed by their designated letters. Group 2 consisted of A7, A8, A9, A10, A11 and A12 and were identified by the number 2, followed by their designated letters.

5.3.1 Group 1 Horses

Each horse was given a dose of 0.05 mg/kg body weight of clenbuterol hydrochloride[2], administered twice daily in their grain, from 18 March until the end of October. Treatment began before any of the horses had developed clinical signs, and just before they would be expected to start showing signs of pruritus (Anderson *et al.* 1988). The horse owners were responsible for administering the drug, and so each horse in Group 1 was chosen because it was known to suffer severely from CH, and because the horse's owner was prepared to administer clenbuterol hydrochloride at the required times.

Each horse was examined visually every week and photographed to assess the extent of the clinical signs, which were ranked with an arbitrary index number, based on severity, as follows :-

0 - no signs.

1 - very mild signs, some scratching, small scabs, slight hair loss. No open lesions.

2 - moderate signs, more scabs, hair loss, horse irritated.

3 - more severe, bald areas, scabs, red open areas, broken hair in mane and/or tail indicating scratching.

4 - very severe, large areas of hair loss, raw, open lesions, horse severely irritated, hair in mane and/or tail rubbed down to skin.

Intradermal skin tests were performed regularly over the trial period in order to provide a quantitative measure of the efficacy of the drug. Skin tests are a measure of the reactivity of the horse to a known allergen. They elicit the same chain of events as the natural bites of the causal insect, presumably including the breakdown of the mast cells and the consequent release of the mediators of allergy. Therefore, if the drug acts as an effective mast cell stabilizer in the skin, regular skin tests should demonstrate this effect.

Each horse was skin tested with 0.1 mL of a 1% extract of *C. obsoletus* prior to the appearance of clinical signs, in February 1988, in order to confirm the diagnosis of CH, and to use the results as a baseline for comparison with later tests. The only difference in protocol from the tests described in Chapter 2.2 was that the horses were not clipped prior to injection each time, as the owners objected to the unsightly aspect of the clipped area, particularly as it would have been repeated so often. Welt width at the widest part of the lesion was measured 20 min and 1, 2, 3, 4, 24, 48, 72, 144, 216 and 288 h after inoculation. In some cases, the reactions were monitored longer if the reaction persisted. Each horse was retested approximately one month later, and then every two months for the duration of the trial.

5.3.2 Group 2 Horses

Group two horses were tested in order to determine whether regular skin testing of affected horses might result in some improvement in clinical signs. Tests began in June, after horses in Group 1 began to develop clinical signs of CH despite the clenbuterol hydrochloride treatment. The original objective for Group 2 was to determine how long it would take for CH-caused lesions to regress after administration of clenbuterol hydrochloride. Because Group 1 horses did not develop clinical signs as early in the year as they had in previous years, it seemed possible that the skin tests, rather than the drug itself, might be responsible for this brief respite. The first two skin tests in Group 1 horses were administered only 4 weeks apart, whereas later skin tests were staggered by 8 weeks. Therefore, the altered objective of the tests on Group 2 horses was to determine whether regular skin tests alone might result in an improvement in clinical signs.

All six horses in Group 2 were showing severe clinical signs before skin tests were started, apart from 2-A11 which was kept in a well-screened stable to prevent development of lesions, which would make its presentation as a show horse impossible. However, it had shown severe clinical signs and pruritis when exposed in previous years.

The skin tests were performed in the same manner as in Group 1 horses, using the same extract, but were administered more frequently, so that each horse was tested every four weeks, instead of every two months. (The first skin test in each horse confirmed the diagnosis of CH).

The welt formed after the first skin test on each horse was measured at its widest part 20 min and 1, 2, 3, 4, 24, 48, 72, 144, 216 and 288 h after injection in order to check that the progress of the reaction followed the normal course. However, due to the inconvenience to the veterinarian in having to be present on different days to inoculate each horse, after the first injection, each horse was only measured 2 and 4 h after injection on the first day, and at the normal intervals on the second and following days. Therefore, several horses could be skin tested on the same day.

As in Group 1, each horse was examined visually on a regular basis and photographed to assess the extent of the clinical signs, which were ranked as for Group 1 horses. Horse 2-A12 had a violent reaction to needles and was, therefore, extremely difficult to handle. It was dropped out after its first skin test and was not replaced, reducing Group 2 to 5 horses.

The width of the skin reactions over time to each injection were compared within each group using a two-way ANOVA with repeated measurements (SAS 1988). Reactions within each group were compared between injections and over time. Reactions between groups for the first four injections were compared using a three-way ANOVA with repeated measurements on two factors, (time and injection). Data from Group 1 at 2, 4, 24, 48, 72, 144, 216 and 288 h post inoculation were used, so that the two groups were comparable.

5.4 RESULTS

5.4.1 Group 1 Horses

All the horses accepted the oral preparation of clenbuterol hydrochloride in their grain, indicating that the drug was palatable. None of the horses developed clinical signs until early to mid April, and, in most cases, signs were mild until May (Table 5.4.1-1). It appeared that the treatment may have delayed the onset of the disease. However, by mid-season, all the horses developed clinical signs, and some were very severe, indicating that the treatment was having little or no effect on the progress of the disease. Horses 1-A1 and 1-A3 were particularly severely affected, with open, bleeding lesions and extreme alopecia. Horse 1-A1 had appeared to show some improvement while on clenbuterol hydrochloride in the preliminary trial, but in this trial showed no improvement and was one of the most severely affected horses. Horse 1-A5, the second participant in the preliminary trial, was the least affected horse, less so than in previous years. The owners of all the test animals, with the exception of Horse 1-A5, concluded that clenbuterol hydrochloride was an ineffective treatment for CH.

Table 5.4.1-1. Development of the clinical signs of CH as indicated by severity index number in horses in Group 1.

DATE	1-A1	1-A3	1-A5	1-A13	1-A14	1-A15
17 March	0	0	0	0	0	0
24	0	0	0	0	0	0
31	0	0	0	0	0	0
7 April	0	1	0	0	0	1
14	1	3	0	1	2	2
21	1	1-2	1	2	4	0
28	1	1-2	1	2	4	1
5 May	4	1-2	0-1	2	4	1
12	4	2-3	0-1	2	4	2
19	4	3-4	0-1	2-3	4	3
26	4	3-4	1	2-3	4	3
2 June	3	3-4	0	2	3	2
9	3	3-4	0	2-3	4	2-3
16	4	4	1	3	3	4
23	4	4	0	3	3	4
30	4	4	0	3	3	4
7 July	4	4	1	3	4	4
14	4	4	0	3	4	4
21	4	4	0	2	4	4
28	4	4	0	2-3	4	4
4 August	4	3-4	0	2	4	3-4
11	4	3-4	1-2	2	4	3-4
18	4	4	0	1-2	4	3
25	3-4	4	0	1-2	4	2-3
1 Sept.	4	3-4	0	3	4	2-3
8	4	3-4	0	2	3	2-3
15	4	3	0	2	2-3	2-3
22	3-4	3	0	1	2	2-3
29	3-4	3	0	0-1	1	2-3
6 Oct.	2	2-3	0	0-1	0-1	2-3
13	2-3	2	0	0	1	2
20	2	1-2	0	0	1	2
27	0	1	0	0	1	2

KEY

0 - no signs.
1 - very mild signs, some scratching, small scabs, slight hair loss. No open lesions.
2 - moderate signs, more scabs, hair loss, horse irritated.
3 - more severe, bald areas, scabs, red open areas, broken hair in mane and/or tail indicating scratching.
4 - very severe, large areas of hair loss, raw, open lesions, horse severely irritated, hair in mane and/or tail rubbed down to skin.

The horses developed raised welts at the site of the injections shortly after inoculation. These reactions were firm and hard to the touch for the first 4 h and became softer and less indurated after 24 h. The reactions peaked at 24 h post-inoculation (Fig. 5.4.1-1). Each individual horse's reaction to each of the five injections is shown in Fig. 5.4.1-2.

There was no interaction between time and reaction to the injection (ANOVA, P=0.2691). Therefore, there was no significant difference in the durations of the reactions after each of the five injections. However, in individual cases, the reactions to the later injections were less prolonged than were those to the early injections (Fig. 5.4.1-2). Horse 1-A5 had a measurable welt 216 h after the first injection, but the welt had completely disappeared by 144 h post-inoculation at all the other injection times, in some cases disappearing by 72 h. Horse 1-A14 had a much more prolonged reaction to the first injection than to subsequent ones and the reactions to injection 4 and 5 disappeared after 72 h. Horse 1-A13 continued to show a reaction to the first injection for >three weeks (504 h), but reacted to the second and subsequent injections for only 288 h and 144h, respectively. This horse had a very unusual delayed reaction to the second injection. The entire neck area developed a large pillow-like welt measuring 349.2 mm at 24 h post-inoculation. The reaction for the first 24 h followed the usual progression. The horse did not appear to be in distress and the large swelling regressed over the following days and disappeared after 72 h. A more normal welt was also visible during this time and was measured and used in the calculations. The horse had no more unusual reactions. Horse 1-A1 had the most prolonged reaction to the first injection, with the welt remaining for more than 18 days (432 h), with subsequent reactions disappearing after 216 h. Horse 1-A3 had reactions lasting for 216 and 288 h post-inoculation after the first and third injections respectively, but by the fifth injection, the reaction had completely disappeared within 48 h. Horse 1-A15 had the most prolonged reaction to the third injection, and had no reaction by 72 h after the fifth injection.

There was a significant time effect (ANOVA, P=0.0038) indicating that the horse's reactions changed over time, in general increasing until approximately 24 h after inoculation,

Figure 5.4.1-1. Mean welt width reactions over time of all six Group 1 horses after five intradermal injections of *Culicoides obsoletus* extract.

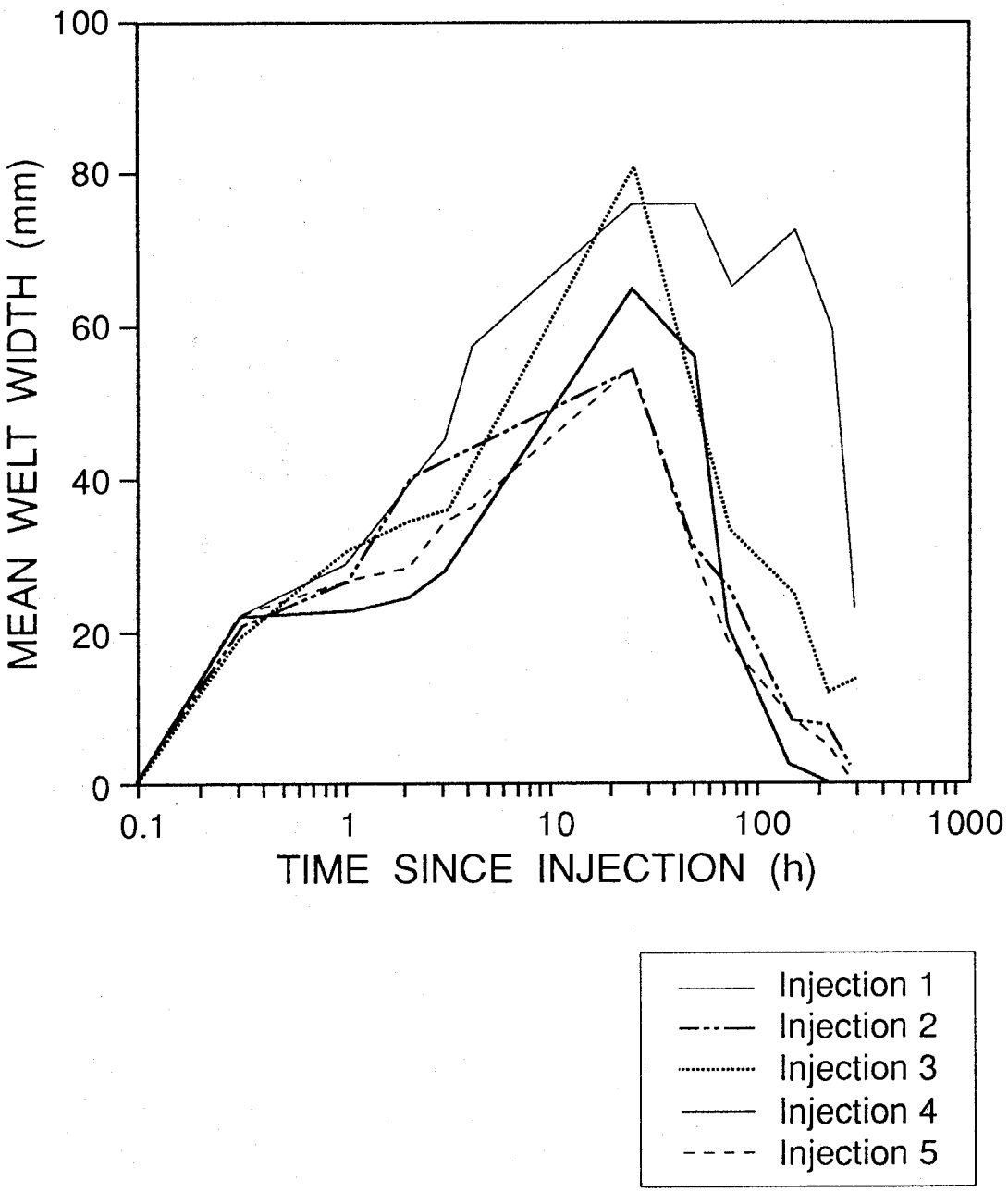
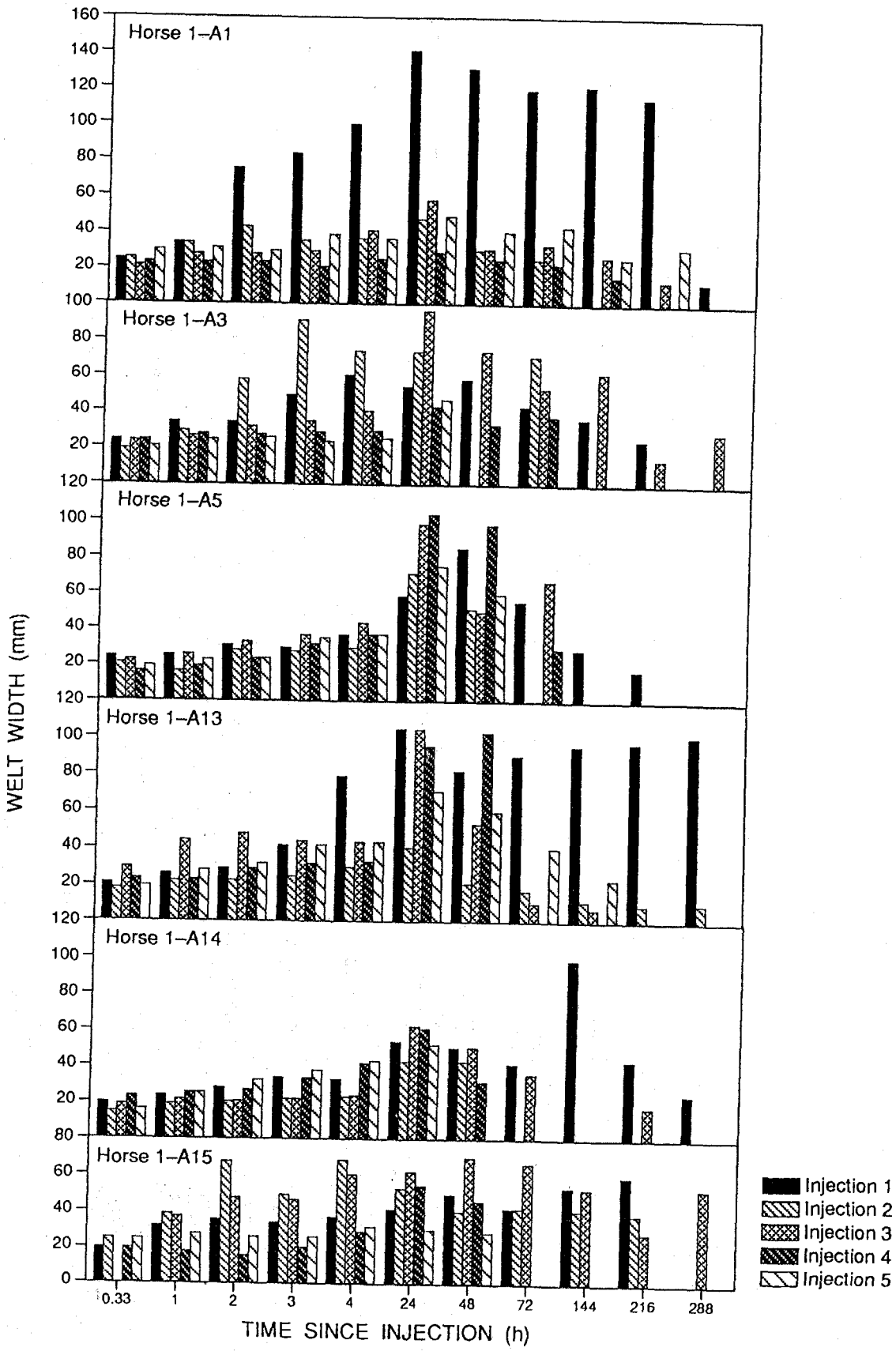

100
80
60
40
20
0
MEAN WELT WIDTH (mm)
0.1
1
10
100
1000
TIME SINCE INJECTION (h)
Injection 1
Injection 2
Injection 3
Injection 4
Injection 5

Figure 5.4.1-2. Reactions over time seen in each horse in Group 1 after five intradermal injections of *Culicoides obsoletus* extract (reaction 20 min after the third injection in horse 1-A15 was not measured).


Horse 1–A1
Horse 1–A3
Horse 1–A5
Horse 1–A13
Horse 1–A14
Horse 1–A15
WELT WIDTH (mm)
TIME SINCE INJECTION (h)
0.33
1
2
3
4
24
48
72
144
216
288
Injection 1
Injection 2
Injection 3
Injection 4
Injection 5

and then gradually subsiding. Horse 1-A5 reached a peak reaction 48 h post-inoculation after the first test, and thereafter peaked at 24 h. Horse 1-A14 reached the peak reaction for the first injection after 144 h, but all subsequent reactions peaked at 24 h. Horse 1-A13's reactions all peaked at 24 h post-inoculation, except for 48 h after the fourth injection. Horse 1-A1's reactions all peaked 24 h after inoculation, but horses 1-A3 and 1-A15 did not show a consistent pattern. Horse 1-A3 peaked 4 and 3 h post-inoculation for the first two injections respectively, but all other reactions peaked at 24 h. Horse 1-A15 reached the peak reaction 216, 4, 48, 24 and 4 h post-inoculation, respectively.

When the mean reactions of the six Group 1 horses to the injections were compared, there was a significant injection effect (ANOVA, P=0.0307), *i.e.* a difference between the horses' reactions to each injection. However, as the difference was not great, I could not statistically determine which injections were different from the others. However, if the mean of all the horses' reactions are pooled for each injection (Fig. 5.4.1-1) the reaction to the first injection was greater than those to the later injections, indicating that clenbuterol hydrochloride may have had some effect in reducing the reaction to the intradermal injection.

Also, if each individual horse's reactions are examined for each injection (Fig. 5.4.1-2), the first injection usually resulted in a much more severe reaction than did later injections. This was particularly obvious in horses 1-A1 and 1-A13, and less so in horses 1-A5 and 1-A14. Thus the reactions to the first injection were both more severe and more prolonged than later reactions. The horses all found the injection irritating, particularly at the start of the trial. In some cases, the horses scratched themselves severely at the injection site. Horses 1-A1 and 1-A13 scratched the welt after the first injection, resulting in open lesions, and horse 1-A1 severely scratched the injection site, resulting in bleeding, after the first injection. The horses appeared to be less irritated by later injections.

5.4.2 Group 2 Horses

The clinical signs of CH did not decrease significantly over the period of the injection treatment (Table 5.4.2-1). As in Group 1 horses, the injections resulted in a welt at the

Table 5.4.2-1. Development of the clinical signs of CH as indicated by severity index number in horses in Group 2.

DATE	2-A7	2-A8	2-A9	2-A10	2-A11
9 June	2	3	4	4	0
16	2	3	4	3-4	0
24	2	3	4	3	0
30	2	3	4	3	0
7 July	2	3	4	3-4	0
13	2	3	4	4	0
21	2	3	4	4	0
27	2	3-4	4	4	0
1 August	2	3-4	4	4	0
7	2	3-4	4	4	0
15	2	3-4	4	4	0
25	2	4	4	3	0
1 Sept.	2	3	4	3	0
15	2	3	4	4	0

KEY

0 - no signs.
1 - very mild signs, some scratching, small scabs, slight hair loss. No open lesions.
2 - moderate signs, more scabs, hair loss, horse irritated.
3 - more severe, bald areas, scabs, red open areas, broken hair in mane and/or tail indicating scratching.
4 - very severe, large areas of hair loss, raw, open lesions, horse severely irritated, hair in mane and/or tail rubbed down to skin.

injection site. Several horses developed small extraneous lesions near the site of injection, but not connected with it, similar to those observed in horse A-4 after the original skin tests with *C. obsoletus* extract (Chapter 2). Horse 2-A8 developed these extra welts 4 h after the third injection; horse 2-A8 developed them 2 h after the second injection and horse 2-A11 developed them within 1 h of the second injection.

The mean welt widths of the five Group 2 horses to the four injections of *C.obsoletus* are shown in Fig. 5.4.2-1. Again, there was no interaction between time and reaction to the injection (ANOVA, P=0.0826), *i.e.* the reactions to the injections were not different from each other over time. Also, there was no difference between the horse's reactions to each injection (ANOVA, P=0.3487). However, if the mean of all the horses' reactions for each injection are pooled, it is obvious that the first injection provoked a greater reaction when compared with the last injection (Fig. 5.4.2-1). If the individual horse's reactions to each injection are examined (Fig. 5.4.2-2), the most notable reactions were those of horse 2-A11, which had a large reaction to the first injection, much smaller reactions to the second and third injections and no reaction at all to the last injection. The hypothesis that this lack of reaction could be correlated with a corresponding regression in lesions could not be tested, as this horse was very well protected from insect attack. This horse was extremely irritated by the first injection and had to be cross-tied so as to prevent it from scratching the injection site. It was less irritated by the second and third injection and showed no irritation whatsoever to the last injection. Horse 2-A7 also had reduced reactions in later tests, with the reaction to the last injection disappearing after 72 h.

The reactions to the injections changed significantly over time (ANOVA, P=0.0029), increasing to a peak, and then gradually disappearing. In several cases, the reaction peaked earlier in the later tests than in the first test. Horse 2-A8 peaked at 48, 24, 24 and 4 h post-inoculation, respectively, for the four injections; horse 2-A9 peaked at 24, 48, 4 and 4 h post-inoculation, and horse 2-A11 peaked at 48, 24, 24 and 0 h post-inoculation. Horses 2-A10 and

Figure 5.4.2-1. Mean welt width reactions over time of all five Group 2 horses after four intradermal injections of *Culicoides obsoletus* extract.

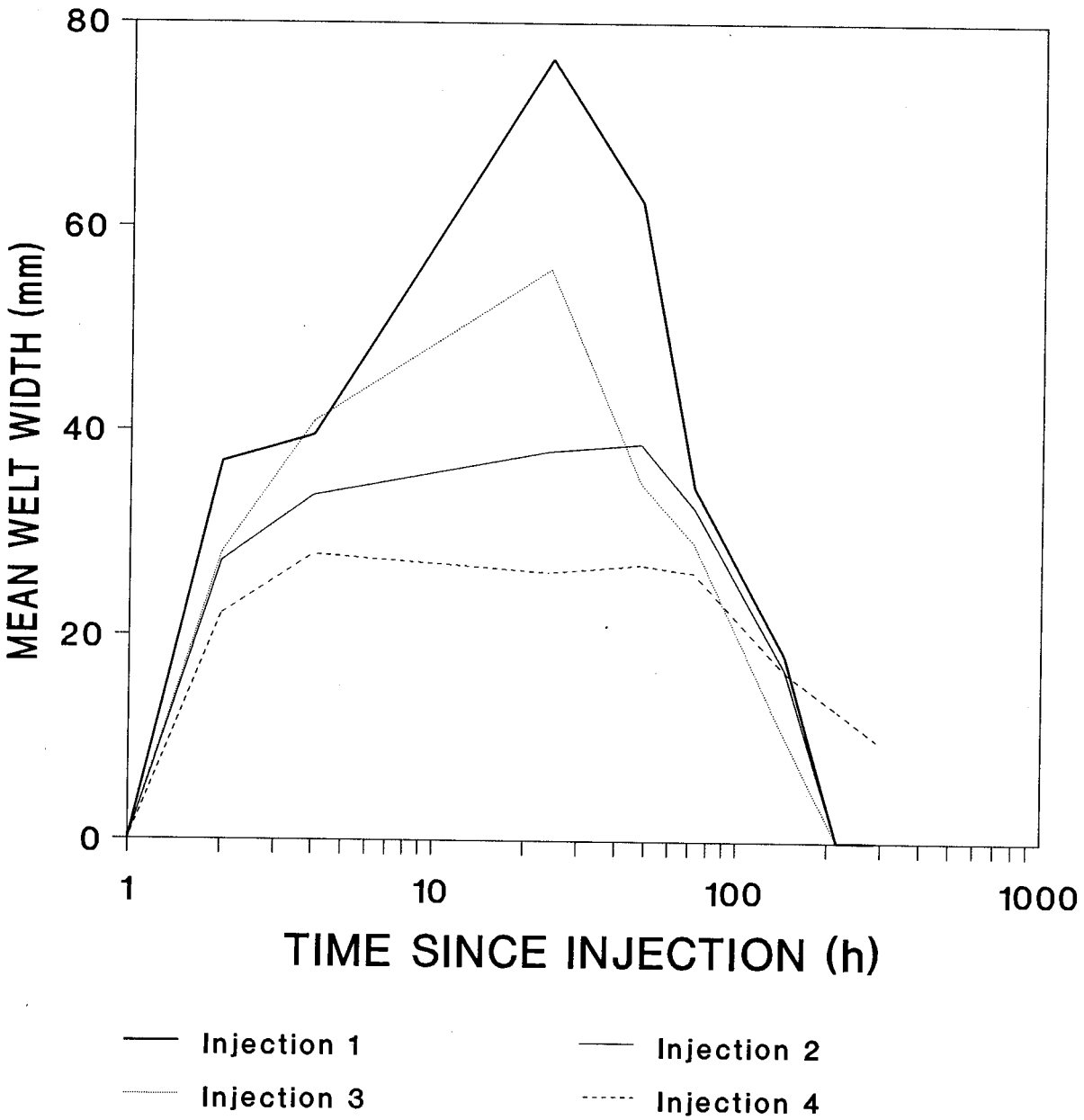
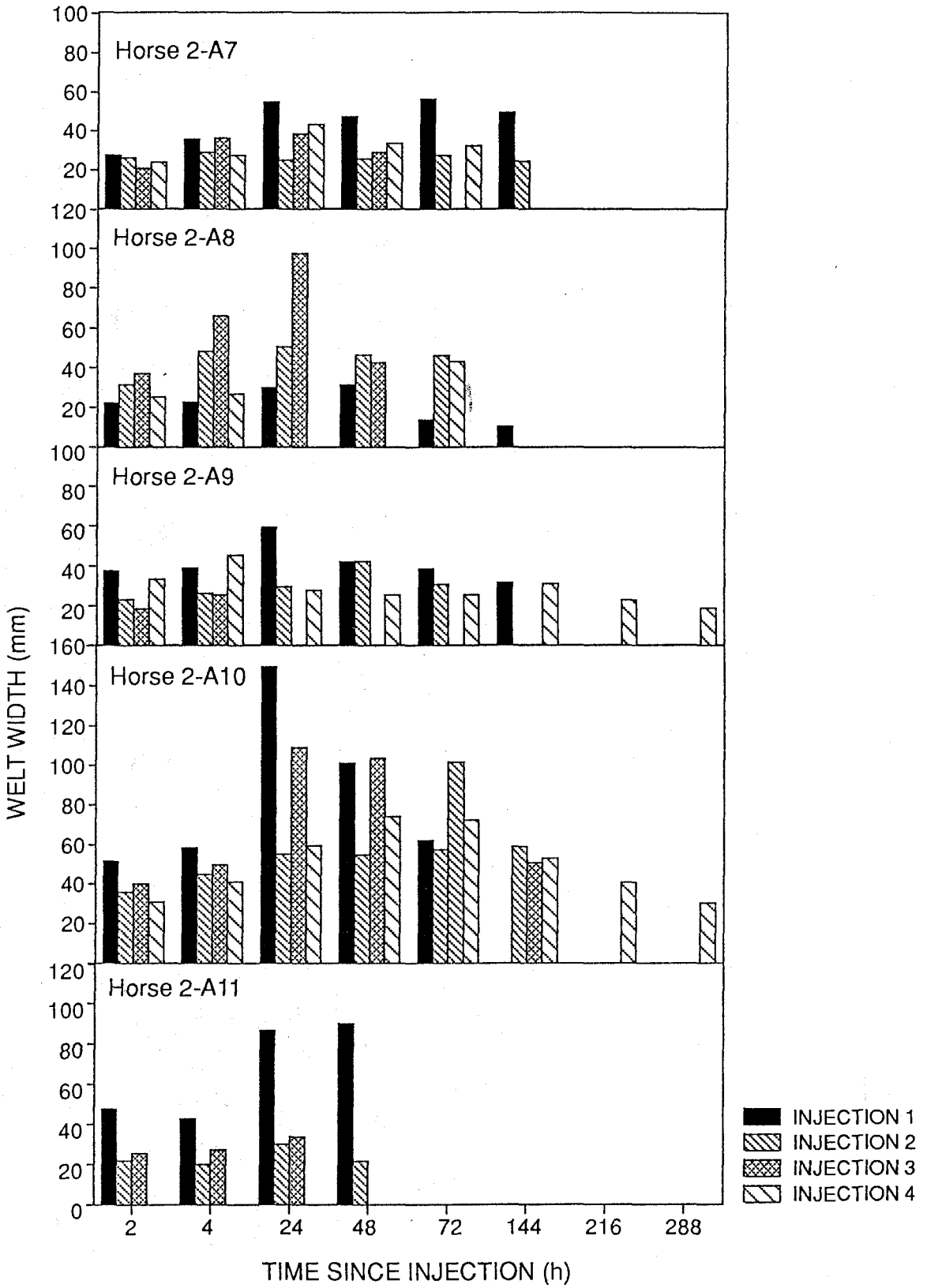

80
60
40
20
0
MEAN WELT WIDTH (mm)
1
10
100
1000
TIME SINCE INJECTION (h)
Injection 1
Injection 2
Injection 3
Injection 4

Figure 5.4.2-2. Reactions over time seen in each horse in Group 2 after four intradermal injections of *Culicoides obsoletus* extract.


Horse 2-A7
Horse 2-A8
Horse 2-A9
Horse 2-A10
Horse 2-A11
WELT WIDTH (mm)
TIME SINCE INJECTION (h)
2
4
24
48
72
144
216
288
INJECTION 1
INJECTION 2
INJECTION 3
INJECTION 4

2-A7 showed no trends, peaking at 24, 144, 24 and 48 h and 72, 4, 24 and 24 h post-inoculation, respectively.

When the reactions of Group 1 and 2 were compared, there was no interaction (ANOVA) between groups, time and injection ($P=0.1679$), no interaction between time and injection (pooling groups) ($P=0.516$), between injection and group (pooling time) ($P=0.5944$), or between time and group (pooling injection) ($P=0.3733$). Therefore, the data for all injections at all times could be pooled. There was no significant difference between the reactions of horses in Group 1 and Group 2 (ANOVA, $P=0.0990$). There was a highly significant effect of time (ANOVA, $P=0.0001$) and a borderline effect of injection ($P=0.0531$), which is probably accounted for by the injection effect seen in Group 1 horses.

5.5 DISCUSSION

5.5.1 Group 1 Horses

Clenbuterol hydrochloride did not appear to be an effective treatment for CH. Horses consistently fed with the manufacturer's recommended dose of clenbuterol hydrochloride over an entire season developed severe and typical clinical signs of CH, although the onset of clinical signs appeared to be somewhat delayed. In all cases, the horses showed both immediate (Type I) and delayed (Type IV) hypersensitivity. If clenbuterol hydrochloride had acted as a mast cell stabilizer (Teitzel 1982) it should not only have prevented or considerably reduced the development of clinical signs, but should also have prevented the development of a reaction following intradermal skin tests with the known allergen. Only when all the horses' reactions, at each time interval were pooled, could an apparent, but not significant difference be seen between the reactions to the first injection, in comparison with any of the later injections, indicating that the drug may have had some slight, mast cell stabilizing effect.

Even if clenbuterol hydrochloride had a slight effect on the release of allergic mediators, it did not have a clinical effect, in contrast to the results seen in humans and horses with respiratory allergies (Sasse and Hajer 1978; Del Bono *et al.* 1979). This discrepancy can

be partially, but not completely, explained by the bronchodilating effect of the drug. However, this is the first time that clenbuterol hydrochloride has been tested in cutaneous allergies and it is possible that mast cells in the skin may act differently from those in the lung tissue.

Disodium cromoglycate is an anti-allergic drug used in both human and veterinary medicine which also acts as a mast cell stabilizer (Foreman and Lichtenstein 1980). Its exact mode of action is not fully understood, but it is thought to act by blocking the antigen-stimulated transport of calcium across the mast cell membrane, possibly by affecting the intracellular levels of cyclic AMP (Foreman *et al.* 1975). It is not a bronchodilator, but acts by preventing mediator release in both human (Cox 1967; Booij-Noord *et al.* 1971) and horse lungs (Murphy *et al.* 1979; Thomson and McPherson 1981). However it does not prevent the release of mediators in skin allergies (Goose and Blair 1969; Assem and Mongar 1970; Pearce *et al.* 1974; Frick 1980). In some cases, it even enhanced the reaction to human allergy skin tests (Garland *et al.* 1978).

As both disodium cromoglycate and clenbuterol hydrochloride act on the cell membrane, and both work well with respiratory allergies, it seems probable that they exhibit a similar tissue specificity. This is no doubt due to the morphological and pharmacological differences between skin and lung mast cells. In the past, it was thought that mast cells comprised a homogeneous population of cells, with a similar morphology. However, it is now known that mast cells can be divided into connective tissue mast cells (CTMC) and mucosal mast cells (MMC). MMC are found only in the gut and lung, whereas CTMC are ubiquitous (Roitt *et al.* 1985). Only CTMC are affected by disodium cromoglycate, but the morphology of these cells in skin is very different from those in other tissues, particularly in terms of the size and number of the internal granules, the density of staining and their pharmacological properties (Roitt *et al.* 1985). Therefore, it is quite possible that clenbuterol hydrochloride acts as a successful mast cell stabilizer in lung tissue, as is claimed, despite the fact that it has little or no effect on the skin.

Although the immediate reaction seen in CH affected horses is due to a Type I hypersensitivity, which is also responsible for the first part of the reaction to the skin test, CH also involves a delayed component or Type IV hypersensitivity. However, clenbuterol hydrochloride only has an effect on Type I hypersensitivity (Corbella 1978) which may also have contributed to its lack of success.

After studying six horses on clenbuterol hydrochloride treatment over an entire season, it would appear that the apparent improvements seen in the horses in the preliminary trial, particularly in horse 1-A5, were not due to the use of the drug, but to some other factor in their environment. The most probable explanation is that horse 1-A5 had moved to an area with a lower *Culicoides* population. This horse continued to have very minor clinical signs throughout the clenbuterol hydrochloride trial. However, the next season, this animal was moved again, to a pasture in the Eastern Fraser Valley, and began to show more severe clinical signs. Therefore, the observed improvement was almost certainly due to location.

5.5.2 Group 2 Horses

Group 2 horses were tested to determine whether the delay in onset of clinical signs was due to the spacing of the first two injections; they showed no apparent improvement in clinical signs. Because the average overall reaction to the first injection was much greater than to the last, a tolerance to the injection may have begun to develop. In particular, horse 2-A11 showed a gradually decreasing reaction to the injections, until finally it did not develop a welt at all after the fourth injection. Unfortunately, this could not be correlated with a concurrent decline in clinical signs, as this horse was well protected from insect bites. As the fourth injection of *C. obsoletus* extract did not provoke a reaction, it seems likely that the horse would be hyposensitized to *Culicoides* bites. The injections may possibly have had some slight effect on clinical signs and so may have been responsible for the delay in onset seen in Group 1 horses.

It is also noteworthy that the third injection in horse 2-A11 provoked only an immediate response, with the welt having disappeared by 24 h post-inoculation. Several other

horses in the trial also began to reach the peak of reaction more rapidly after several injections. This follows the sequence of reactions which has been described in guinea pigs following arthropod bites and is assumed to occur in other animal species (Larrivee *et al.* 1964; Chapter 2). Originally, an animal shows no reaction to an insect bite (Stage 1). After several bites it becomes sensitized and develops a delayed reaction to later bites (Stage II). After further exposure, it develops both immediate and delayed reactions (Stage III). Later it loses this delayed reaction and develops only the immediate reaction (Stage IV). Finally, it no longer reacts to the insect bite (Stage V) (Benjamini and Feingold 1970; Feingold 1973a). Horse 2-A11 developed immediate and delayed reactions to the first injection, indicating that it was in Stage III at the start of the trial. It then appeared to go through Stage IV, with only an immediate reaction after the third injection, followed by Stage V, no reaction, after the fourth injection.

It is unlikely that long-term hyposensitization could be induced after so few injections, particularly as they were administered intradermally in order to provoke a local reaction, as opposed to subcutaneously, where they would provoke a systemic reaction. Human hyposensitization is carried out using subcutaneous injections. Possibly a local hyposensitization of the injection area took place in horses. It may be possible to hyposensitize affected horses using an extract of *C. obsoletus*, but frequent subcutaneous injections with large doses need to be given to a large number of test animals before this hypothesis can be tested.

6. AN IMMUNOTHERAPY TRIAL IN HORSES AFFECTED WITH *CULICOIDES* HYPERSENSITIVITY IN BRITISH COLUMBIA

6.1 INTRODUCTION

Immunotherapy is a broad term used to describe injection therapy aimed at the reduction or eradication of the clinical signs associated with a particular allergen. It is frequently used synonymously with the term 'hyposensitization'. To hyposensitize an individual means to make it less sensitive to an antigen which has previously induced an allergic reaction in that individual. It is accomplished by treating the allergic individual with small, but gradually increasing doses of the allergen. These doses are too small to initiate a severe allergic reaction, but large enough to stimulate the immune system. An animal which has been hyposensitized has reached Stage Five in Benjamini and Feingold's (1970) stages of sensitization.

Hyposensitization has been successfully used as a therapy in allergic humans. It was used to treat patients with Hymenoptera sting allergies in the 1920's and 1930's and became standard treatment for the next forty years (Graft 1987). Although, for many years, this treatment was deemed at least partially successful, these early studies had no controls, and most patients said to do 'better' on being re-stung by the causal Hymenoptera still required adrenaline treatment (Lichtenstein *et al.* 1979). It was later determined that the use of whole body extracts of Hymenoptera was little better than placebo (Hunt *et al.* 1978). However, treatment using only venom was shown to be highly successful (Lichtenstein *et al.* 1974), and is now considered the treatment of choice in patients who have previously developed a systemic anaphylactic reaction to Hymenoptera stings (Reisman *et al.* 1985; Graft 1987).

The first use of hyposensitization, or immunotherapy, was in the treatment of hayfever (Noon 1911). This procedure was advanced and refined over the following decades, with particular emphasis on dosage regimens and mechanism of action (Patterson *et al.* 1978). Later, more controlled studies were developed and *in vivo* and *in vitro* methods of measuring

antibody levels were established, leading to the development of clearly defined immunologic parameters for monitoring the results of treatment. It is now considered to be one of the most satisfactory and effective methods of treating pollen allergies.

Hyposensitization has also been used sporadically to treat people allergic to insect bites (McIvor and Cherney 1941a; 1941b; Hatoff 1946; Dubin *et al.* 1948; Feingold 1973b; Rohr *et al.* 1984). Moreover, Collins (1966) reported using intradermal injections of flea antigen to successfully treat dogs and cats suffering from flea dermatitis, and also using intradermal injections of inhalant allergens to treat animals allergic to dust, pollens and moulds, with one to three injections resulting in relief. In Australia, 160 flea-allergic dogs were treated with a total of three intradermal injections of whole ground fleas, *Ctenocephalides felis*, at seven day intervals, resulting in 98% success (Keep and Taylor 1967). Similar trials were conducted in the United States, with feline flea-allergy dermatitis, again involving only three injections (Reedy 1975). Seventy-five percent of cases reported good to excellent results. The treatment was supplemented by flea control by a professional exterminator on the premises at the same time as the injections, plus twice-weekly applications of flea powder to all dogs and cats in the household. Therefore, it is quite probable that much of the improvement associated with the injections was due to the removal of the causal allergen. Michaeli and Goldfarb (1968), however, successfully hyposensitized flea-allergic dogs and cats with both intradermal and subcutaneous injections of flea saliva, without concurrent flea control. A later study showed that hyposensitizing flea-allergic dogs with three intradermal or subcutaneous injections of flea antigen was 85.6% effective, but that continuous flea control and maintenance of a flea-free environment was almost as effective (84%) (Nesbitt and Schmitz 1987). Five to seven injections prevented the development of seasonal clinical signs of flea dermatitis (Saunders 1977), but flea control was also practiced concurrently. Intradermal injections were more effective than subcutaneous injections (Saunders 1977).

A double-blind study involving placebos and several commercially available flea antigens was conducted using flea-allergic dogs (Halliwell 1981). There was no change in

previous flea-control measures. The use of flea antigens seemed only marginally better than placebos in this subjective study. Hyposensitization was also found to be unsuccessful in another double-blind, subjective study in cats with the injections being administered over 20 weeks, although several animals did show an improvement (Kunkle and Milkarsky 1985).

In human hyposensitization, injections are given over a long period, sometimes several years, so it is possible that many of the unsuccessful or equivocal animal trials would have had greater success if carried out for a longer duration. Also, the injections are administered to humans subcutaneously rather than intradermally. Intradermal injections are painful, and promote a local response, hence their efficacy in diagnostic skin tests. Subcutaneous injections, on the other hand, are relatively painless and provoke a systemic immunological response which is far more desirable when attempting to hyposensitize an individual, than a local response. The flea antigen is unusual in that it is a hapten, which conjugates with the host's skin collagen to form a complete antigen (Michaeli *et al.* 1965). This may explain why intradermal injections have proved more successful than subcutaneous injections in the treatment of flea allergies in animals.

All the animal trials mentioned above involved repeated administration of the same dose of allergen, whereas human therapy involves increasing the dose of allergen regularly up to a maintenance level. It is, therefore, probable that the differences between human and other animal therapy have resulted in the successful treatment of humans using hyposensitization and the more variable results seen in animal trials.

'Hyposensitization' is often used interchangeably with the somewhat older term 'desensitization'. True desensitization involves the complete elimination of specific Immunoglobulin E (IgE) antibodies, which is very rarely achieved, even when clinical signs are no longer apparent (Terr 1976), whereas hyposensitization refers to the decreased sensitivity to allergens observed clinically in response to the gradual administration of allergenic extracts (Lockey and Bukantz 1976). Immunotherapy is a broader term which

recognizes that the response to injection therapy is the product of several immunologic events, some of which are still not clearly understood.

Immunotherapy might be a desirable approach for the treatment of horses suffering from CH. Most drug therapies are expensive, time-consuming to administer and have adverse side effects, especially when used over a long period of time. To date, there is no truly effective drug treatment available. Corticosteroids are the most successful (Kleider and Lees 1984), but most horses still show some clinical signs and many show no improvement. Immunotherapy could offer a safe and relatively permanent treatment for the disease.

6.1.1 OBJECTIVES

A two-year trial was conducted with the following objectives :

1. to determine in the first year whether it was possible to hyposensitize affected horses and to develop a dosage regime, and

2. to prevent the development of clinical signs in the affected horses by treating all the animals with a maintenance dose throughout the season.

6.2 METHODS AND MATERIALS

6.2.1 First year - 1989

Ten horses severely affected by CH were chosen - A3, A7, A8, A9, A13, A14, A15, A16, A17, and A18. All had been affected for several years. Each showed typical clinical signs and ranged in age from 2-24 yrs. The group was made up of 8 mares and 2 geldings, and consisted of a range of heights, breeds and colours (Appendix I). Most of the horses had been skin tested at some point prior to the start of this trial to confirm the diagnosis of CH, and those that were new to the trial were skin tested before the immunotherapy began.

6.2.1.1 Preparation of Extract

The insects used to make up the extract were *C. variipennis*, mass-reared by the United States Department of Agriculture (U.S.D.A.) in Wyoming. The colony from which the insects were obtained was established in 1957 from specimens collected in Texas. I had previously established that horses in British Columbia suffering from CH reacted equally to skin tests with *C. obsoletus* and to skin tests with *C. variipennis* (Chapter 4). The insects were freeze-dried and kindly donated to me at regular intervals by Dr. Gregg Hunt, U.S.D.A., Laramie, Wyoming.

The extract was prepared by crushing a known weight of dried insects in 0.9% physiological saline. The resulting extract was filtered through Millipore filters of 0.8 μm, 0.45 μm and 0.22 μm pore size, respectively and finally filtered through a sterile 0.22 μm Millipore filter. Preparation of the extract was performed aseptically under a laminar air-flow hood. Sterility was checked by incubating an aliquot of the freshly made extract with nutrient broth gently agitated at 37^{o}C for 48 h. The extract was frozen at -18^{o}C in appropriate aliquots until needed. One milligram of *C. variipennis* contained approximately 168 insects. Dosage was measured as the starting weight of the insects before preparation *i.e.* a dose of 1 mg meant that 1 mg dry weight of insects (168 insects) was extracted for that injection.

Each injection was administered with an adjuvant, Immunostim[1]. An adjuvant is any agent that acts non-specifically to increase an immune response to a specific antigen (Allison 1973). The addition of an adjuvant will slow the release of the antigen and stimulate the immune system, making each injection of allergen more effective. Immunostim is a non-pathogenic mycobacterial cell wall fraction which acts as an immunostimulant without the usual side effects associated with biologically active products such as Freund's Complete Adjuvant (Alkemade 1988).

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[1] registered trade name. Immunostim was kindly donated by Dr. S.J. Alkemade and Vetrepharm Research Inc, 69 Bessemer Rd., Unit 27, London\, Ontario.

The adjuvant was received as a liquid with 400 μg mycobacterial cell fractions per mL. It was diluted with PBS, pH 7.2 and 20 μg of active ingredient were included with each extract injection, irrespective of the dose.

6.2.1.2 Method of injection

The horses were injected subcutaneously by a veterinarian using a 23 gauge needle to minimize trauma. Subcutaneous injections were administered in order to provoke a general, rather than a local, response. The injection was given in the neck region and the side of the neck was alternated with each subsequent injection. The neck was not clipped. The needle was inserted into the skin at an oblique angle and the plunger pulled back slightly to ensure that the needle had not entered a vein, as direct injection of the allergen into the blood stream would have entailed a high risk of anaphylactic shock. After ensuring that the needle was placed correctly, the injection was given. Each horse was observed for any adverse reactions for 10-15 min after injection.

The injections were administered as close to one week apart as possible, depending on the veterinarians' commitments. Overall, they were 6-10 days apart.

6.2.1.3 Dosage Regime

The first injections were given on 18 April 1989. Each horse was started with an extract derived from 2 mg of insects (dry weight), except for horses A9 and A13, which were started with 1 mg due to pregnancy and small size, respectively. The dose was kept at 2 mg for the first 5 injections. Horse A13 was increased to 2 mg at the second injection, and dosage was increased more slowly for Horse A9 than for the rest, as done for safety in human immunotherapy (Patterson *et al.* 1978). The dose was then increased by 0.5 mg, and held at that level for two injections, then increased again by 0.5 mg. Three consecutive injections of 3 mg were administered; then the dose was increased to 3.5 mg, then 4 mg.

The doses were increased so gradually at first as I was concerned about the risk of fatal anaphylactic shock in response to the injections, despite the presence of a veterinarian, with appropriate drugs to counteract anaphylactic shock. However, anaphylactic shock is frequently

fatal, despite resuscitation attempts. Therefore, it was considered that slight increases in the dose of allergen were safest. Large increases in dose are considered best in human immunotherapy, but equine immunotherapy is largely unknown. However, the horses showed no ill-effects whatsoever after the injections. Therefore, dosage was increased thereafter by 1 mg per week, depending on the individual horse's reaction to the previous weeks injection. If the horse had a particularly large local reaction to the previous dose, that horse was left at the previous dose until the reaction was less severe. This meant that although the increase in dosage followed a trend, individual horses were treated with different dosage regimes, depending on their own reactivity. This is consistent with human immunotherapy.

When the dose reached 10 mg in August, an improvement in clinical signs in many of the horses was noted. Therefore, this possible maintenance dose was maintained for 4 consecutive injections. However, some of the horses started to scratch again, so the dose was again increased by 1 mg per week up to 20 mg and then maintained until December, when clinical signs could no longer be monitored. It was considered risky to increase the dose further as each injection consisted of an extract made from 3360 insects. Although it is possible that a horse could be bitten by this many insects in one night, the bites would not all be at the same site, and would be only intradermal. Also, each insect injects a minute amount of protein during its bite, whereas the extract consisted of the entire insect, which bears the allergen throughout its body (Riek 1954). The addition of the adjuvant would also increase the effect of the allergen.

Some horses were maintained at a slightly lower dose than 20 mg. Horse A16 was maintained at 19 mg as it was entered into the program later than the rest, on 15 July 1989. Horse A8 was maintained at 16 mg as its immunotherapy program was interrupted for several weeks when it suffered a severe leg injury and was treated with many pain-killer and anti-inflammatory drugs which may have affected the immunotherapy. Horse A7 appeared to do well at a dose of 9 mg, so was maintained at this dose for 8 injections. This horse was later increased to 16 mg and maintained at that dose.

The frequency of injections over the winter was reduced to every two then three weeks, as the horses did not have clinical signs, and due to the difficulty of obtaining a veterinarian during this time.

6.2.1.4 Reaction to injection

The horses developed raised welts after the injections. These reactions were measured at their widest point using vernier calipers at 4 and 24 h after injection.

To ensure that the reactions were due to the injection of extract rather than the adjuvant, two normal horses, N7 and N8, were injected subcutaneously with 20 μg of adjuvant in physiological saline, the same amount of adjuvant as used in each extract injection.

6.2.1.5 Assessment of Reaction

As a measurable welt developed after the injection, the progress of each horse could be objectively measured. All horses were injected on the same day, so the reaction was first measured 4 h after injection, when all ten horses had been injected. This would indicate the presence of an immediate, or Type I, reaction. The reaction was again measured 24 h after injection to measure the delayed, or Type IV, reaction. The welt was measured at its widest part using vernier calipers.

Each horse's reaction at each time interval, 4 and 24 h, to each injection, was divided by the dosage, in insects (168/mg), given to that particular horse for that injection. This resulted in a measure of welt width per insect equivalent. This meant that, despite the horse's different dosage regimes, they could be directly compared.

Each horse's clinical signs were visually assessed each week, at the time of the first welt measurement. Detailed notes were taken, and clinical signs were assigned an arbitrary index number, based on severity, as in Chapter 5.2. The key is repeated below :

0 - no signs.

1 - very mild signs, some scratching, small scabs, slight hair loss. No open lesions.

2 - moderate signs, more scabs, hair loss, horse irritated.

3 - more severe, bald areas, scabs, red open areas, broken hair in mane and/or tail indicating scratching.

4 - very severe, large areas of hair loss, raw, open lesions, horse severely irritated, hair in mane and/or tail rubbed down to skin.

Affected horses pastured in the same area, which were not treated in the immunotherapy trial were observed, to show that any improvements seen in the horses in the trial were due to the injection therapy, rather than to a lack of *Culicoides*. No trapping was performed during the trial, but the presence of severely affected horses would indicate a normal population.

6.2.2 Second year, 1990

Once the maintenance dosage in the first year was reached and safely administered for several weeks for each horse by a veterinarian, it was considered safe for the horse owners to administer the injections themselves. This was deemed necessary in the second year as it became increasingly difficult to obtain veterinary assistance. Maintenance doses very rarely result in adverse reactions in human immunotherapy (Norman 1981). Also, it was considered useful to determine whether injection therapy could be successfully placed in the hands of horse owners, as the expense of employing a veterinarian to perform weekly injections would be prohibitively high.

Each owner was given instructions by a veterinarian on how to administer the injections and was monitored by the veterinarian the first time that they injected their horse.

The frequency of injections was increased to every two weeks and then weekly by Spring 1990. Each owner was given syringes made up to the appropriate dose for each horse, with adjuvant added. The owners were instructed to keep the syringes under refrigeration until just before use. They were asked to take careful note of the very slightly cloudy appearance of the extract and to check its appearance for any change before injecting it. If the extract did not look perfect, it was to be discarded. Each owner received approximately three injections at a time from me.

The most convenient time for injecting the horse weekly varied from owner to owner, so it was not possible for me to measure the reactions after injection. Each owner was, therefore, provided with a set of vernier calipers and carefully instructed on their use and how to take a reading. One owner, who found reading the calipers extremely difficult, was asked to take the measurement, then mark the distance between the measuring tips of the calipers on a piece of paper. Each owner was also given a table of results to fill in with the measurements and dates of injection. Any comments were also solicited. One owner was greatly disturbed at the thought of administering the injections so, with the owner's permission, I performed all injections on this horse (A17).

The owners were instructed that the injections should be performed as close to 7-day intervals as possible. Some owners were extremely prompt, and showed concern when they were only a few hours late, whereas others frequently forgot, so administered the injections at very erratic time intervals. Thus, some horses had a low cumulative dose and the number of total injections that each horse received varied greatly from 26 in horse A8 to 54 in horse A17, which I treated.

Two horses were dropped from the trial. Horse A8 did not receive an injection for 61 days because of its severe leg injury. When injections were resumed, the local reaction was painful and persisted for several days. As its tolerance for a higher dose appeared to have been lost, and as a veterinarian was no longer available, it was not considered advisable for the owner to begin again with escalating doses. Therefore, this horse did not receive injections after 13 April 1990. Horse A9 was dropped due to pregnancy after 25 March 1990. Human immunotherapy is considered safe during pregnancy (Feingold 1973b), but if the individual were to go into anaphylactic shock, it might have a serious effect on the foetus. As this horse had been a successful racehorse, any foal would be extremely valuable, and also as this mare had previously resorbed several foetuses, it was considered prudent to discontinue immunotherapy.

The clinical signs of all horses, including A8 and A9 were visually assessed on a weekly basis, as in the first year of the trial, until October 1990. Affected but untreated horses were again checked to confirm that untreated horses were developing clinical signs as usual.

6.2.3 Statistical analysis

The width of the horses' reaction, in insect equivalents (original reaction in mm divided by dosage in insects, times 100) for each of the first 26 injections, were compared using a two-way ANOVA with repeated measurements on both factors, injection and time, with time at only two levels, 4 and 24 h (SAS 1988). After the first 26 injections, two horses dropped out, so injections 28 to 36 were analyzed separately with only 8 horses. Injection 27 was eliminated as another horse (A3) was injected but unavailable for welt measurements afterwards. After injection 36, no statistical analysis was made as only a few horses received >36 injections.

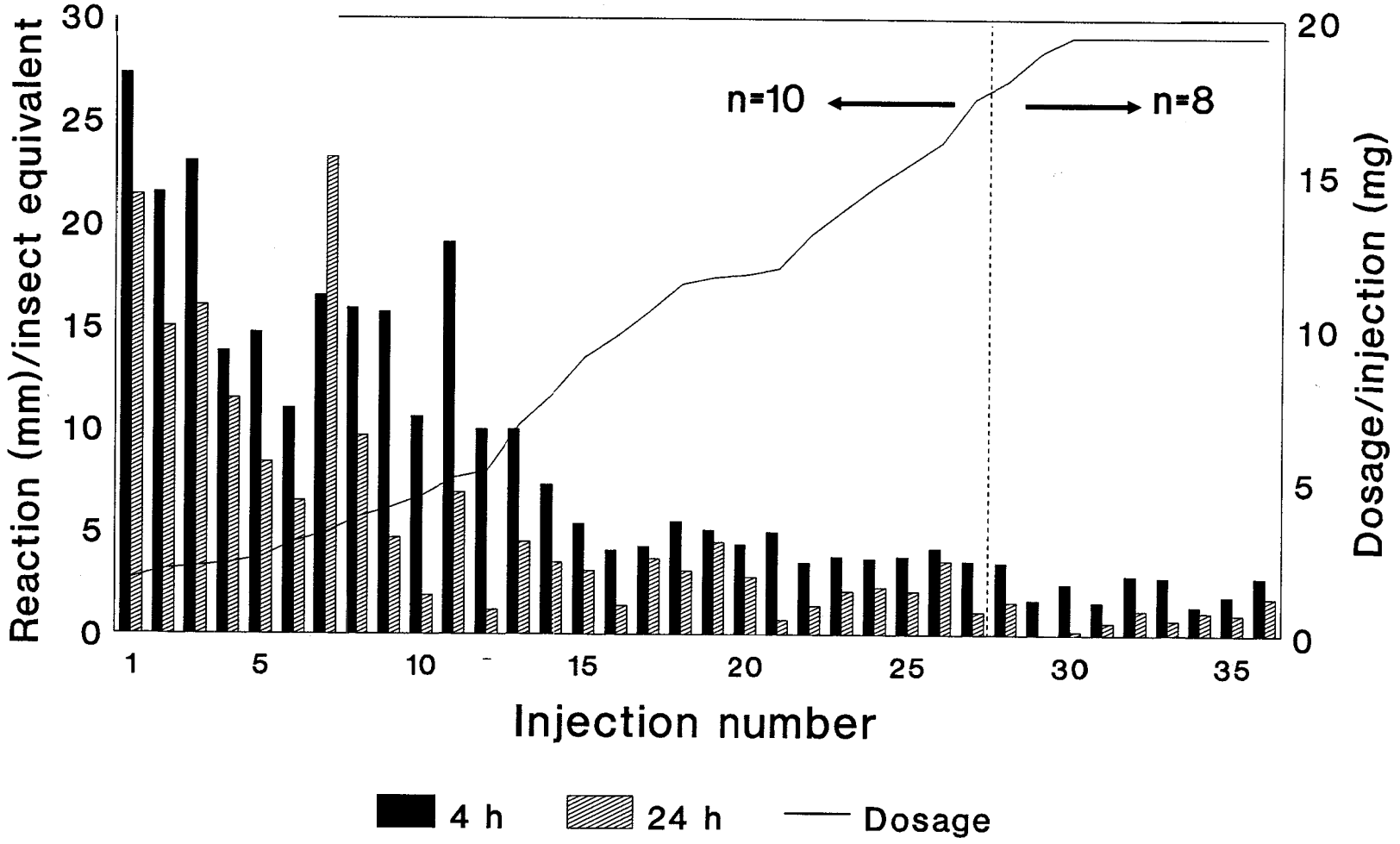
6.3 RESULTS

6.3.1 Reaction to injection therapy

Each horse reacted to the injections at the injection site, showing a welt at the needle site within 4 h of injection. The welt was oval in shape, and frequently hard to the touch. In some cases, the horses appeared to find the welt irritating.

The mean reactions of 10 horses to the first 26 injections, and the mean reactions of eight horses to injections 27 to 36, together with mean dosage in mg, is shown in Fig. 6.3.1-1. There was no interaction between time and injection ($P=0.1089$), so the reactions 4 and 24 h after injection followed the same pattern. There was a highly significant effect of time, indicating a statistical difference between the reactions at 4 h, which were greater than the reactions at 24 h ($P=0.0045$). There was a highly significant injection effect ($P=0.0001$) indicating that the horse's reactions to the injections significantly decreased over time, despite the increase in dosage.

Figure 6.3.1-1. The mean reactions of ten affected horses to 26 injections of increasing dose of *Culicoides variipennis* extract, and eight horses to injections 27-36, together with mean dosage/injection in mg.


Reaction (mm)/insect equivalent
30
25
20
15
10
5
0
n=10
n=8
20
15
10
5
0
Dosage/injection (mg)
1
5
10
15
20
25
30
35
Injection number
4 h
24 h
Dosage

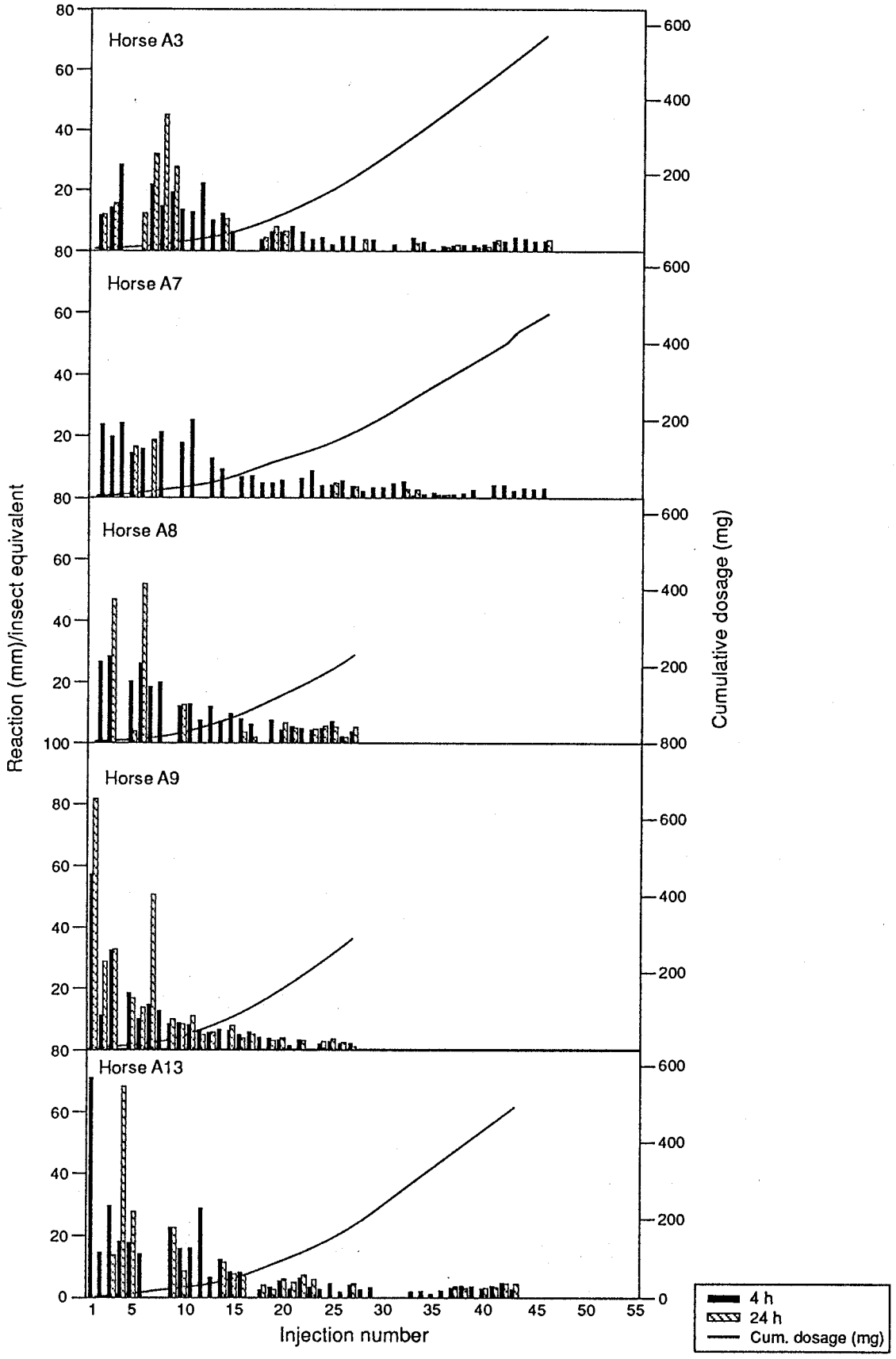
When the mean reaction to each injection is compared with the reaction to the previous injection, a gradual decrease in reaction can be seen graphically. In most cases, the reaction to each subsequent injection was slightly less than to the previous injection, and overall this is highly significant.

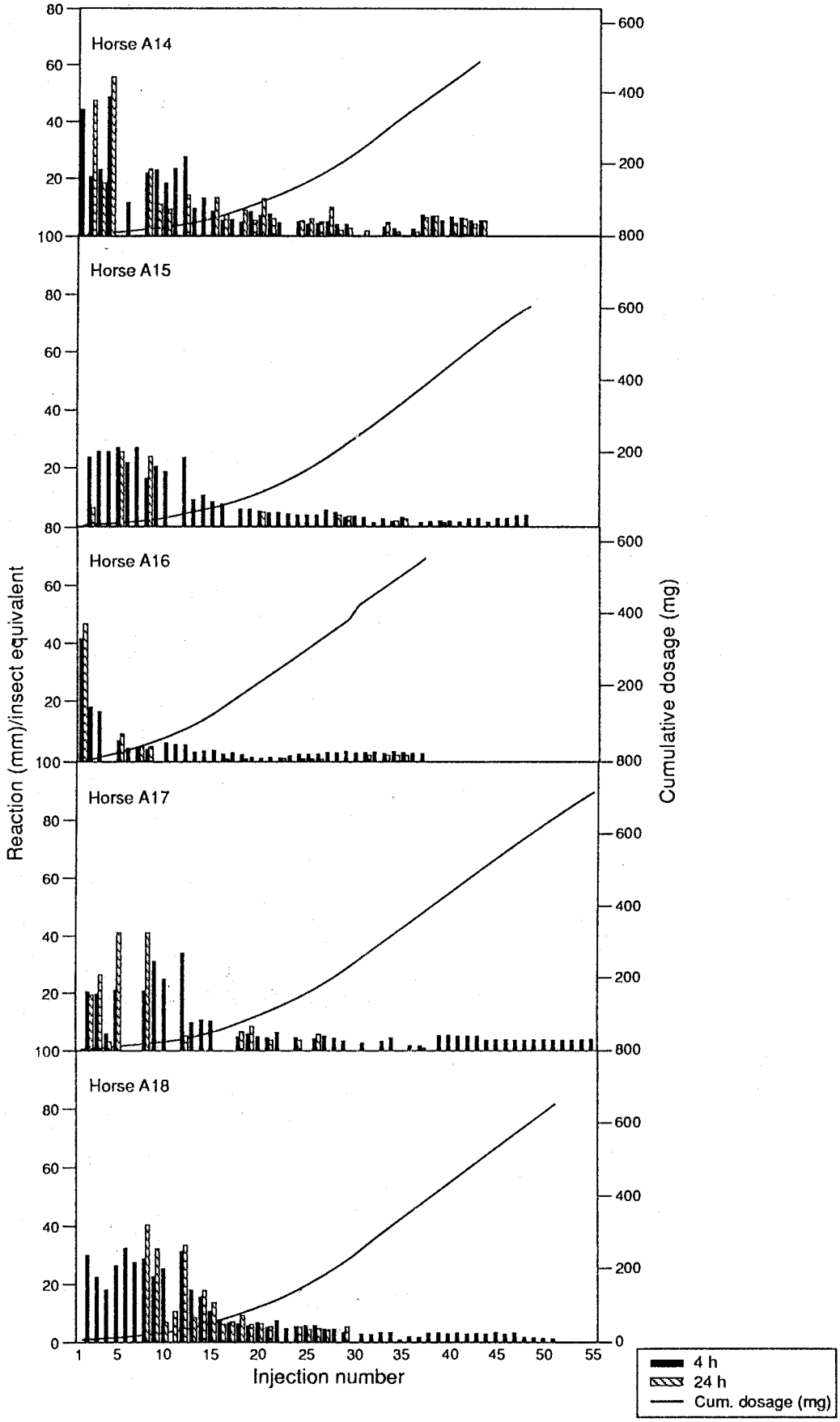
The mean reactions of 8 horses to injections 28 to 36, analyzed separately, again disclosed no interaction between time and injection (P=0.2407). The reaction at 4 h was again significantly greater than that at 24 h (P=0.0004), but the difference between the reactions at 4 and 24 h was much greater for injections 28 to 36, than for the first set of injections (P=0.0045 *vs.* P=0.0004). There was no further effect of injection after injection 28 (P=0.1411) indicating that the reactions to the injections had plateaued.

The reactions of each individual to the injections over both years (Fig. 6.3.1-2) is similar to that of the treated population (Fig. 6.3.1-1). Most horses reacted strongly to the first injections, then their sensitivity dropped rapidly, then more gradually, until it eventually stabilized. In many cases, reactions were recorded at both 4 and 24 h after injection for the first few injections, but gradually the 24 h reaction became less evident until, in many cases, the reaction had disappeared within 24 h. Several horses, notably A3, A8, A13, A17 and A18, appeared to reach a peak of reactivity after a few injections, then their reactions rapidly decreased. Horse A16 entered the program 5 months after most of the other horses. As none of the previous injections had resulted in adverse reactions, this horse was advanced more rapidly through the injections of increasing dosage. Horse A16's reaction decreased much more rapidly than those of the other horses and reached a plateau very quickly.

Two unaffected horses, which were injected subcutaneously with 20 μg adjuvant in physiological saline, did not develop a welt immediately after injection, 4 or 24 h after injection, indicating that the reaction seen in the affected horses was entirely due to the insect extract.

Figure 6.3.1-2. Reactions of individual horses to all injections of *Culicoides variipennis* extract, together with cumulative dose in insect equivalents.


Horse A3
Horse A7
Horse A8
Horse A9
Horse A13
Reaction (mm)/insect equivalent
Cumulative dosage (mg)
Injection number
4 h
24 h
Cum. dosage (mg)


Horse A14
Horse A15
Horse A16
Horse A17
Horse A18
Reaction (mm)/insect equivalent
Cumulative dosage (mg)
Injection number
4 h
24 h
Cum. dosage (mg)

6.3.2 Progress of disease

All the horses began to develop clinical signs as usual in late April/early May 1989 (Table 6.3.2-1). In most cases, the disease appeared to progress normally, and all horses were moderately to severely affected by June or July, with the exception of Horse A7. During this time, the dose that each horse received was low, but gradually increasing. However, between late July and early August, 8 of the 10 horses began to show a distinct improvement. Although all the horses still exhibited lesions and hair loss, the hair was beginning to grow back, and the lesions were clearly healing. The horses, which were normally severely irritated at this time of year, did not appear irritated and were not scratching at the lesions.

Improvement was noted in most cases when the dose reached 10 mg. Therefore, the dose was maintained at this level for several weeks. However, after several further injections, some horses began to show evidence of pruritis, so the dose was again increased. Most of the horses continued to show an improvement until the end of the season. Overall, nine of the ten horses reacted positively to the treatment, during the first year. Other affected horses in the area which were not involved in the trial continued to exhibit severe clinical signs until early November.

Six of the ten horses (A7, A8, A13, A16, A17 and A18) were affected much less severely in 1990 than in previous years, with three of these (A8, A16 and A17) being virtually free of clinical signs (Table 6.3.2-1). Two horses (A3 and A14) showed some improvement, and two appeared unchanged (A9 and A15).

Horse A3 was normally extremely severely affected, with open bleeding lesions approximately 30 cm wide, throughout the entire ventral midline region, with complete hair loss in this region. The chest, rump and face also exhibited open, bleeding lesions. The mane and tail were usually completely rubbed out, with ruggae in the tail. This horse had begun to show an improvement in August of the first year of therapy, with the open lesions beginning to heal, and fine hair growing back in the ventral midline, mane and tail region. However, this horse began to show clinical signs in late April of the second year. The ventral midline region

Table 6.3.2-1. Clinical signs of all the horses during the immunotherapy trial.

YEAR	DATE		HORSE A3	A7	A8	A9	A13	A14	A15	A16	A17	A18
1989	Apr.	18	0	0	0	1	0	0	0	na	0	0
		26	1	0	1	2	1	0	0	na	0	0
	May	4	1	0	na	2	1	1	0	na	0	1
		10	2	1	na	3	2	1-2	1	na	1	2
		19	3	1	na	3	3	2-3	1	na	1	3
		25	3	1	2	3	3	2-3	2	na	2	3-4
		31	3	0	2	na	3	3	3	na	3	4
	Jun.	7	3-4	0	2	na	3	3	3	na	3	4
		15	4	0	2	na	3	3-4	3	na	3	4
		21	4	0	2	na	3	3-4	3	na	3	4
		29	4	0	2	na	3	3-4	3	na	3	4
	Jy.	6	4	0	2	na	3	4	3	na	3	4
		15	4	0	na	na	3	4	3	3	3	4
		21	4	0	3	3	3	4	2-3*	3	3	4
		27	4	0	3-4	3-4	3	4	2	3	3-4	4
	Aug.	2	3*	0	3	3-4	3	3	2	3	3	4
		11	3	0	1-2*	3-4	1	3	2	3	2	3*
		16	2-3	0	1	3-4	0-1*	2*	2	2	1*	3
		23	2-3	0	1	3-4	0-1	1-2	2	1-2*	0-1	2-3
		30	2-3	0	1	3-4	0-1	1-2	2	1	0-1	3
	Sep.	6	2-3	0	1	3-4	0-1	1	2	1	0-1	2
		14	2-3	0	1	3-4	0-1	1	2	1	0-1	2
		20	2	0	0-1	3-4	0-1	0-1	2	1	0	1-2
		28	2	0	na	4	0-1	0-1	2	0	0	1
	Oct.	3	1-2	0	na	4	0-1	2	2	0	0	1
		12	1-2	1	na	4	0	2-3	2	0	0	1
		17	1	1	na	3-4	0	2	2	0	0	1
		31	1	1	0	4	0	2	2	0	0	0-1
	Nov.	8	0	1	0	4	0	2	2	0	0	0
		23	0	0	0	1	0	2	1	0	0	0
	Dec	8	0	0	0	0	0	0	0	0	0	0
1990	Jan.	4	0	0	0	0	0	0	0	0	0	0
		23	0	0	0	0	0	0	0	0	0	0
	Feb.	18	0	0	0	0	0	0	0	0	0	0
	Mar	11	0	0	0	0	0	0	0	0	0	0

Table 6.3.2-1. contd.

YEAR	DATE		HORSE A3	A7	A8	A9	A13	A14	A15	A16	A17	A18
1990	Apr.	8	0	0	0	0	1	2-3	1	0	0	0
		23	3	0-1	1	3	1	4	3	0	0	0
	May	1	3	0	0	3	1	4	1-2	0	1	0
		7	3	0	0	3	1	4	1-2	0	0-1	0
		15	3	0	0-1	3	1-2	3	1	0	0	1
		23	3	0	0	3	1-2	3	2-3	0	1	1
		31	3	1	0	3	1-2	3	3	0	0-1	2
	Jn.	5	3	1	0-1	3	1-2	3	3	0	0-1	2
		12	3	1	0	3	1	3	2-3	0	0	2
		19	3	2	0	3	0-1	3	2-3	0	0	2
		26	3	1	0	3	0-1	3	2-3	0	0	2
	Jy.	3	3	2	0-1	3	0-1	3	3	0	0	2-3
		10	3	1	0	3	0-1	3	3	0	1	3
		18	3	1	0	3	0-1	2-3	3	0	1	3
		23	3	1	0	4	0-1	2-3	2-3	0-1	1	2-3
		30	2	1	0	4	0	2-3	2	0	1	2-3
	Aug.	7	2	1	0	4	0	2	2-3	0	0	2
		14	2	1	0	4	0	2	2	0	0	2
		21	2	1	0	3-4	0	2	2	0-1	0	2
		28	1-2	1	0	3-4	0	2	2	0	0	1
	Sep.	4	1-2	1	0	3-4	0	2-3	1-2	0	0	1
		13	1	1	0	3	0	2	1-2	0	0	0-1
		18	1	1	0	3	0	2	1	0	0	0-1
		25	0-1	0	0	0	0	2	0	0	0	1
	Oct.	5	0-1	0	0	0	0	2	0	0	0	0-1

KEY

0 - no signs.

1 - very mild signs, some scratching, small scabs, slight hair loss. No open lesions.

2 - moderate signs, more scabs, hair loss, horse irritated.

3 - more severe, bald areas, scabs, red open areas, broken hair in mane and/or tail indicating scratching.

4 - very severe, large areas of hair loss, raw, open lesions, horse severely irritated, hair in mane and/or tail rubbed down to skin.

* - Time at which the horse began to show a visible improvement, usually in the form of the hair beginning to grow back, indicating that the lesion was no longer being scratched.

na Horses were unavailable for examination due to owner's activities.

developed some scabs and a few bleeding areas, but was not as severely affected as in previous years. The tail remained generally long throughout the second year, but was rubbed short in several areas. The tail was rubbed bald at the beginning of the season, but grew back slightly, and although continuously irritated, was not bald for the rest of the season, and did not develop scabs or bleeding lesions. In general, although still quite severely affected, this horse did show an improvement from previous years. Horse A3 reacted mildly to the injections at first (Fig. 6.3.1-2). This reaction increased, with strong immediate and delayed reactions, then decreased as the injections proceeded. The delayed reactions became very mild, or were absent after later injections.

Horse A7 was normally only moderately affected, with clinical signs in the ventral midline region, and some scratching in the tail region. It was not as severely affected as other horses in this study. During the first year of therapy, it began to develop mild signs in May, however these healed and it appeared that the injection therapy, even at the low dose employed at this stage, was having a beneficial effect on this horse, possibly due to the fact that this animal was the least affected of all the horses prior to treatment. The dose was increased until August, then maintained, as it appeared that the treatment was effective enough at that low dose with this animal. However, it began to show mild clinical signs again in early October, so dose was again increased, but it was never as high as in other horses. During the second year, this horse developed mild clinical signs, with some scabs, and some bald areas in the ventral midline region. Overall, horse A7 was only mildly affected throughout the immunotherapy trial, but had been more severely affected previously (Table 5.3.2-1). This horse did not react as strongly to the injections as other horses. In general, it developed only an immediate reaction after injection (Fig. 6.3.1-2).

Horse A8 normally developed moderate to severe clinical signs in the ventral midline region. The mane and tail were also affected. This horse began to show an improvement in early August during the first year of immunotherapy. Due to its leg injury, it did not receive injections after April in the second year. However, the improvement seen during August and

the rest of the first year of therapy continued, despite the lack of injections. This horse did not develop clinical signs during the second year, and was considered completely sound. This horse developed strong reactions after the first six injections, but these gradually decreased (Fig. 6.3.1-2).

Horse A9 normally developed severe clinical signs in the mane and tail region, although the ventral midline region was never affected. The mane and tail were usually rubbed bald. This horse developed severe clinical signs during the first year of immunotherapy, and unlike all the other horses, did not show an improvement in late July/early August, when the other horses began to recover. As this horse was away for a period of time at the beginning of the trial, it did not reach the dose of 10 mg until mid September. It was at this dose that the other horses showed an improvement. Horse A9 was not treated after March of the second year and continued to develop clinical signs as before. However, the tail was not rubbed bald as in previous years. This horse had a very strong reaction to the first injection (Fig. 6.3.1-2), but the reactions steadily declined over time, with the exception of injection 7 which provoked a small immediate, but a large delayed response. Overall, this horse did not appear to respond to therapy.

Horse A13 normally showed severe clinical signs in the ventral midline region, and also rubbed the lower part of its mane so that the hairs were very short, and also rubbed its tail. During the first year of therapy, it began to show an improvement in mid August, with the hair growing back along the ventral midline, and no further scratching in the mane and tail. It began to show very slight clinical signs in late April of the second year. Some hair was rubbed out in this region, but the area did not become scabbed or bleeding. However, these mild signs healed, and hair grew back over the rubbed areas. From June onwards, this horse had virtually no clinical signs, although it did appear pruritic on some occasions. This horse had strong reactions to the first few injections (Fig. 6.3.1-2), but these gradually decreased. At first, the reactions peaked 24 h after injection, but this changed until the reaction at 4 h was similar to

that 24 h after injection. Horse A13 continued to develop both an immediate and a delayed reaction after each injection for the duration of the study.

Horse A14 normally rubbed out its mane entirely and scratched the tail until it was completely bald. The tail usually showed ruggae, and sometimes bled. The shoulder and rump regions were often scratched so severely that they bled, and developed large open lesions. This horse did not scratch its ventral midline region. During the first year of therapy, this horse was severely affected with bleeding lesions, but began to show a clear improvement by mid August. This continued until October, when it started to scratch its tail and shoulder again. Dosage was increased regularly during this period until a maintenance dose was reached in December. During the second year of therapy, this horse began to show clinical signs by April. Signs were very severe at first, with large bleeding lesions, but began to heal in late May. The lesions were not as severe for the rest of the season, but were still clearly present. This horse continued to rub its tail and mane, although after May, the affected areas were never raw. Horse A14 reacted strongly to the first four injections, then the reaction decreased. In most cases, the reaction peaked at 24 h, rather than at 4 h after injection throughout most of the trial (Fig. 6.3.1-2). However, after more than 30 injections, the delayed reaction was slightly less than the immediate reaction, although this horse continued to exhibit a delayed reaction throughout the study.

Horse A15 normally exhibited severe clinical signs in the ventral midline region and chest, with open, bleeding lesions. The mane and tail were usually completely rubbed out. This horse began to show an improvement in the first year of therapy in late July, with the lesions healing, and hair growing back. However, it developed severe clinical signs during the second year of therapy, and did not appear to be benefitting from the treatment. However, as with the other horses, this horse's sensitivity to the injections did appear to decrease over time (Fig. 6.3.1-2).

Horse A16 normally rubbed out the mane and tail entirely and also scratched the ventral midline region, resulting in bleeding lesions. During the first year, an improvement

was noted by late August, with the mane beginning to grow back. During the second year, this horse remained completely clear of clinical signs. This horse was considered completely sound. This horse exhibited a large reaction to the first injection, but this dropped very rapidly (Fig. 6.3.1-2). In most cases, the immediate reaction was small, and the delayed reaction was even less, or was non-existent.

Horse A17 normally showed severe clinical signs in the ventral midline region and mane, with the ventral midline exhibiting large, bleeding lesions and the mane completely rubbed out, down to the crest. The tail was also frequently rubbed until bald areas appeared. During the first year of immunotherapy, this horse began to show a clinical improvement by early August, and from that point on showed only very minor signs. In the second year of the trial, horse A17 was virtually free of clinical signs. On one or two occasions, it showed very minor signs of irritation, but in general could be considered completely sound. At first, this horse reached peak reaction 24 h after injection (Fig. 6.3.1-2), but after 8 injections, the 24 h, or delayed, reaction was small or non-existent. After 26 injections, it did not develop any delayed reaction, and only exhibited a small immediate (4 h) reaction.

Horse A18 normally showed severe ventral midline and chest lesions, with open, bleeding areas. The entire ventral area was usually severely affected. The mane and tail were both severely scratched, with ruggae in the mane. The face area was also affected. Horse A18 showed severe clinical signs in the first year of therapy until mid August, when the lesions began to heal, and hair began to grow back. In the second year of therapy, it began to show clinical signs by mid May. However, these signs were extremely mild in comparison with past years. The chest area developed some bald patches, and the mane and tail showed some signs of having been rubbed, with some broken hairs, although they were not bald. Some small scabs were seen in the ventral midline region, but these were minor. The most serious lesions were in the chest area, but were still much less severe than in past years. In general, the horse was greatly improved, although still showing slight clinical signs. The owner considered that the horse was much healthier overall, probably due to the relief from irritation. It is interesting to

note that the hooves on this horse began to grow normally during the trial. Prior to the start of the trial, the hooves grew only slightly, but as the horses' general well-being improved, the hooves began to grow healthily. Horse A18 at first developed only immediate reactions after injection (Fig. 6.3.1-2), but after 8 injections, it developed both immediate and delayed. The delayed reactions reduced over time, but, although small, were always present.

Other affected horses in the area which were not treated continued to develop severe clinical signs as usual in both years, indicating that any improvements were due to treatment, and not to a lack of the causal agent.

During the first year of the trial, each horse received the injections on the same day, meaning that the number of days between injections did not vary from horse to horse. However, during the second year, the injections were performed by the horse's owner, so the regularity of the injections varied greatly. The number of days since the last injection, during the time that the owners performed the injections, is shown in Table 6.3.2-2. Horses A16, A17 and A18 received the injections most regularly, and were also three of the six horses which showed a great improvement with treatment, in particular A16 and A17 which did not develop any clinical signs during this time. The other horses received fewer injections and they were administered less frequently and irregularly.

6.3.3 Follow-up, 1991

In the year following the cessation of immunotherapy, horse A3 showed moderate clinical signs, with scabs in the ventral midline region, face and shoulders. Both the tail and mane were rubbed, but not bald. This horse was not as severely affected as it had been prior to treatment. Horse A7 showed mild clinical signs, similar to those seen in 1990, but milder than before treatment. Horse A8 showed no visible clinical signs in 1991. The owner stated that it had shown slight irritation in the shoulder and wither region. Horse A9 had rubbed the top 25 cm of the tail, and most of the mane. There were no open lesions. In general, the clinical signs were similar to those seen in the past. This horse had a three-month-old foal at foot when examined, which was already beginning to show clinical signs. Horse A13 rubbed the mane

Table 6.3.2-2. The number of days between injections, during the second year of immunotherapy.

Recommended interval (days)	NUMBER OF DAYS SINCE LAST INJECTION									
	A3	**A7**	**A8**	**A9**	**A13**	**A14**	**A15**	**A16**	**A17**	**A18**
14	14	14	61	14	21	21	14	14	14	13
14	14	14	19	-	21	21	14	14	15	16
14	23	23	-	-	14	14	23	14	15	19
7	9	9	-	-	15	15	9	7	7	7
7	7	7	-	-	15	15	7	7	6	5
7	11	11	-	-	12	12	11	9	8	7
7	15	15	-	-	7	7	15	7	7	10
7	25	25	-	-	13	13	25	7	9	5
7	8	8	-	-	-	-	8	13	5	9
7	9	9	-	-	-	-	9	7	7	5
7	8	8	-	-	-	-	8	7	8	8
7	34	34	-	-	-	-	34	7	6	8
7	-	-	-	-	-	-	-	7	7	15
7	-	-	-	-	-	-	-	7	7	13
7	-	-	-	-	-	-	-	7	7	12
7	-	-	-	-	-	-	-	-	7	9
7	-	-	-	-	-	-	-	-	14	-
7	-	-	-	-	-	-	-	-	14	-
7	-	-	-	-	-	-	-	-	7	-
7	-	-	-	-	-	-	-	-	7	-

down to 5 cm long for the first 30 cm above the withers. The tail was unaffected and the ventral midline region exhibited dry skin, but no lesions. The midline region had not lost hair. In general, this horse was slightly worse than when on immunotherapy, but was not as severely affected as it had been prior to therapy. Horse A14 had rubbed the mane down to 5 cm and the tail to 2.5 cm. There were no open lesions. It appeared to show signs similar to those seen in 1990. Horse A15 showed much more severe clinical signs in 1991 than in 1990. The neck, mane and tail were rubbed raw and the ventral midline and shoulders were scabbed. Horse A16 continued to be sound. It did not exhibit any clinical signs during 1991. Horse A17 showed mild clinical signs, with some small scab formation and dry areas in the ventral midline region, and slight hair breakage in the tail. The signs were worse than in 1990, but still extremely minor in comparison with those prior to treatment. Horse A18 developed lesions in the chest and shoulder region early in the season, which partially healed. However, lesions were always present in the chest area. The ventral midline region had dry skin, but no lesions. In general, this horse exhibited clinical signs similar to those seen in 1991, and still showed a great improvement over previous years.

6.4 DISCUSSION

This study showed that immunotherapy can be a successful and feasible treatment for CH in horses, with six horses greatly benefitting from the treatment, three of which were completely sound. No horse's condition worsened due to treatment.

The use of whole-body insect extracts was evidently justified. In Hymenoptera sting allergies, treatment involves only the venom itself. Venom is found only in the venom sac, so whole body extracts were not effective (Hunt *et al.* 1978). However, in insect allergies the whole body is usually used, as the allergen, although no doubt found in the salivary glands, is found throughout the body (Chapter 2).

The mechanism of immunotherapy in horses has not been explored, but can be extrapolated from human immunotherapy work. CH has been shown to be IgE-mediated using

passive transfer experiments (Riek 1954; Baker 1978; Braverman *et al.* 1983; Matthews *et al.* 1983a; Quinn *et al.* 1983; Morrow *et al.*1987). In human allergy, affected individuals have an abnormally high level of Immunoglobulin E (IgE). In human immunotherapy, it has been observed that the titer of IgE rises immediately after immunotherapy is begun, often tripling in the first few months of venom immunotherapy (Sobotka *et al.* 1978), and then dropping to below pretreatment levels (Reisman *et al.* 1975; Evans *et al.* 1976; Lockey and Bucantz 1976; Yunginger 1986). Immunotherapy was also found to suppress the seasonal rise in IgE titer normally seen in individuals suffering from seasonal rhinitis (Lichtenstein *et al.* 1973; Norman 1980; 1981).

This decrease in IgE titer corresponds with a rise in the titer of Immunoglobulin G (IgG) (Devey *et al.* 1976; Mokry *et al.* 1985; Yunginger 1986; Camazine 1987), and it is this IgG, together with the decrease in IgE levels, which is thought to be responsible for the clinical efficacy of immunotherapy. The IgG level may rise 2-30 times above the original level after a few months of immunotherapy (Magnusson *et al.* 1983). This rise in IgG was long considered to be 'blocking' the allergic reaction in some manner, but it was not until Lessof *et al.* (1978) demonstrated that bee-sting allergic individuals could be passively protected from allergic reactions by injections of IgG from immune beekeepers, that a direct link was proved. Since this time, most studies have reported that an increase in the IgG level results in an improvement in clinical signs, although in most cases the actual level of IgG could not be correlated with the degree of improvement (Levy *et al.* 1971). As the levels of IgE, IgG and the severity of clinical signs vary from one allergic individual to another, it is likely that different individuals can be protected by different levels of IgG, depending on their particular circumstances. It is, therefore, probable that it is not the individual's level of IgG that is important, but the ratio between IgE and IgG levels. Hoffman *et al.* (1981) found that several treated individuals had adverse reactions to a sting challenge despite high levels of IgG. However, when the IgE:IgG ratio was studied rather than actual IgG levels, a clear correlation between ratio and level of protection was found.

Blocking IgG is IgG that is specific to the allergen. Human IgG can be broken down into subclasses 1-4. In a normal human, the distribution of these IgG subclasses is approximately 65% IgG_1, 25% IgG_2, 6% IgG_3 and 4% IgG_4 (Aalberse *et al.* 1983). However, it has been determined recently that in individuals with allergies, the IgG subclass distribution directed against the relevant allergen is significantly different from this ratio and is composed mainly of IgG_1 and IgG_4 (Nakagawa 1991). In individuals successfully treated with venom immunotherapy, 50% of their IgG belonged to the IgG_4 subclass, indicating that IgG_4 may act as the 'blocking antibody'. IgG_4 appears to have a blocking action in human allergies to grass, pollen, venom and dust mites (Devey *et al.* 1976; Van der Giessen *et al.* 1976; Nakagawa and Miyamota 1985; Urbanek and Dold 1986; Nakagawa *et al.* 1987; Ohashi *et al.* 1987; Nakagawa 1991). In treated individuals, the rise in IgG_4 may be as high as 703% of the pretreatment levels (Urbanek and Dold 1986). It has been shown that IgE-mediated basophil degranulation could be inhibited by passive sensitization with allergen-specific IgG_4 antibodies in bee-venom allergy (Nakagawa and de Weck 1983). In some cases, the level of IgG_1 has also been shown to increase, particularly at the start of immunotherapy and may also play a role in allergy suppression, although, in tests, the level of IgG_4 showed a sharper and more prominent increase than IgG_1 (Nakagawa 1991). In house dust mite allergy, the level of mite-specific IgG_1 did not correlate with patient response to therapy, but the level of IgG_4 correlated well with an improvement in clinical signs (Ohashi *et al.* 1987).

The mechanism of action of blocking antibodies is not fully understood, but it is thought that the blocking IgG competes with the allergen-specific IgE attached to the mast cells and basophils, by combining with the antigen in the fluid phase, before it reaches the target cells, thereby reducing the effective concentration of antigen that can react with the cell-bound IgE (Lichtenstein *et al.* 1968; Halliwell and Schwartzman 1971; Patterson *et al.* 1978; Hsieh 1984). The blocking antibody and the allergen form a complex which is then removed by the reticulo-endothelial system (Frick 1980).

Three IgG subclasses a, b and c, have been identified in horses (Helms and Allen 1970). The relative concentrations of these subclasses vary from breed to breed (McGuire *et al.* 1972) and their functional abilities differ in the course of an immune response (Wells *et al.* 1981). It is possible that one of these subclasses acts in the same manner as human IgG_4.

The complete mechanism of action of human immunotherapy is not yet understood, but is a result of many immunological changes including decrease in IgE antibody formation by a suppression mechanism, probably by suppressor T cells (Foreman and Lichtenstein 1980); production of IgG blocking antibodies, and other factors, probably including activation of antigen-specific suppressor T cells.

The efficacy of immunotherapy may also involve host immunity. *Anopheles stephensi* Liston had higher mortality rates when fed on rabbits which had previously been injected with whole mosquito antigens (Alger and Carbrera 1972). Death is probably related to antibodies produced in the rabbit to the mosquito antigens. Decreased fecundity, reduced feeding rates and increased mortality were seen in tse-tse flies (*Glossina* sp.) fed on guinea pigs and rabbits immunized with whole body fractions of the tse-tse fly (Kaaya and Alemu 1982; 1984; Matha *et al.* 1986). Similar studies have shown that tick feeding also induces a form of host immunity that results in lower tick weight (Rechav *et al.* 1989; Need and Butler 1991; Need *et al.* 1991). Whether such antibodies could prevent the insect from successfully taking a blood meal is unknown, and it is this feeding that provokes the allergic reaction.

Equine immunology is understood far less than human, but as this study has shown that immunotherapy is a successful treatment of an equine allergic disease, it is possible that the mechanisms behind the efficacy of the treatment are similar in both species.

The regularity of the injections had a strong effect on the efficacy of the program. Weekly injections are thought to provoke a higher IgG titer than less frequent injections (Feingold 1973b). During the first year of therapy, all the horses were injected on the same day, at the same time interval and 9 of the 10 horses showed a distinct improvement. During the second year, the regularity of injections varied greatly from horse to horse, depending on

the animal's owner. Of the three horses that were completely sound (A8, A16 and A17), two received the most regular, evenly spaced injections of all the horses, indicating that regular, weekly injections are most effective. Horse A18 also received fairly regular injections (Table 6.3.2-2), and showed a great clinical improvement over past years. Horses A13 and A14 both showed a distinct improvement in the first year, with regular injections, but received widely spaced and erratic injections which terminated early, during the second year. Although horse A13 showed a great improvement over previous years, it did still develop minor clinical signs and horse A14 was only slightly improved over previous years, and showed moderate to severe clinical signs on occasions during the season. As both animals did show some improvement, it is probable that a more regular injection regime would have resulted in a much greater improvement. This may also have been true with horses A3, A7 and A15, only two of which (A3 and A7) showed an improvement, as all these animals improved with regular, weekly injections during the first year of therapy. An increased interval between injections has been linked with treatment failure in human immunotherapy (Kemeny *et al.* 1983). The regularity of the injections depended on the owners, who frequently forgot to give injections. In some cases, this was due to a reluctance to perform the injection itself. These owners were very concerned about their horses' welfare, and were interested in the research aspects of the study. Therefore, they were, at least in principal, highly motivated to continue with the therapy. Despite this, several of these owners were unreliable in performing injections. Therefore, it is probably unrealistic to expect owners to perform injections if such treatment were to become publicly available. Also, a veterinarian would still have to perform the first injections which involve dose increases, because of the risk of anaphylactic shock. Therefore, such a program of weekly injections is unlikely to be feasible on a large scale, and would only be effective with individual animals of particular value. However, slow-release polymers that can be coupled to a protein or protein complex have recently been developed (Alkemade, pers. comm.). The advent of such polymers makes a large scale immunotherapy program in horses perfectly feasible. Such polymers can be developed to release a small amount of protein gradually over a

3 month period or, alternatively, to release prescribed amounts on a regular basis over 3 months. This would mean that a veterinarian would only have to be called 4 times a year, and most owners would be prepared to pay for such a service if it resulted in an improvement in clinical signs. Research needs to be conducted to determine optimum rates of allergen release and safety factors, but this area shows promise.

In some cases in human immunotherapy, a 'rush' program has been advised over the conventional method of weekly injections. The rush method involves attempting to hyposensitize individuals in a matter of hours or days, as opposed to months or even years, by administering up to 23 injections of increasing doses in the first day, 6 injections in the second and 2 on the third (Nataf *et al.* 1984). Although often effective, in many cases, the rush method entailed a much higher risk of anaphylactic shock so should only be performed on hospitalized patients (Gillman *et al.* 1980; Malling *et al.* 1985; Adolph *et al.* 1986). A modified rush, with maintenance dose achieved in 8 weeks was equally as effective as conventional therapy, with no increase in adverse reactions (Clayton *et al.*1983). Although such a rapid immunotherapy program has obvious attractions for equine allergies, these are outweighed by the disadvantage of the higher risk of anaphylactic shock. It might be possible to use a hospital-type situation with particularly valuable animals, but in general such a method would not be feasible.

In human immunotherapy, cumulative dose injected is correlated with efficacy. IgG levels have been found to be highest in individuals that have received the largest cumulative dose (Evans *et al.* 1976; Lockey and Bukantz 1976). In this study, Horses A16, A17 and A18 received the largest cumulative doses (Fig. 6.3.1-2). Two of these horses recovered completely, and the third was vastly improved, changing from being very severely affected, with the owner considering having the horse euthanased, to being only mildly affected. However, in some cases, the horses received relatively high cumulative doses and showed no improvement (A15), or only slight improvement (A14), despite receiving the same or higher cumulative doses than other horses which responded to therapy (A3 and A13). Although

cumulative dose is probably an important factor in achieving success, too few horses were tested to make any firm conclusions. It is probable that no particular cumulative dose will be effective for every horse as there is great individual variance in clinical signs and allergic state from horse to horse. Also, although important, cumulative dose is only one of many factors that influence efficacy.

Most of the horses in this study benefitted from the treatment. However, the degree of improvement in each horse varied from slight to complete. This is consistent with human immunotherapy programs and is to be expected in any allergy treatment. The degree to which an individual reacts to an allergen varies, depending on many factors, both intrinsic and extrinsic. In particular, the efficacy of immunotherapy will depend on the stage of development of the individual's immune system to the allergen.

In the past, animal immunotherapy has involved a low number of low dose injections and has frequently proved unsuccessful or equivocal. However, studies in human immunotherapy have shown that such low dose treatment is ineffective (Patterson 1979), and actually may result in a worsening of the condition as it causes an increase in the levels of IgE, without provoking an increase in IgG (Aas 1978; Creticos *et al.* 1984; Graft 1987). As my study was successful using high dose injections over a two year period, it is possible that animal immunotherapy could be equally as successful as human immunotherapy if appropriate dosage regimes are employed. An attempt to treat horses suffering from CH with immunotherapy using a crude extract of *Culicoides* in Florida, was unsuccessful, with only one horse benefitting over a six month period, although treated horses were improved over untreated, but this could not be verified statistically (Barbet *et al.* 1990). However, this study began when the horses were already affected, and it is likely that allergic state has an effect on the impact of immunotherapy. In human treatment, therapy is begun prior to the expected seasonal exposure. In my study, no improvement was noted for the first five months of treatment, then a steady regression of lesions was noted. It is possible that had the Florida study been continued, it may have been eventually successful.

In any immunotherapy program, the question of when, if ever, to terminate therapy is an important consideration. IgE is very persistent, but levels of IgG drop in a few months if immunotherapy is discontinued (Golden *et al.* 1981). In many cases, human immunotherapy is carried on for life, with booster injections being given every few months, once a regular maintenance dose has been reached. It must be remembered that immunotherapy is a treatment, not a cure, and is not usually expected to be permanent, without regular boosting. However, repeated booster injections are not always necessary, and some individuals may remain protected for years. This again varies from individual to individual.

In this study, when horses were observed one year after the discontinuation of therapy, the three horses that completely recovered (A8, A16 and A17) still failed to develop any clinical signs and, in general most horses were only slightly worse than during immunotherapy, if at all. Horse A15, which did not appear to benefit from the treatment, exhibited more severe clinical signs one year after therapy, than during therapy, so it is possible that the treatment did have some positive effect. Of the horses that benefitted from the treatment, none regressed to the level of clinical signs seen prior to treatment. This indicates, that while probably not permanent, the effects of this immunotherapy are long-lasting. Therefore, it is probable that after a course of immunotherapy, a CH affected horse could be maintained symptom-free with one or two booster injections per year.

Two horses, A8 and A9, only received injections for one year, and consequently had the lowest cumulative doses and received the least number of injections. Horse A9 was pregnant during treatment, which may have affected its immune system in some way, making it less receptive to treatment, and showed no improvement at any time, but horse A8 showed a great improvement, and was free of clinical signs throughout 1990 and 1991, despite being taken off treatment early in 1990. This horse had previously been severely affected but has remained symptom-free to date. This clearly indicates the individual differences between each horses' immunological state, as this horse was successfully treated with a much lower dose than the other test animals.

Immunotherapy is a successful and safe method of treating CH, with no noticeable side effects. Research is needed on a large scale to determine whether slow-release polymers would be feasible to use in conjunction with the allergen, and to determine how rapidly dose can be increased without adverse side effects. As none of the horses in this trial suffered from a systemic, anaphylactic shock reaction, it is probable that dose could be safely increased more rapidly, resulting in faster improvement in clinical signs.

7. IDENTIFICATION AND CHARACTERIZATION OF THE ALLERGEN RESPONSIBLE FOR *CULICOIDES* HYPERSENSITIVITY

7.1 INTRODUCTION

Immunotherapy is an effective treatment for CH. However, the previous trial was conducted using a crude extract of *C. variipennis*. This involved obtaining very large numbers of the insect. When the ten horses were on a maintenance dose of 20 mg (dry weight of insects before extraction), this equated to approximately 33,600 insects which were extracted to produce one weeks' dose. Therefore, although feasible for a two year study involving only ten horses, such a method would be impractical on a larger scale. Also, even if such vast numbers of insects could be obtained on a regular basis, injecting the crude extract is undesirable. The crude extract contains a large number of proteins, many of which are potentially allergenic. Therefore, it is possible that injecting large quantities of these extraneous proteins may sensitize the animal, and enzymes injected may cause stress. This is unlikely to create a large risk as the horses are not exposed to the body proteins in a natural situation. However, if immunotherapy is ever to be feasible on a large scale, the actual allergen itself must be identified, with the hope that it can be characterized and produced on a large scale. Nearly all mammalian allergens are acidic proteins (Halliwell 1971; Ohman and Sundin 1987).

Therefore, the objectives of this section were to identify the allergen in the insect using immunoblotting techniques, and to characterize it.

7.2 METHODS, MATERIALS AND RESULTS

The identification of human allergens is usually performed using a Western blotting technique (Burnette 1981; Diano *et al.* 1987). For my study, detection of *Culicoides* allergens involved separating insect extract into its constituent proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). The proteins in the gel were then electro-

blotted onto a nitrocellulose membrane (Lin and Kasamatsu 1983), which could then be processed using an immunoassay kit.

7.2.1 General protocol

A 7.5% to 15% T, 2.6% C gradient polyacrylamide gel was found to achieve the best separation of the insect proteins. A 10 x 15 x 1.5 mm^3 gel was poured using a gradient gel-maker. A 3% T, 20% C acrylamide stacking gel was used. An extract of whole, dried *C. variipennis* from the same source as those insects used to prepare the hyposensitizing extracts for immunotherapy (Chapter 6) was made in phosphate buffered saline (PBS), pH 7.0 using a tissue homogenizer. One μM of phenylmethyl sulfonyl fluoride (PMSF) (Sigma), a protease inhibitor, was added. The resultant extract was centrifuged at 13000 xg for 5 min, then filtered through a 0.45μm Millipore syringe filter. Protein content was determined with Bradford's reageant (Bradford 1976). Generally, following electrophoresis, half the gel was stained with Coomassie Brilliant Blue (Thorne 1978), and the other half was electro-blotted onto a nitrocellulose membrane in a semi-dry unit (LKB Nova Blot).

The nitrocellulose was processed as outlined in the immunodetection kit (Amersham). Briefly, after blocking the membrane in a milk powder solution, antigens were detected with specific antibodies. These antibodies were then, in turn, detected with secondary antibodies which were biotinylated. The nitrocellulose membrane was then incubated in a solution of streptavidin-labelled alkaline phosphatase followed by a solution containing enzyme substrate (nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP)). This resulted in development of a coloured product which indicated the presence of antigen. This particular system is sensitive in that it combines the specificity of antibody probe with the high affinity of biotin to streptavidin.

As CH is IgE-mediated, the obvious primary antibody to use to detect the allergen was equine IgE which would bind to the allergen. This IgE-allergen complex would then be detected by the biotinylated secondary antibody, anti-equine IgE. The streptavidin-alkaline

phosphatase conjugate binds the biotin, and would then be visualized by incubation with the substrate. The allergen should show up as a single dark band against a lighter background.

However, equine IgE and, hence, anti-equine IgE are not commercially available. Therefore, alternatives were tested.

7.2.2 Preparation of equine serum

Serum from allergic individuals presumably contains elevated levels of IgE. This level should decrease after immunotherapy (Chapter 6). Therefore, serum collected from affected horses prior to the immunotherapy trial were used. However, due to the minute amounts of IgE present, serum alone did not contain enough IgE to be detected. Therefore, 2 L of whole blood were collected from an affected horse, A19, which had been skin tested to conclusively diagnose CH, but had not been used in the immunotherapy trial. The blood was allowed to clot at room temperature overnight, and then the serum was collected by centrifugation at 1000 xg for 10 min. The immunoglobulins were precipitated using saturated ammonium sulfate solution (Carson *et al.* 1975; Phelps 1978; Dunbar 1987). Saturated ammonium sulfate, pH 7.2-7.4, was added very slowly to serum while stirring up to a ratio of 60:40, ammonium sulfate:serum. The solution was then centrifuged at 5000 xg for 30 min, and the pellet was dissolved in PBS and dialysed against PBS overnight to remove the ammonium sulfate. The resultant solution, which should have an elevated level of equine IgE, was used at a 1:100 dilution as the primary antibody in detecting allergen (s) on an immunoblot. However, as anti-equine IgE was unavailable, an alternative was considered.

7.2.3 Using anti-human IgE as the secondary antibody

Although IgE is normally considered to be homocytotropic, several workers have suggested that there is a certain amount of cross-reactivity between equine IgE and anti-human IgE (Suter and Fey 1981; 1983; Matthews *et al.* 1983b; Magro *et al.* 1988). It has also been shown that there are similarities between human and equine IgE (Matthews *et al.* 1983a; Morrow *et al.* 1987). Therefore, anti-human IgE developed in goat (Sigma Chemical Company) was used as the secondary antibody at dilutions of 1:100 and 1:1000. As this was

not available in a biotinylated form, biotinylated anti-goat IgG developed in rabbit adsorbed with human serum proteins (Sigma) was used in addition. Human IgE (Helix Laboratories, Richmond, B.C.) was used as a positive control, and a 1 mg/mL solution of bovine serum albumin (BSA) was used as a negative control.

However, no reaction occurred with the insect extract. The positive control showed up clearly. This may have been due to a lack of cross-reactivity between equine IgE and anti-human IgE, or to too low cross-reactivity, or may have been because the level of equine IgE in the affected horse serum was still too low, despite the purification of the immunoglobulins.

7.2.4 Isolating equine IgE

While equine IgE is unavailable in a commercial form, workers in Switzerland have reported a method for purifying IgE, which resulted in an 81% pure solution (Suter and Fey 1981; 1983). If IgE could be purified in the laboratory, it might be used to develop anti-equine IgE in rabbit, which could then serve as the secondary antibody. The methods of Suter and Fey (1981; 1983) were followed, with some modifications.

7.2.4.1 Anion exchange

The concentrated immunoglobulin solution (7.2.2) was equilibrated with 0.01 M PO_4 buffer, pH 8.0 then run on an anion exchange column. Originally a DEAE Sephadex A25 column was used. This column was 2.5 x 13 cm and 30 mg of protein was applied for each run. The pump was run at 0.5 mL/min. The first peak was eluted off with the equilibration buffer, and the peak obtained with 0.75 M PO_4 buffer was collected. However, this column took 24 h to equilibrate and several hours to perform one run. Therefore, a smaller DE51 column was used to which 50 mg of protein could be applied. The pump was run at 0.5 mL/min. However, this column also proved unsatisfactory, and was replaced by a Bio-Rad Econo-Q Cartridge which proved to be far superior. This column was 1 x 5 cm and allowed a load of up to 75 mg per run. This column could be run much faster. It could be equilibrated in 15 min, and allowed up to 4 runs per day. The sample was applied to the column at 0.5 mL/min, then the buffers were applied at 2 mL/min. The profile obtained from a typical run is

shown in Fig. 7.2.4.1-1. This column was run repeatedly until enough protein eluted with the 0.75 M PO_4 buffer was collected to purify further. The stored fractions were pooled and concentrated using an ultrafiltration cell (Amicon).

7.2.4.2 Gel filtration

The concentrated protein obtained from the anion exchange column was further purified on a Sephacryl S200 column. The column was 2.5 x 150 cm and was equilibrated with PBS, pH 7.2 for 24 h. 53.5 mg of protein was applied at 0.22 mL/min and the column was run for 24 h. The profile obtained is shown in Fig. 7.2.4.2-1.

According to Suter and Fey (1981), relatively pure IgE was obtained in the first part of the peak. However, when the resultant fractions were run on a polyacrylamide gel, they were found to contain many proteins, and it appeared unlikely that IgE was a major component of the fractions. Other workers have also been unable to reproduce this work (Matthews, pers. comm.). To develop a new purification scheme for horse IgE would be very time consuming and beyond the scope of this thesis.

7.2.5 Using equine IgG

An allergic animal has elevated levels of IgE and low levels of allergen-specific IgG, or blocking antibody. As hyposensitization progresses, either through natural exposure, or immunotherapy, the IgE level will drop and the level of allergen-specific IgG will rise. So a hyposensitized horse should have high levels of specific IgG. However, in the immunotherapy trial in this study, the whole insect as opposed to the specific allergen, was used, so the horses involved will have not only developed allergen-specific IgG, but may also have developed IgG specific to many other proteins. However, all animals have some specific IgG, even before immunotherapy (Norman 1975). Therefore, an affected horse, which has not been treated with immunotherapy, such as horse A19 from which the serum was obtained, will have some allergen-specific IgG.

Normal human serum contains approximately 5mg/mL of IgG compared with 0.1-0.7 μg/mL IgE, and 20μg/mL in allergic serum (Bennich and Johannson 1970; Norman 1975).

Figure 7.2.4.1-1. The profile obtained when 75 mg of purified immunoglobulins from an affected horse were run on a Bio-Rad Econo-Q Cartridge anion exchange column at 2 mL/min.

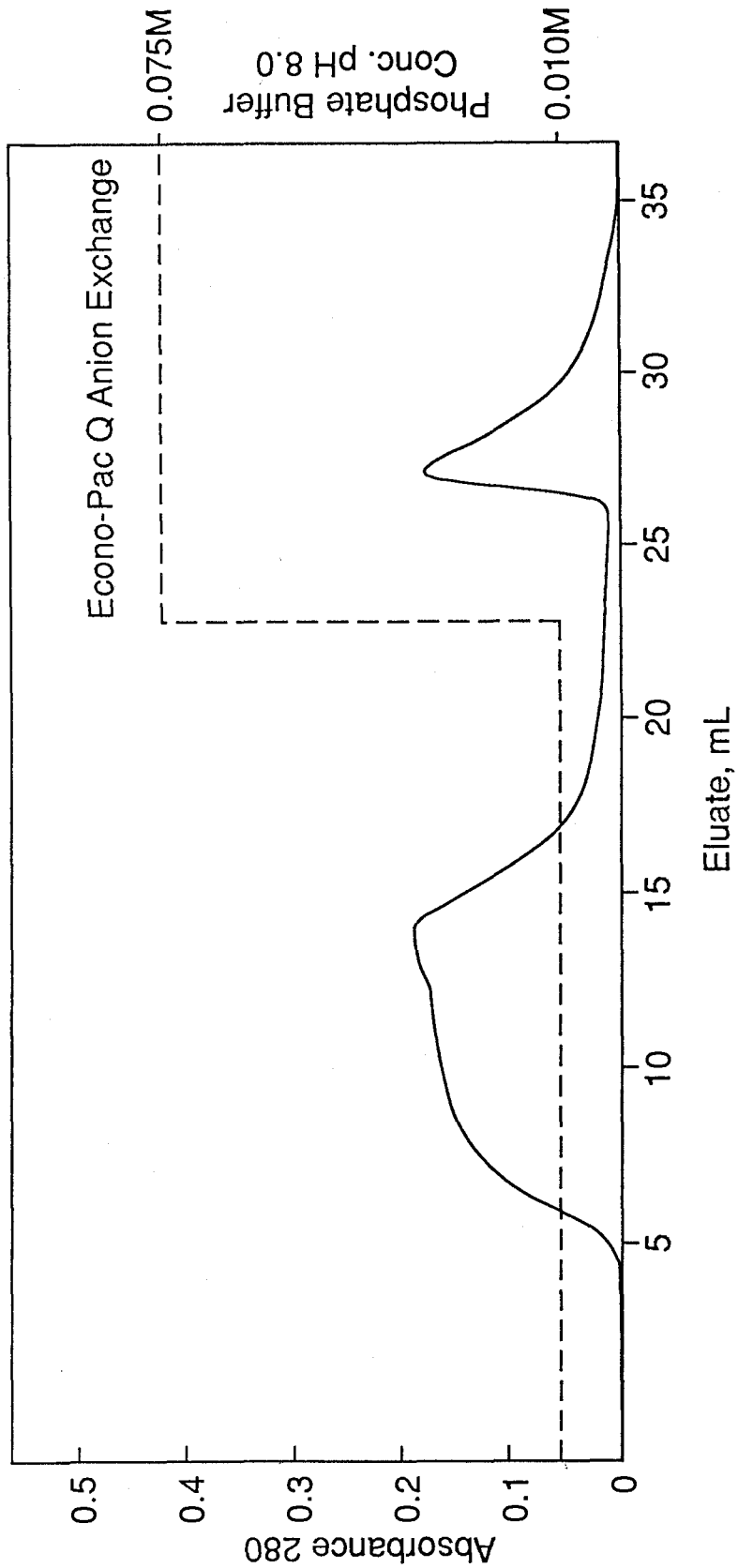
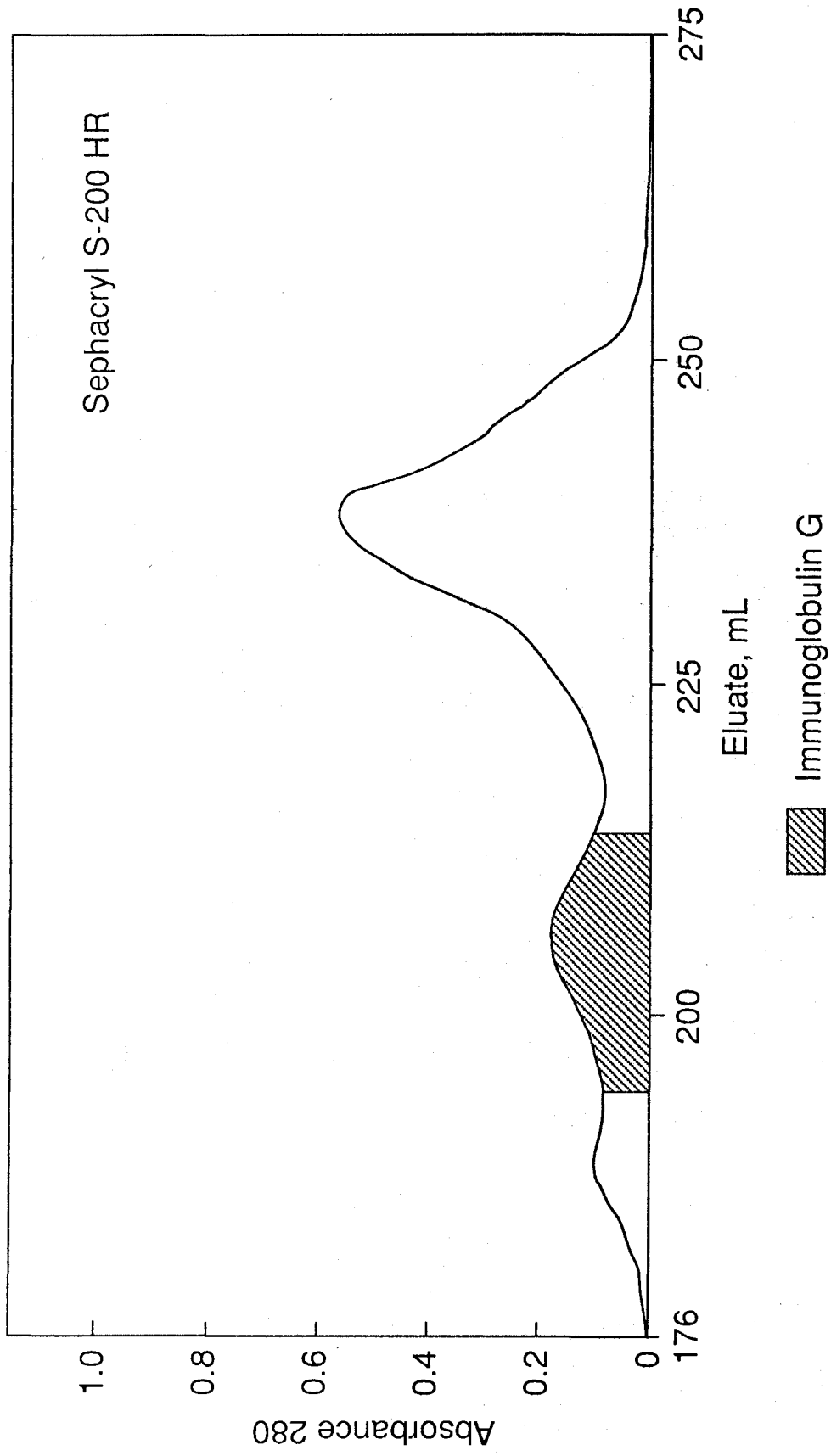

Econo-Pac Q Anion Exchange
Phosphate Buffer
Conc. pH 8.0
0.010M
0.075M
Absorbance 280
0
0.1
0.2
0.3
0.4
0.5
5
10
15
20
25
30
35
Eluate, mL

Figure 7.2.4.2-1. The profile obtained when 53.5 mg of protein obtained after anion exchange was run on a Sephacryl S200 column at 0.22 mL/min. The shaded area indicates the peak in which IgG was detected.


Sephacryl S-200 HR
Absorbance 280
1.0
0.8
0.6
0.4
0.2
0
176
200
225
250
275
Eluate, mL
Immunoglobulin G

Even though IgE might be slightly higher in the horse, due to a response to parasite load as seen in dogs (Halliwell 1973), it still should be extremely low in comparison with IgG. In allergic human serum, even before immunotherapy, the IgG is mostly composed of IgG_1 and IgG_4 (Nakagawa 1991). It therefore, follows that although a horse affected by CH will have very high levels of IgE and very low levels of IgG, the actual quantity of IgG will be much higher. Therefore, it seemed possible that allergen-specific IgG could be used in place of IgE as the primary antibody.

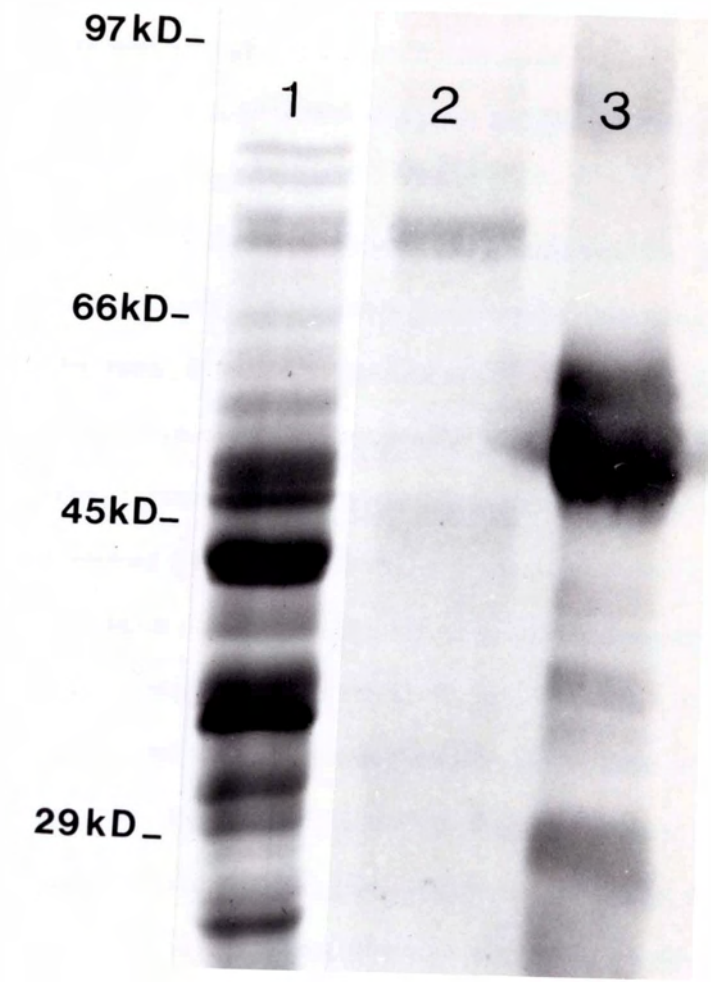
Although my purification attempts described earlier did not result in pure IgE, it did result in greatly concentrating the IgG, and probably also the IgE. Therefore, the fractions collected after gel filtration with Sephacryl S200 purification (Fig. 7.2.4.2-1) were tested for the presence of IgG specific to *C. variipennis* proteins. Insect proteins separated by SDS PAGE, were immobolized on polyvinylidene fluoride membrane (PVDF) (Millipore) which was found to give a clearer result than nitrocellulose, and incubated with enriched IgG fractions. Using anti-equine IgG antibodies (biotinylated anti-equine IgG, Sigma), I was detect a single strongly reacting band of approximately 80,000 D (Fig. 7.2.5-1). Occasionally, other bands would show up as well, but these were fainter than that detected at 80,000 D, and did not show up consistently. They are propably unspecific proteins. The single allergen detected by this method was not the strongest protein band (Fig. 7.2.5-1), which supports the specificity of this procedure.

An N-terminal sequence was performed for further characterization. However, the N-terminus proved to be blocked. Therefore, it was not possible to compare the N-terminus with the protein database.

7.3 DISCUSSION

Anti-human IgE did not appear to cross-react with equine IgE. Other workers have suggested at least partial cross-reactivity, but it is probable that if so, it was too low to detect using these methods.

Figure 7.2.5-1. SDS polyacrylamide gel stained with Coomassie Brilliant Blue showing insect extract in lane 1, and a PVDF membrane blot with insect extract in lane 2 indicating the causal allergen and equine IgG as a positive control in lane 3. Molecular weights are shown on the left.


97kD
66kD
45kD
29kD
1
2
3

It was not possible to purify equine IgE to a level which could be used to develop anti-equine IgE, using the methods that have previously been described. Due to the vast difference in quantities of IgE and IgG, IgE is often masked by IgG. Any attempt to remove IgG invariably removes IgE as well. However, although IgE could not be used to determine the allergen, allergen-specific IgG was found to be equally effective. This may be useful for future research in species where IgE and anti-IgE are not readily available.

Allergies are sometimes a result of several allergens. In CH affected horses, however, there is apparently a single major allergen.

Even if indeed this allergen is the major cause of the allergic reaction, isolating the protein for use on a large scale is clearly not a feasible possiblity for developing a hyposensitizing 'vaccine'. However, it should be possible to produce a cDNA library from a *Culicoides* mRNA preparation, which could then be screened using an oligonucleotide probe. However, since the N-terminus is blocked, it is not possible to use this sequence to make the oligonucleotide probe for screening this library. Limited proteolysis may give access to internal amino acid sequences which could be used to design an appropriate probe. Alternatively, antibodies could be employed to screen the library. The cDNA for the allergen could then be cloned. Ultimately, this could be overexpressed in *Escherichia coli* or a Baculovirus expression system. The latter would be preferable as it involves an insect cell line, capable of post-translational modification such as glycosylation *etc.* Therefore, the potential for immunotherapy on a large scale would be feasible. Although successful, a crude extract can only be used on a small scale due to the enormous number of insects required. Also, the use of a pure allergen as opposed to a crude extract made of many proteins, which include the allergen, is bound to be a more effective treatment, and will probably promote more rapid production of specific IgG.

It is probable that such an allergen could be linked to the new slow-release polymers (Chapter 6) so that fewer injections would be needed. It is hoped that with the evidence that

immunotherapy can be successful, and the identification of a single allergen, immunotherapy will become the treatment of choice in the future for horses affected with CH.

8. CONCLUSIONS

Culicoides hypersensitivity is a severe, debilitating disease which affects a large number of horses worldwide, affecting recreational riding, racing and stock horses. It is my hope that this thesis will contribute in some way towards treating this unpleasant disease. In working toward that goal, my research has led to the following specific conclusions :

1. *Culicoides* Hypersensitivity (CH) in horses in British Columbia is a severe allergic reaction caused by the bites of *Culicoides* species. In British Columbia, the major causal species is *C. obsoletus*.

2. Light-trap catches showed that the seasonal distribution of *C. obsoletus* corresponds well with the appearance and regression of lesions. Other *Culicoides* species are present in the Lower Mainland, but *C. obsoletus* represents the majority. Only one other species, *C. cockerellii*, was collected in any numbers, and this species was captured at only one site, and only in large numbers on two occasions.

3. *C. obsoletus* do enter buildings, contrary to popular belief. Although significantly more *C. obsoletus* were caught outside stables, specimens were caught inside stables every night and, once horses are sensitized, a few bites can cause clinical signs. Stabling has frequently been recommended as a preventative for CH; however, it will probably be ineffective as a complete control unless the stable is well screened.

4. Different *Culicoides* have been found to be responsible for CH in different parts of the world. Using skin tests, I have shown that the allergen is common to several species, indicating that any treatment based on the allergen itself, such as immunotherapy, would have worldwide applications. I have also shown that a laboratory-raised species, *C. variipennis*, also contains the allergen.

5. A mast cell stabilizer effective in equine respiratory allergies, clenbuterol hydrochloride, was not effective in preventing the clinical signs of CH, indicating an inherent difference between respiratory and cutaneous allergies. Repeated intradermal skin tests, although not resulting in a clinical improvement, did appear to result in the development of slight hyposensitization and one horse ceased to react to the extract, indicating that immunotherapy has potential.

6 A two-year immunotherapy trial using subcutaneous injections of increasing doses of *C. variipennis* extract was successful. In the first year, nine out of ten horses improved, with three completely recovering. In the second year, when the horses were on a maintenance dose, two remained free of clinical signs all year, three were greatly improved over previous years, two showed some improvement and one was unchanged. Of the two horses that only received treatment for one year, one remained free of clinical signs, and one was unchanged. A year later, the three horses which completely recovered were still symptom-free, and the others were the same or only slightly worse than during therapy. This trial indicated that immunotherapy can be a successful treatment of CH, and that its effects are relatively long-lasting.

7. The allergen responsible was identified using immunoblotting techniques. Anti-equine IgE is unavailable, and anti-human IgE does not cross-react with equine IgE. Allergen-specific IgG was purified and found to be effective in determining the causal allergen. The hypersensitivity is caused by a single allergen with an approximate molecular weight of 80,000 D. Once further characterized and sequenced, it should be possible to synthesize this protein, to use in large scale immunotherapy. The recent advent of slow-release polymers makes immunotherapy a feasible and attractive treatment for CH in horses.

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APPENDIX I

Table AI-1. Individual details and disease history of all horses used in this study.

DESIGNATED LETTERS	AGE[a] (years)	SEX	HEIGHT (m at withers)	COLOUR	BREED	AGE WHEN FIRST AFFECTED[b]	LESION SITES
A1	7	mare	1.52	bay	Hackney X Quarterhorse	2	ventral midline, mane, tail, chest, face, between hind legs
A2	16	gelding	1.63	chestnut	Quarterhorse	<8	ventral midline, withers, tail
A3	22	mare	1.50	cream	Appaloosa X Mustang	<9	ventral midline, mane, tail, face, rump, withers
A4	19	gelding	1.70	dun	Thoroughbred X Quarterhorse	12	ventral midline, tail
A5	16	gelding	1.52	bay	Quarterhorse	6	ventral midline, mane, tail
A6	11	mare	1.52	grey	Arabian	5-6	ventral midline, mane, tail
A7	4	mare	1.42	strawberry roan	Appaloosa Mustang X Morgan	3	ventral midline, mane, tail

Table AI-1 contd.

DESIGNATED LETTERS	AGE[a] (years)	SEX	HEIGHT (m at withers)	COLOUR	BREED	AGE WHEN FIRST AFFECTED[b]	LESION SITES
A8	8	gelding	1.63	liver chestnut	Quarterhorse	7	ventral midline, mane, tail
A9	7-8	mare	1.60	bay	Standardbred	6	mane, tail, withers, chest
A10	8-9	mare	1.47	bay	Arabian	2	ventral midline, mane, tail, face, nose
A11	7	mare	1.60	chestnut	Morgan	2-3	mane, tail, inside of back legs
A12	5	mare	1.57	palomino	Arabian	4	ventral midline, mane, tail
A13	17	mare	1.12	dark bay	Shetland X	11	ventral midline, mane, tail
A14	23	mare	1.70	chestnut	Clydesdale X	12	mane, tail, withers
A15	5	mare	1.42	strawberry roan	Appaloosa, Mustang X Morgan	3	ventral midline, mane, tail, chest, withers

Table AI-1 contd.

DESIGNATED LETTERS	AGE[a] (years)	SEX	HEIGHT (m at withers)	COLOUR	BREED	AGE WHEN FIRST AFFECTED[b]	LESION SITES
A16	2	gelding	1.52	grey	Arabian	1	ventral midline, mane, tail
A17	10	mare	1.52	dun	Quarterhorse	<4	ventral midline, mane, tail
A18	22	mare	1.47	black	Quarterhorse	17	ventral midline, mane, tail, chest, withers
A19	11	gelding	1.47	grey	Appaloosa	4	ventral midline, mane, tail, chest, sheath
N1	13	mare	1.50	chestnut	Arabian X Welsh	-	Control horse, not affected
N2	8	mare	1.37	dapple grey	Arabian X Welsh	-	Control horse, not affected
N3	16	gelding	1.52	palomino	Arabian X	-	Control horse, not affected
N4	10	gelding	1.55	chestnut	Quarterhorse X Thoroughbred	-	Control horse, not affected
N5	10	gelding	1.52	strawberry roan	Polish Arabian	-	Control horse, not affected

Table AI-1. contd.

DESIGNATED LETTERS	AGE[a] (years)	SEX	HEIGHT (m at withers)	COLOUR	BREED	AGE WHEN FIRST AFFECTED[b]	LESION SITES
N6	18	gelding	1.60	chestnut	Half Arabian	-	Control horse, not affected
N7	11	gelding	1.60	bay	Standardbred	-	Control horse, not affected
N8	7	mare	1.57	bay	Arabian X Quarterhorse	-	Control horse, not affected

[a] age at start of tests.
[b] In some cases, the age of the horse at onset of disease can only be stated as less than a specific age as, before this, the horse belonged to a previous owner who could not be contacted.

APPENDIX II

Table AII-1. *Culicoides* light-trapping sites

SITE	ADDRESS
1	14518 60th Ave. Surrey
2	347 208th St. Langley
3	6990 240th St. Langley
4	19579 5th Ave. Surrey
5	17514 32nd Ave. Surrey