# Congenital myasthenic syndrome of Brahman cattle

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# Congenital myasthenic syndrome of Brahman cattle

## Congenitaal myastheen syndroom bij het Brahman rund

(met een samenvatting in het Nederlands)

Proefschrift

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door

Peter Neil Thompson

geboren op 31 mei 1966 te Johannesburg, Zuid-Afrika

Promotoren: Prof. Dr. J.A.P. Heesterbeek

Prof. Dr. J.A.M. van Arendonk

# Contents

# Chapter 1

# General introduction

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# Introduction

This thesis tells the story of the discovery of a "new" disease in cattle, from its initial recognition and description of the first clinical case, through the elucidation of the molecular basis of the disease, the development of a screening test, investigation of the epidemiology of the disease and its impact in the population, to the evaluation of options for its control or eradication.

More than 250 inherited defects have been described in cattle, of which the majority are inherited in an autosomal recessive manner (Nicholas, 2005). However, in only 28 conditions has the causative mutation has been described at the DNA level. In contrast, several thousand Mendelian diseases have been described in humans, and in almost 400 of these the mutation has been described at the DNA level (OMIM, 2006). However, with the recent advances in genetic methodology, it is expected that the proportion of genetic defects fully characterised will increase rapidly.

## The Brahman breed

> "Brahmin, Zebu or sacred cattle of India. – While these cattle are usually thought of as exhibits in zoölogical gardens and menageries, they have some economic importance. In the South, especially Texas, Brahmin bulls are bred to native or even grade beef-bred cows in the belief that the one-half- or three-quarter-breds do better than the improved cattle on account of their greater resistance to parasites, flies, ticks and the heat."
>
> – (Gay, 1920)

The Brahman is a *Bos indicus* breed of cattle developed in the southern United States, largely from the Ongole (Nellore), Kankrej (Guzerá), Gir and Krishna Valley breeds imported from India in the mid to late 19th century, and from India via Brazil in the early 20th century (Porter, 1991). Approximately 290 imported animals formed the basis of the American Brahman breed. It is a medium-sized beef breed, with typical zebu traits such as long, drooping ears, a large hump, loose skin in the dewlap and sheath, and well-pigmented skin (Figure 1). The Brahman possesses good heat-

tolerance and insect- and tick-resistance and has therefore been exported to many tropical and subtropical countries. Coat colours range from light to dark grey, or red, sometimes spotted with white.

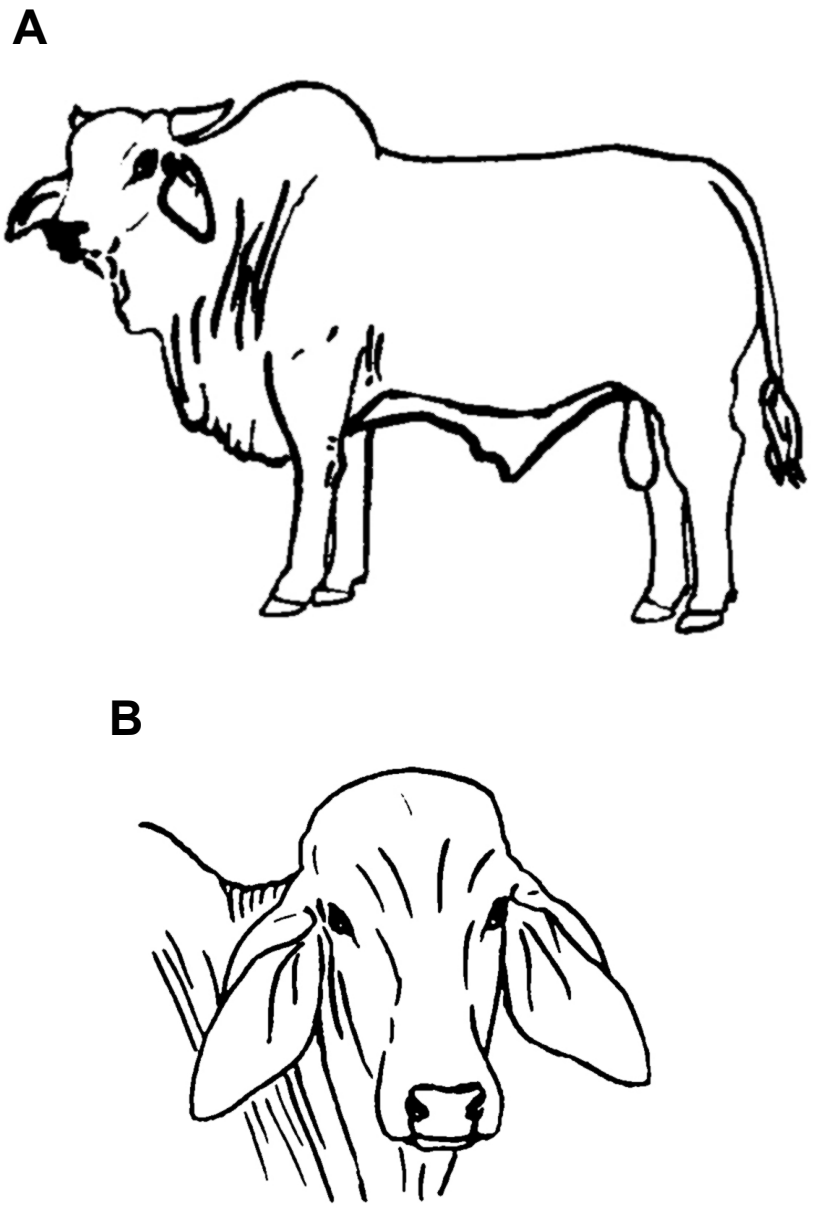


**Figure 1.** Typical appearance of the Brahman, showing (A) the bull, and (B) the head with pendulous ears.

The first American Brahmans were imported into South Africa in 1954 from Texas, and the Brahman Cattle Breeders Society of South Africa (BCBSSA) was founded in 1957. Over the years imported animals, mainly from the U.S.A., have played an important role in establishing the breed in South Africa. In total, more than 3,000 Brahmans were imported into South Africa, mainly during the 1960s and 1970s. Apart from the U.S.A. and South Africa, significant Brahman populations also exist in Australia and Argentina, with smaller populations in a number of other countries. In South Africa, five "sub-breeds" fall under the umbrella of the Brahman breed, namely Guzerat, Gir, Nellore, Indu Brazil and American Brahman (white and red), with the latter accounting for more than 95% of registered animals. The "white" and "red" classification reflects slightly different ancestries, with white (or grey) Brahmans resulting mainly from Guzerá (Kankrej) and Nellore (Ongole) crossings, and red Brahmans from Gir, Indu Brazil and, to a lesser extent, Guzerá crossings (Sanders, 1980). Apart from the pedigree Brahman population, the breed is widely used in crossbreeding, particularly with British and European beef breeds, taking advantage of its adaptability to a subtropical climate and poor nutritional conditions. In addition, the Brahman has been used in the development of many synthetic beef breeds such as the Santa Gertrudis, Braford, Beefmaster, Brangus, Charbray, Simbra and Droughtmaster.

Currently there are approximately 60,000 registered Brahmans in South Africa (BCBSSA, 2006). The complete registered South African Brahman pedigree is maintained on behalf of BCBSSA by the Agricultural Business Research Institute at the University of New England, Australia. It contains the records of approximately 612,000 animals and includes up to five generations of pedigree information for imported animals, the majority of them American Brahmans.

## Congenital myasthenic syndromes

Congenital myasthenic syndromes (CMS) in humans are a heterogeneous group of genetic diseases, characterised by the dysfunction of neuromuscular transmission at skeletal muscle motor endplates. They are rare conditions, with an estimated prevalence in Europe of one in 50,000 (Hantai et al., 2004). Congenital myasthenic syndromes, sometimes termed "congenital myasthenia gravis", have also occasionally

been described in domestic animals, including dogs (Trojaborg and Flagstad, 1982; Miller et al., 1983) and cats (Indrieri et al., 1983). Like the more commonly seen autoimmune myasthenia gravis (Drachman, 1994), CMS is characterised by muscle weakness that worsens with exercise but, unlike myasthenia gravis, the symptoms of CMS usually start early in life (Zafeiriou et al., 2004). Congenital myasthenic syndromes in humans may be classified, based on the location of the specific molecular anomaly, into presynaptic, synaptic and postsynaptic, of which postsynaptic are the most frequent and presynaptic the least frequent (Hantai et al., 2004; Engel and Sine, 2005). Presynaptic CMSs are characterised most commonly by defects in choline acetyltransferase, the enzyme that catalyses acetylcholine (ACh) production. Synaptic CMSs are caused by defects in acetylcholinesterase (AChE) in the synaptic cleft, usually involving its collagen tail. Postsynaptic CMSs most frequently involve either a deficiency or kinetic abnormality of the acetylcholine receptor (AChR), situated on the crests of the postsynaptic membrane folds, but may also be due to defects in rapsyn or MuSK, enzymes that help to concentrate AChR in the postsynaptic membrane, or in $Na_V1.4$, the sodium channel in the depths of the postsynaptic folds. The result of all the various CMSs, at the level of the neuromuscular junction, is that the safety margin of neuromuscular transmission is reduced (Engel and Sine, 2005). Activation of AChRs by ACh normally triggers an endplate potential; the safety margin of neuromuscular transmission is determined by the difference between depolarisation caused by the endplate potential and the depolarisation threshold required to activate the sodium channels and give rise to a propagated action potential that results in muscle contraction.

The nicotinic AChR (nAChR) is a pentaoligomer consisting of five radially arranged subunits and forming a central trans-membrane ion channel (Hucho et al., 1996). In the foetus, skeletal muscle nAChR contains four different subunits: alpha2, beta, gamma and delta, whereas in the adult the gamma subunit is replaced by an epsilon subunit (Mishina et al., 1986) (Figure 2). In contrast, neuronal nAChRs consist of only one ($\alpha$) or two ($\alpha$, $\beta$) subunit types (Utkin et al., 2000).

The foetal ($\gamma$-AChR) and adult ($\varepsilon$-AChR) receptor subtypes have been shown to have differing functional properties. The foetal subtype is important in the initial development of the neuromuscular junction and ensures an orderly innervation pattern of skeletal muscle by the motor nerve (Koenen et al., 2005), while presence of $\varepsilon$-AChR

is necessary for the maintenance of the highly organised postsynaptic structure (Schwarz et al., 2000). The $\gamma$-to-$\varepsilon$ switch occurs gradually over the first 2–3 weeks after birth in mice (Yumoto et al., 2005).

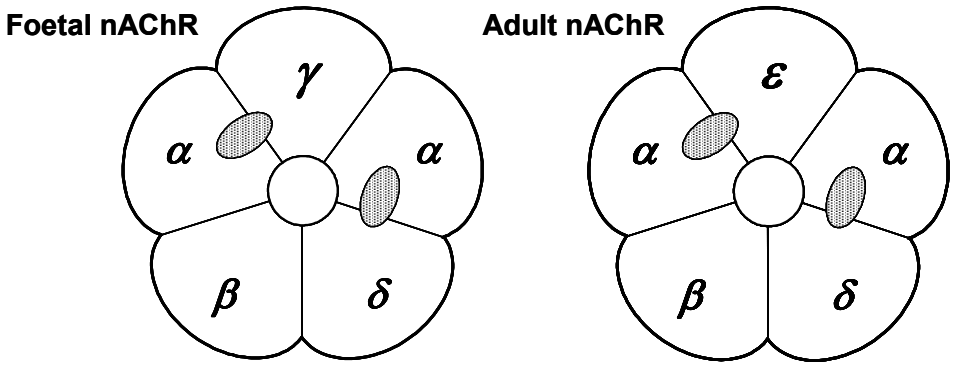


**Figure 2.** Schematic representation of the foetal and adult forms of the skeletal muscle nicotinic acetylcholine receptor (nAChR), showing the different subunit types, the central ion channel, and the acetylcholine binding sites (shaded areas).

Numerous mutations affecting the genes encoding the AChR subunits have been described in humans. Such mutations may either result in kinetic abnormalities of the receptor, or be null mutations resulting in deficiency of AChR. In mutations resulting in AChR deficiency in humans, the gene encoding the AChR $\varepsilon$-subunit (*CHRNE*) is the most commonly affected (Engel et al., 1996). The likely reason for this is that continued, low level expression of the foetal-type $\gamma$-AChR may partially compensate for the absence of $\varepsilon$-AChR, whereas when the other subunits are affected there is no such substituting subunit (Engel and Sine, 2005).

## Overview of the thesis

The postsynaptic congenital myasthenic syndrome that is the subject of this thesis is caused by a deficiency of nAChR at the neuromuscular junction, resulting from homozygosity for a null mutation in the $\varepsilon$-subunit gene (*CHRNE* 470del20). To date,

this represents the only naturally occurring CMS in animals to have been described at the DNA level. This thesis covers all steps from the initial discovery and case description, to the diagnosis, development of a diagnostic test, and screening of the population, to predictive modelling and assessment of its impact on the Brahman breed.

In Chapter 2, the first presentation of calves with CMS is described. The clinical presentation was quite unlike that of any disease previously described in cattle, but reminiscent of myasthenia gravis in dogs and other animals. This prompted the author to pursue appropriate diagnostic and therapeutic approaches, resulting in a presumptive diagnosis of postsynaptic "congenital myasthenia gravis", or CMS.

The publication of the first case report led to collaboration with the German group of Steinlein and Sieb in order to identify the underlying mutation. Although the bovine *CHRNE* complementary DNA (cDNA) sequence had previously been described (Takai et al., 1985), its genomic sequence had not. The description of the genomic structure of bov*CHRNE* and the discovery of the mutation responsible for the disease, a 20 base pair deletion in exon 5 of the gene, is the subject of Chapter 3.

In Chapter 4, the development and validation of a PCR screening test for the mutation is described. The chapter discusses the results of initial screening for the mutation, both amongst relatives of the then known carrier bulls and amongst unrelated animals, to obtain an initial estimate of the frequency of the mutation. Clinical findings from another affected calf are also described.

Wider screening of the South African registered Brahman cattle population was then undertaken, in collaboration with the BCBSSA. The results of this screening to date are presented in Chapter 5. A more precise estimate of the mutation frequency in the population, and its change over time, is thus obtained. Chapter 5 also addresses the question of whether there is any selective advantage associated with an animal being heterozygous for the mutation. In addition, pedigree analysis is carried out in order to identify ancestral carriers of the mutation and thus determine its origin in the South African population.

In order to control a recessive disease-causing mutation such as CMS in a livestock population, it is necessary to know how the frequency is expected to change over time. In Chapter 6, a spreadsheet model is described that predicts the change in mutation frequency over time in a population, under certain assumptions. The model allows for fitness to vary between genotypes, between sexes and over time. It can thus be used to model mutation frequency where there is heterozygote advantage or disadvantage and/or when various control measures are implemented.

Finally, Chapter 7 contains a general discussion of what is currently known about CMS in Brahman cattle. The most important issues arising from each chapter are highlighted, and the implications of CMS for the Brahman breed in South Africa are discussed.

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# Chapter 2

# Suspected congenital myasthenia gravis in Brahman calves

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Veterinary Record (1998) 143:526–529

## Abstract

Four, three to four-week-old Brahman calves, with a common sire and a common maternal grandsire, developed progressive muscular weakness which was exacerbated by exercise and improved with rest. Although the calves remained alert and continued sucking, their neuromuscular condition deteriorated, and they had to be euthanased within four to six weeks. The one calf in which an attempt was made to diagnose the condition responded quickly, but transiently, to the intravenous administration of edrophonium chloride and for a longer period to oral pyridostigmine bromide. Repetitive peripheral nerve stimulation resulted in a decrease in the amplitude of the evoked muscle action potential. A definitive diagnosis could not be made but the condition showed many similarities to congenital myasthenia gravis, which has not previously been described in cattle.

# Introduction

Myasthenia gravis is a defect of neuromuscular transmission which has been described in humans, dogs and cats. In humans, acquired myasthenia gravis is an autoimmune disease characterised by the presence of antibodies which target nicotinic acetylcholine receptors at neuromuscular junctions, resulting in a reduction in the number of acetylcholine receptors (Drachman, 1994). An analogous disease occurs in dogs (Dewey et al., 1997) and cats (Joseph et al., 1988). Several genetically determined congenital myasthenic syndromes have been described in humans (Engel, 1993) and the analogous conditions in dogs and cats are referred to as congenital myasthenia gravis (Indrieri et al., 1983; Miller et al., 1983) or congenital myasthenic syndrome (Flagstad et al., 1989). This paper describes a case of suspected congenital myasthenia gravis in a Brahman calf, which was one of four calves similarly affected.

# Case description

### *History*

A five-week-old, female Brahman calf developed progressive muscle weakness, beginning at three to four weeks of age. According to the farmer, the calf had been normal until that stage, but then started to lose the ability to stand for long periods. Within approximately a week, it was no longer able to rise without assistance, but remained alert and continued to suck from its dam while it was recumbent. Two months previously, the farmer had had two female calves showing the same clinical signs; they had remained alert and sucked strongly until they were destroyed at nine weeks of age. Later, a male calf showed identical clinical signs and was also destroyed by the farmer at about two months of age. All four calves had been treated for the condition with dexamethasone, phenylbutazone and oxytetracycline, both by the farmer and the referring veterinarian, without apparent effect.

The breeding records revealed that the four affected calves all had the same sire and the same maternal grandsire. Only two other calves on the farm had the same ancestors in common, but they were apparently completely normal. No other similar cases

occurred amongst the approximately 50 calves born on the farm during that calving season.

### *Clinical examination*

When first examined, the calf was in sternal recumbency, with a slight tremor of the limb muscles, but was alert, in good body condition and sucked strongly from a bottle. A few, small, poorly defined, non-painful lumps could be palpated in the gluteal and semimembranosus muscles. The calf's temperature, pulse rate and respiratory rate were within normal limits and no other clinical abnormalities were detected. A blood smear revealed no parasites and a faecal flotation revealed no helminth eggs or coccidial oocysts.

When it was lifted into a standing position, the calf initially stood steadily and strongly, without assistance. However, after approximately a minute, all four limbs suddenly started to tremble and shake for several seconds, before the calf collapsed back into sternal recumbency. This procedure was repeated several times during the following seven days, and each time the calf was able to stand for between 20 and 60 seconds before it collapsed.

### *Haematological and blood chemistry examinations*

The results of the haematological and serum chemistry examinations are summarised in Table 1. The abnormalities detected were a mild neutrophilia and hyperglycaemia. All other parameters measured were within, or very close to, normal limits. The serum cholinesterase activity was also measured and found to be normal. The serum glucose level was measured again one month later and was virtually within normal limits. A serum sample taken one month after the first examination was tested for botulism antibodies, using a biological test in mice. The test failed to reveal the presence of any antibodies to the toxins of *Clostridium botulinum* types C or D.

**Table 1.** Haematological and serum chemistry findings in a calf with suspected congenital myasthenia gravis

| Measurement | Age of calf (weeks) | | Reference range |
|---|---|---|---|
| | 5 | 10 | |
| Haematocrit (%) | 28 | –[1] | 24–40 |
| Erythrocyte count ($10^6/\mu$l) | 8.0 | – | 5.0–9.0 |
| Leucocyte count (/$\mu$l) | 10,900 | – | 4,000–10,000 |
| Neutrophils (mature) | 6,540 | – | 600–4,000 |
| Neutrophils (immature) | 110 | – | 0–120 |
| Lymphocytes | 3,490 | – | 2,500–7,500 |
| Monocytes | 500 | – | 30–840 |
| Eosinophils | 110 | – | 0–2,400 |
| Basophils | 0 | – | 0–200 |
| Thrombocyte count ($10^3/\mu$l) | 700 | – | 200–600 |
| Serum protein (g/litre) | 66.3 | 62.1 | 65–78 |
| Albumin (g/litre) | 41.2 | 39.4 | 28–37 |
| Globulins (g/litre) | 25.1 | 22.7 | 28–42 |
| Sodium (mmol/litre) | 143 | 145 | 132–152 |
| Potassium (mmol/litre) | 4.9 | 4.5 | 4.1–5.6 |
| Calcium (mmol/litre) | 2.6 | 2.6 | 2.0–2.9 |
| Phosphate (mmol/litre) | 2.4 | 2.4 | 1.2–2.3 |
| Glucose (mmol/litre) | 7.3 | 4.8 | 3.1–4.7 |
| Urea (mmol/litre) | 3.3 | – | 3.6–10.7 |
| Creatinine ($\mu$mol/litre) | 83.3 | – | <133 |
| Aspartate aminotransferase (U/litre) | 10 | – | <80 |
| Creatine phosphokinase (U/litre) | 31 | – | <60 |
| Whole blood selenium (ng/ml) | 134 | – | >40 |

[1] Not done

***Further diagnostic tests***

Under local analgesia, small muscle biopsies were taken from one quadriceps muscle and from a palpable lump in the contralateral semimembranosus muscle. No histological abnormalities could be detected in the quadriceps muscle by light microscopy. The biopsy was stained for bovine immunoglobulin using an immunoperoxidase method; there was no evidence of the deposition of immune complexes in the muscle. Fibroplasia and a mild leucocyte infiltration were visible under the epimysium of the semimembranosus muscle, but the deeper lying muscle tissue appeared normal.

Because of the similarity of the clinical signs to those of myasthenia gravis in small animals, an anticholinesterase test was applied to the calf. Edrophonium chloride (0.1 mg/kg) (Tensilon; Roche), a very short-acting anticholinesterase, was administered intravenously, the calf was lifted to its feet and the effects of the drug were observed. The calf immediately appeared stronger and began walking without difficulty. After approximately 15 minutes, during which its strength appeared to remain constant, the calf suddenly developed muscle tremors, particularly in its limbs. Within ten seconds it was no longer able to remain standing, and collapsed into sternal recumbency. Four days later the same test was repeated, using 0.08 mg/kg edrophonium chloride. The result was similar, with the calf able to stand and walk for ten minutes before collapsing in the same way. No muscarinic side-effects were observed at any time after the administration of the anticholinesterase drug.

With the calf under general inhalation anaesthesia, repetitive stimulation of a peripheral nerve was performed. The peroneal nerve, on the lateral aspect of the stifle, was stimulated with a needle electrode at a rate of 27 per second, and the evoked compound muscle action potentials were measured over the peroneus tertius muscle. The evoked action potentials started at 0.5 mV, and within three pulses had decreased to 0.425 mV (a decrease of 15%), where they remained.

***Treatment***

Treatment with oral pyridostigmine bromide (Mestinon; Roche), a longer-acting anticholinesterase drug, was started. An initial dose of 20 mg was given twice daily in the form of tablets crushed and mixed with the calf's milk. The dose rate was gradually increased to 40 mg twice daily over 11 days, at which point the treatment was stopped due to unavailability of the drug. Initially, the calf reacted well to the treatment and was able to rise to its feet unassisted and walk for between 15 and 25 minutes, two or three times a day. The improvement in muscle strength appeared to start 30 to 45 minutes after the administration of pyridostigmine and to last for several hours. However, on the last two days of treatment, the calf was once again weaker and unable to stand for longer than a minute at a time. The gradual increase in the dose rate did not appear to result in a corresponding clinical improvement.

Three days later another Tensilon test was performed, using 0.125 mg/kg edrophonium chloride intravenously. This time the calf was able to stand strongly for only two minutes after the administration of the drug.

When a further supply of pyridostigmine bromide had been obtained, treatment was started with 30 mg being given twice daily. Prednisolone (0.5 mg/kg) (Sanvet) was also administered twice daily by intramuscular injection. The dose rate of pyridostigmine was steadily increased over three weeks to 600 mg twice daily. The dose rate of prednisolone was increased to 1.0 mg/kg after one week and dropped back to 0.5 mg/kg for the last five days of treatment. Initially, the calf did not respond to the treatment, but once the dose exceeded 120 mg twice daily, it became stronger. Further increases in dose resulted in little change, but a marked improvement occurred when the dose reached 540 mg twice daily. By this stage, the calf showed approximately the same degree of muscular strength as it had shown seven weeks previously at a dose rate of 30 mg twice daily, and was able to stand and walk for 15 minutes at a time. No muscarinic side-effects were observed in the calf during the treatment with pyridostigmine.

However, during the last ten days of treatment, the calf developed a severe, cranioventral bronchopneumonia. A trans-tracheal aspirate was taken, from which *Actinomyces pyogenes* was cultured. Daily treatment with danofloxacin (1.25 mg/kg)

(Advocin; Pfizer Animal Health) and subsequent twice daily treatment with trimethoprim and sulphamethoxypyridazine (24 mg/kg) (Sulfatrim; Logos Agvet) did not resolve the pneumonia. The calf was euthanased ten weeks after it was first examined.

***Post-mortem examination***

The post-mortem examination revealed a severe, purulent, bacterial broncho-pneumonia of the cranioventral pulmonary lobes. Apart from those associated with the pneumonia, there were no other lesions. The oesophagus and thymus were macroscopically normal. No histological lesions were found in samples of the cerebrum, cerebellum, spinal cord or skeletal muscles.

## Discussion

In this calf, as well as in its three similarly affected herd mates, the most important clue to a defect of neuromuscular transmission was increasing weakness on sustained exertion (Engel, 1993). No evidence could be found for any other muscular or neuromuscular disease in the affected calves or in any other animals in the herd. There was no history of treatment with any drugs which could have resulted in neuromuscular blockade, for example, aminoglycosides. The neutrophilia and the hyperglycaemia which were detected initially, were considered to be due to the endogenous release of corticosteroid and catecholamines. The lumps palpable in the hind limb muscles were probably due to previous intramuscular injections. The severe pneumonia which developed was probably at least partially precipitated by the daily injections of prednisolone. Mega-oesophagus or pharyngeal atony are common findings in dogs with acquired myasthenia gravis and often result in the development of aspiration pneumonia (Dewey et al., 1997). In this calf, no regurgitation or dysphagia were observed at any time and no evidence of mega-oesophagus was found post-mortem. However, it is possible that weakness of the pharyngeal and/or oesophageal striated muscle may have led to the aspiration of feed, and resulted in pneumonia.

The positive response of the calf to treatment with an anticholinesterase is consistent with a diagnosis of myasthenia gravis, but does not differentiate between the congenital and acquired (autoimmune) syndromes. It does, however, rule out presynaptic congenital myasthenia gravis as described in dogs (Flagstad et al., 1989) and endplate acetylcholinesterase deficiency (Engel, 1993). The failure to detect immunoglobulin bound to muscle tissue by immunoperoxidase staining does not rule out acquired myasthenia gravis, because there may have been no neuromuscular junctions present in the sections examined.

The decrease in the amplitude of the compound muscle action potential on repetitive peripheral nerve stimulation is characteristic of myasthenia gravis and most human congenital myasthenic syndromes (Dewey et al., 1997; Drachman, 1994; Engel, 1993; Flagstad, 1993). It also differentiates the condition from botulism in humans, which shows an increase in the amplitude response (Drachman, 1994). The decrease in the amplitude is due to a reduced safety margin of neuromuscular transmission, which is defined as the difference between the actual amplitude of the endplate potential and the amplitude required to trigger a muscle fibre action potential (Engel, 1993).

A large proportion of human patients with myasthenia gravis have thymic abnormalities, consisting of either thymic hyperplasia or thymoma (Drachman, 1994). Thymomas have also been reported in a few cases of canine acquired myasthenia gravis (Poffenbarger et al., 1985). In this calf, however, there was no evidence of thymic hyperplasia or neoplasia, either clinically or post-mortem.

The facts that this condition affected four out of six calves with a common sire and maternal grandsire, and that no other calves were affected, suggested a hereditary cause. Although the mode of inheritance could not be determined, the history is consistent with an autosomal recessive mode of inheritance, like that described for congenital myasthenia gravis in the Jack Russell terrier (Wallace and Palmer, 1984) and for several congenital myasthenic syndromes in humans (Engel, 1993).

The clinical signs and the results of the diagnostic tests were thus consistent with a diagnosis of congenital or acquired myasthenia gravis, with the history suggesting a congenital condition. The onset of clinical signs in congenital myasthenia gravis in dogs is delayed, and usually occurs between six and nine weeks of age (Miller et al.,

1983). The signs are often progressive, the response to treatment erratic and the prognosis guarded to poor. All these characteristics are consistent with the clinical findings in this calf.

In dogs, congenital myasthenia gravis may occur as either a postsynaptic disorder (Dewey et al., 1997) or a presynaptic disorder (Flagstad et al., 1989). The postsynaptic disorder is associated with failure of postsynaptic acetylcholine receptor insertion. Ultrastructural examination of motor endplates in Jack Russell terriers with congenital myasthenia gravis revealed an increase in the density of postsynaptic membranes and a decrease in secondary fold length (Wilkes et al., 1987). The presynaptic disorder does not respond to anticholinesterase treatment and may be due to the impaired synthesis or release of acetylcholine, to abnormal acetylcholine-induced ion channels, or to endplate acetylcholinesterase deficiency (Flagstad et al., 1989). The various human congenital myasthenic syndromes, most of which are known, or suspected, to have an autosomal recessive mode of inheritance, are due to a variety of ultrastructural and biochemical defects (Engel, 1993). These include, among others, kinetic abnormalities and/or deficiency of acetylcholine receptors, paucity of synaptic clefts, paucity of synaptic vesicles, and endplate acetylcholinesterase deficiency.

The definitive diagnosis of these conditions requires further electrophysiological and cytochemical studies, which were not available in this case. However, on the basis of the history and clinical findings, and the response of the calf to anticholinesterase treatment and repetitive nerve stimulation, a presumptive diagnosis of postsynaptic congenital myasthenia gravis was made. The most likely underlying defect was considered to be an acetylcholine receptor deficiency. The definitive diagnosis of this condition in cattle would require further tests, namely an assay for acetylcholine receptor antibody (if available for cattle), a quantitative assay for acetylcholine receptors in muscle and an ultrastructural examination of endplate morphology.

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# Chapter 3

# Congenital myasthenia in Brahman calves caused by homozygosity for a *CHRNE* truncating mutation

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# Abstract

To elucidate the genetic defect in four previously reported related Brahman calves with severe myasthenic weakness, we determined the genomic structure of the gene encoding the bovine ε-subunit (bov*CHRNE*) of the acetylcholine receptor (AChR). Amplification of DNA isolated from paraplast-embedded tissue samples from one of the myasthenic calves and subsequent sequencing of all bov*CHRN*E exons revealed a homozygous 20-bp deletion within exon 5 (470del20). The deletion causes a frame shift followed by a premature stop codon in the predicted bov*CHRNE* protein. Thus, the 470del20 mutation reported here leads to a non-functional allele, explaining the impairment of neuromuscular transmission observed in the affected Brahman calves. With a survival time limited to only several months, the effect on neuromuscular transmission was more pronounced in the calves than that observed in humans homozygous for truncating *CHRNE* mutations. This may be due to a different capacity to express the foetal-type AChR after birth.

# Introduction

Impairment of neuromuscular transmission can be either acquired or inherited. Myasthenia gravis is an autoimmune disorder characterised by fluctuating weakness and fatigability of ocular, facial, bulbar or limb muscles. Congenital myasthenic syndromes are due to gene mutations causing presynaptic, synaptic or postsynaptic defects at the neuromuscular junction (Engel et al., 1999; Sieb et al., 2002). Both myasthenia gravis and the congenital myasthenic syndromes have been studied extensively in humans, and acquired myasthenia has also been described repeatedly in domestic animals (Indrieri et al., 1983; Shelton et al., 1997; Dewey et al., 1999; Lipsitz et al., 1999; Ridyard et al., 2000; Shelton et al., 2001). Myasthenia gravis is one of the most common neuromuscular disorders diagnosed in dogs (Shelton et al., 1997), where some breeds appear to have a genetic predisposition for developing this autoimmune disease (Shelton et al., 1997; Lipsitz et al., 1999). Much less is known about the molecular basis of congenital myasthenia in animals. There are some reports of inherited myasthenic weakness in domestic animals (Flagstad, 1982; Indrieri et al., 1983; Miller et al., 1983; Wallace and Palmer, 1984; Wilkes et al., 1987; Joseph et al., 1988; Flagstad et al., 1989; Thompson, 1998), but so far the molecular basis in these cases has remained elusive. The analysis of such spontaneous animal models can contribute to our understanding of the pathophysiological mechanisms underlying congenital myasthenia in humans. We have therefore determined the genomic structure of the bovine gene for the $\varepsilon$-subunit of the muscular acetylcholine receptor (bov*CHRNE*). This has enabled us to elucidate and describe the genetic defect in calves that suffered from a severe congenital myasthenic syndrome (Thompson, 1998).

# Materials and methods

### *Calves with congenital myasthenia*

The clinical phenotype of one of four South African Red Brahman calves with suspected congenital myasthenia has been published previously in detail (Thompson, 1998). In summary, the calf developed progressive muscle weakness, beginning at 3–4 weeks of age. Within a week it was no longer able to rise without assistance, and

collapsed after standing for between 20 and 60 s. Haematological and serum chemistry examinations were normal, and a muscle biopsy taken from one quadriceps muscle showed no abnormalities (Thompson, 1998). Peripheral nerve stimulation at 27 Hz revealed a decreased response of 15% at the peroneus tertius muscle. Acetylcholinesterase inhibitors improved the condition transiently. At the age of 15 weeks the calf was humanely killed due to severe pneumonia. Another three similarly affected half-siblings were similarly sacrificed at between nine and 21 weeks of age, after showing no clinical improvement. All four calves shared the same sire and maternal grandsire (Figure 1). Tissue samples from the first calf embedded in paraplast were available for molecular studies.

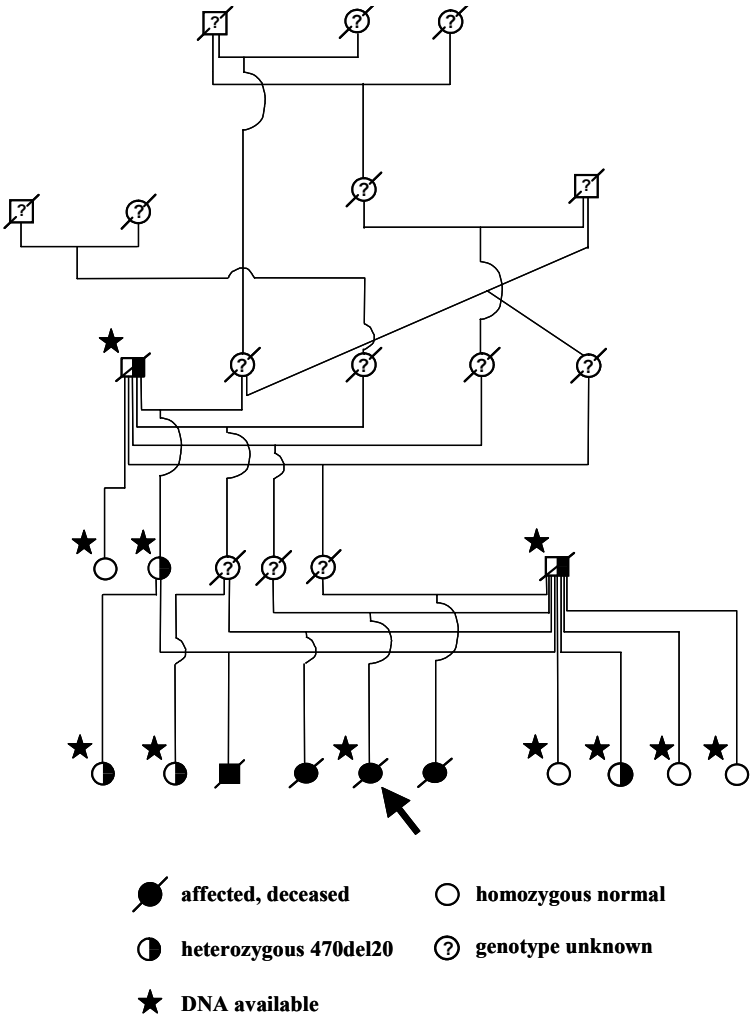


**Figure 1.** Pedigree of the myasthenic calves. The index calf investigated in the present study is marked by an *arrow*. Identification numbers for each individual are given. *Circles*, females; *squares*, males

***Reference bovCHRNE genomic sequence***

The published bov*CHRNE* cDNA sequence (accession number X02597) was used for a BLAST search (Madden et al., 1996). Two genomic clones (accession numbers BE664236 and BE681911) containing parts of bov*CHRNE* were identified. This approach enabled us to identify the boundaries of intron 5 and intron 8. The remaining introns were amplified by exon-exon PCR. For this, the putative positions of exon-exon boundaries were determined by comparison with the human genomic *CHRNE* sequence, and primers were chosen that did not overlap with the predicted boundaries. The template DNA was extracted using the Dneasy Tissue kit (Quiagen) from bovine meat purchased from a local butcher. PCR was carried out in a total volume of 25 $\mu$l in a PTC 200 (MJ Research), containing 50 ng of genomic DNA, 5 pmol of each forward and reverse primers, 200 $\mu$M of each dNTP, 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.3), and 0.1 U Taq DNA polymerase (Invitrogen, Mannheim, Germany). PCR parameters were as follows: denaturation at 95ºC for 5 min followed by 33 cycles of 95ºC for 30 s, annealing at 58–64ºC for 30 s, extension times at 72ºC varying between 30 s and 80 s, followed by a final extension step of 5 min at 72ºC. Approximately 300 bp of each end of the obtained PCR products were directly sequenced on an ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

***Mutation screening***

Genomic DNA was extracted from paraplast-embedded tissue samples (skeletal muscle and spinal cord) of one of the previously described myasthenic calves (Thompson, 1998), using the Dneasy Tissue kit (Quiagen). The DNA obtained from the tissue samples consisted mostly of fragments shorter than 650 bp. Therefore the amount of DNA used for amplification was increased to 150 ng in a total PCR volume of 25 $\mu$l. The PCR was carried out as described above, except for the following parameters: $MgCl_2$ concentrations 1–2 mM, annealing temperature 58–68ºC, annealing time 30–60 s, and extension times 30–60 s. PCR was performed using primer sets amplifying bov*CHRNE* exons and adjacent exon-intron boundaries (Table 1). Probably due to the high GC-content of intron 9, no suitable forward primer could be designed, and exon 10 was therefore not included in the mutation analysis. The amplification products were directly sequenced as described above.

**Table 1.** Primers used for amplification of bovine *CHRNE*

| Exon | Primer forward | Primer reverse |
|---|---|---|
| 1 | 5′ -ACCTGGGGGACCAGACAGC-3′ | 5′-TCTCAGTCGGTCTCTGTCCTGT-3′ |
| 2 | 5′-TAGACCCGGAGGCAGAGCA-3′ | 5′-AGTTGAGCGCTTGGGGCC-3′ |
| 3 | 5′-TGGCCCCAAGCGCTCAACT-3′ | 5′-TGTGCCGCGCTGCCTGCGA-3′ |
| 4 | 5′-TCGCAGGCAGCGCGGCACA-3′ | 5′-GACCTCGGCTTCGCTCCAGT-3′ |
| 5 | 5′-CCAACTCTCGGTTTCCTGGA-3′ | 5′-GGGCCTGCGAACAAGTAAGT-3′ |
| 6 | 5′-TCGGGGTTGGGATCCTGGCT-3′ | 5′-GTCAGTCAGTCCCAGCATCGA-3′ |
| 7 | 5′-AGAGCTGACTGACCGCGCCT-3′ | 5′-GAGGGAGTGCCTGGTGAGGA-3′ |
| 8 | 5′-GGTGCTGCTCGCCTACTTCCT-3′ | 5′-GCCATGCCCCGCGCAGTCA-3′ |
| 9 | 5′-GAGCAGCTCCACGCCTGCG-3′ | 5′-AAGCCCCACCTCCTTCCCGA-3′ |
| 10 | ND[a] | 5′-TGATCAAGAACCTAGCCCTTGA-3′ |
| 11 | 5′-ACCTGGACGGGTGAGCGGA-3′ | 5′-AGATGCGCAAGAGTCAGGGAT-3′ |
| 12 | 5′-CTTGCGCATCTGGCTCCTACA-3′ | 5′-AATTCTTGTCGGTGTGGCTGTG-3′ |

[a] Not determined

***Unrelated control animals***

When sequence differences were found between the DNA obtained from the myasthenic calf and the reference bov*CHRNE* sequence, a random sample of 23 red or black-and-white German slaughterhouse cattle was obtained. Five Red Brahman and five Grey Brahman cattle from South Africa, unrelated to the myasthenic calves, were also screened as controls. Genomic DNA from the control animals was extracted from 10-ml aliquots of EDTA-anticoagulated blood using a salting-out method (Miller et al., 1988). Exon 5 was amplified using primers given in Table 1, and the PCR products were separated on 10% polyacrylamide gels. The exon 5 amplicon from the DNA of the myasthenic calf was run on the same gel as a positive control for detection of 470del20.

### *Control animals related to the myasthenic calves*

DNA was extracted from stored semen samples of the sire and maternal grandsire of the affected calves, using the Chelex DNA extraction method [for details see Forensic Science Laboratory (FSL) of the South African Police Service (SAPS) Quality Manual no. BIO/WI/C009 Revision 3 1999]. In addition, DNA from eight cattle related to the affected calves was extracted from whole blood using the Wizard Genomic DNA Purification kit (Whitehead Scientific). These ten animals were then screened for the presence of the 470del20 deletion. The positions of the tested individuals within the pedigree are shown in Figure 1.

## Results

### *Genomic structure of bovCHRNE*

The bov*CHRNE* gene consists of 12 exons, encoding a predicted protein of 405 amino acid residues. In silico cloning and exon-exon PCR showed that the localisations of the exon-boundaries are conserved compared with the human *CHRNE* gene structure. The gt/ag rule of conserved splice sites (Breathnach and Chambon, 1981) is followed in all introns. The sizes of bov*CHRNE* introns obtained by sequencing (introns 1–2, 4, 6–7, 9–12) or estimation of fragment sizes from gel electrophoresis (intron 3), were comparable to the known intron sizes of the human *CHRNE* gene (Table 2). The ATG start codon is localised in exon 1, and the TAG stop codon in exon 12. The four transmembrane regions are encoded by exons 7, 8, 9 and 12, respectively. At the amino acid level, the human and bovine *CHRNE* genes have 89% identity and 91% similarity. The genomic bovine *CHRNE* sequences have been submitted to GenBank under accession number AF457656.

### *Detection of a 20-bp deletion*

PCR amplification and subsequent direct sequencing of bov*CHRNE* exons from the DNA of the myasthenic calf revealed a loss of 20 bp within the coding sequence of

exon 5 (Figure 2), between nucleotide 469 and 490 (nucleotide numbering referring to the cDNA sequence published under accession number X02597). The myasthenic calf was homozygous for the mutation 470del20. The sequence change was present in DNA samples extracted from two different tissues (skeletal muscle and spinal cord). It is therefore highly unlikely that the 20-bp deletion was artificially caused by DNA degradation during tissue fixation or DNA preparation. The 470del20 mutation was found neither in 46 chromosomes from German red or black-and-white control cattle, nor in 20 chromosomes from South African Red Brahman or Grey Brahman control animals. Thus the 470del20 is not part of the normal bov*CHRNE* gene sequence, nor is it a variation typically found in Brahman genomes. Mutation screening showed that both the sire and the maternal grandsire shared by all four affected calves were heterozygous for the 470del20 mutation. The heterozygous deletion genotype was also detected in the dam of one of the calves, as well as in three healthy half-sisters. Another three half-sisters and a half-sister of the dam of an affected calf were tested and found to be homozygous for the bov*CHRNE* wild-type allele (Figure 1).

**Table 2.** Sizes of introns[a]

| Intron | Bovine (bp) | Human (bp) |
|---|---|---|
| 1 | 259 | 254 |
| 2 | 119 | 126 |
| 3 | 150–200[b] | 123 |
| 4 | 142 | 139 |
| 5 | ND | 306 |
| 6 | 379 | 334 |
| 7 | 77 | 82 |
| 8 | ND | 1,210 |
| 9 | 71 | 83 |
| 10 | 84 | 90 |
| 12 | 117 | 109 |

[a] Accession number for bov*CHRNE* genomic sequences: AF457656

[b] Estimated by gel electrophoresis of amplified fragments

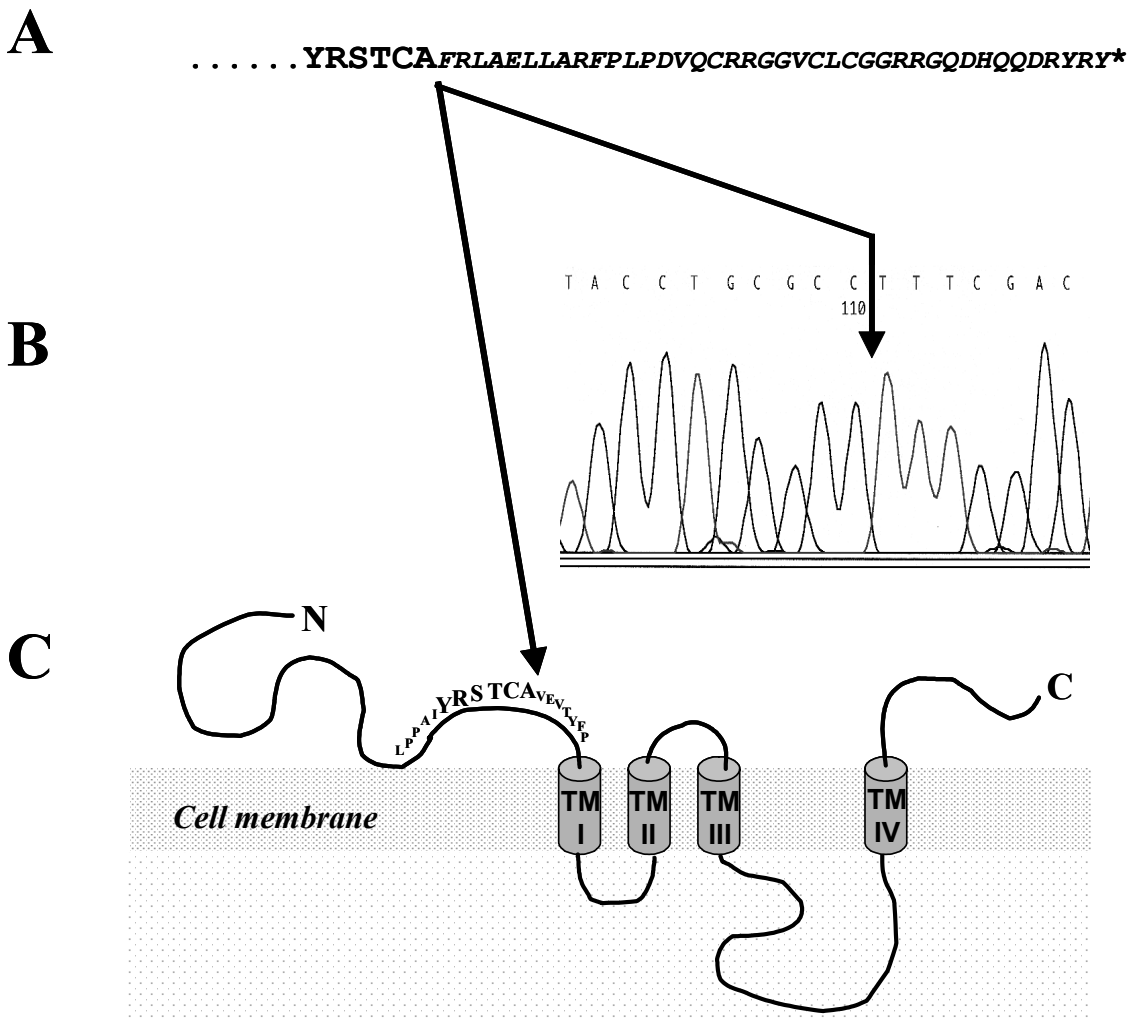


**Figure 2.** Detection of bov*CHRNE*/470del20. A Predicted amino acid sequence of the bov*CHRNE*/470del20 allele. The missense amino acids caused by the deletion are given in *smaller letters*. The premature stop codon is indicated by an *asterisk*. B Sequencing of the affected Brahman calf. The position of the homozygous deletion is indicated by an *arrow*. C Schematic drawing of the putative bov*CHRNE* protein. The four transmembrane regions (TM) are shown, and the position of the 470del20 mutation is indicated by an *arrow*.

***Confirmation of bovCHRNE cDNA sequence***

The cDNA sequence obtained from the cattle control DNA and the affected Brahman calf differed at position bp 317 from the published bov*CHRNE* sequence (accession number X02597). The observed A/C exchange did not affect the predicted amino acid sequence, but deleted a *Dde*I restriction site in our cDNA sequence. The expected fragment sizes of the 193-bp exon 4-PCR product were 105 bp and 88 bp for the published sequence. Only the 193-bp fragment was observed after *Dde*I digestion of amplification products from 90 chromosomes of red, black and white, Red Brahman and Grey Brahman cattle. Thus the published adenine in position bp 317 is either a rare variant or a sequencing error.

## Discussion

Human congenital myasthenic syndromes are a heterogeneous group of disorders which can be caused by presynaptic, synaptic or postsynaptic defects at the neuromuscular junction. Mutations have been found in all four subunits of the muscular nicotinic acetylcholine receptor (AChR), in the choline acetyltransferase gene, and in the *COLQ* gene encoding for the collagen tail of the acetylcholinesterase (Engel et al., 1999; Sieb et al., 2002). Detailed endplate studies including morphology and in vitro electrophysiology were not performed in the Brahman calves. Therefore, it was unclear which subtype of neuromuscular transmission defect was present in the affected animals. However, the beneficial effect of anticholinesterases and the decreased response were consistent with a reduced AChR expression. Thus far in humans, most AChR mutations have been found within the *CHRNE* gene (Engel et al., 1999; Sieb et al., 2002). This guided us to select the bov*CHRNE* gene to start searching for the genetic defect in the myasthenic Brahman calves.

We identified a homozygous 20-bp deletion within exon 5 of the gene. The predicted bov*CHRNE* protein, encoded by the RNA carrying the 470del20 mutation, would be truncated upstream from the four transmembrane domains. Sequence analysis showed that the 470del20 mutation causes a frame shift in the predicted bov*CHRNE* protein after 129 codons, substituting 342 wild-type amino acid residues with 40 aberrant

amino acids, followed by a stop codon. The frame shift occurred 90 amino acids residues N-terminal of the first transmembrane region. Thus, the bov*CHRNE* mutation reported here leads to a non-functional allele, which is likely to be the primary cause for myasthenia in the affected Brahman calves. All four myasthenic calves were related to each other by sharing the same sire and maternal grandsire (Thompson, 1998). Furthermore, the calf investigated in the present study was homozygous for the 470del20 mutation. Thus it is likely that the common ancestors were also related to each other. The prevalence of the 470del20 mutation in the Brahman breed is unknown, and warrants further investigation.

The myasthenic calves do not have a functional copy of the *CHRNE* subunit, and it can therefore be assumed that mature AChR was absent from their neuromuscular junctions. The most likely explanation why the condition did not result in immediate perinatal lethality, would be phenotypic rescue by foetal-type AChR, due to persistent expression of the $\gamma$-AChR subunit encoded by the *CHRNG* gene (Engel et al., 1996). Normally, a switch from *CHRNG* to *CHRNE* gene transcription occurs, either late in pregnancy or in the postnatal phase. Low-level persistent *CHRNG* expression has been demonstrated in healthy humans, as well as in humans carrying two truncating mutations in their *CHRNE* genes (MacLennan et al., 1997; Croxen et al., 2001). Although the true survival times of the affected calves were unknown, as they were killed humanely, all four calves were severely affected at a very young age and would have only been able to survive by hand-feeding (Thompson, 1998). This is in contrast to human myasthenic patients with truncating *CHRNE* mutations, most of whom show a less severe course of the disease (Engel et al., 1996; Ohno et al., 1998; Middleton et al., 1999; Sieb et al., 2000). One possible explanation for this could be a rapid and complete downregulation of bov*CHRNG* gene expression after birth. It would therefore be interesting to study the peri- and postnatal time course of *CHRNG* expression in healthy cattle. Immunohistochemistry of endplates from bovine muscle samples, collected at different ages between the last month before birth and the end of the first six months of life, could be used to compare human and bovine *CHRNG* expression patterns. If no differences are found between humans and cattle, this would argue against a rescuing role of persistent *CHRNG* expression, but for the existence of some other, unknown factors that are able to influence the severity of the phenotype. Breeding of homozygous calves would be helpful to further establish the phenotype-genotype correlation, and would provide the possibility to study the AChR distribution

and composition at the muscular endplate of the affected calves. Thus, future comparative studies including animal models such as the one described, will contribute to our understanding of the complex genotype-phenotype relationship in congenital myasthenic syndromes.

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# Chapter 4

# Congenital myasthenic syndrome of Brahman cattle in South Africa

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# Abstract

A congenital myasthenic syndrome in Brahman cattle is caused by a homozygous 20 base pair deletion (470del20) in the gene coding for the epsilon subunit of the acetylcholine receptor at the neuromuscular junction. It causes a progressive muscle weakness, starting either at birth or within the first month. A PCR-based DNA test, using blood or semen stored on FTA paper, was developed and validated; the test makes it possible to differentiate rapidly and accurately between homozygous wild-type, heterozygous and homozygous affected animals. Preliminary testing of Brahman cattle in South Africa has revealed several carrier animals, some of them influential animals in the breeding population.

# Introduction

Congenital myasthenic syndromes in human beings are a group of heterogeneous disorders of neuromuscular transmission, arising from presynaptic, synaptic or postsynaptic defects. Postsynaptic disorders, resulting from mutations in the genes coding for the various subunits of the nicotinic acetylcholine receptor (nAChR), are most frequently described, with the epsilon subunit being most commonly affected (Engel and Ohno, 2002). The syndromes, which are also referred to as congenital myasthenia gravis, have been reported in dogs (Johnson et al., 1975; Flagstad, 1982; Miller et al., 1983; Wallace and Palmer, 1984) and cats (Indrieri et al., 1983), but their genetic basis remains unknown. Very few of the many inherited defects recorded in domestic animals have been characterised at the molecular level (Nicholas, 1999; Fyfe, 2002).

A suspected congenital myasthenic syndrome was recently reported for the first time in cattle (Thompson, 1998), and the underlying genetic defect was later identified as a homozygous 20-base pair (bp) deletion in the gene (bov*CHRNE*) coding for the $\varepsilon$-subunit of the nAChR at the neuromuscular junction (Kraner et al., 2002). This mutation (470del20) is predicted to result in a non-functional allele and the inability to produce functional adult-type nAChR, thus explaining the severe muscle weakness observed. It appears to be inherited in an simple recessive manner and heterozygotes are clinically normal. Two bulls whose semen had been widely used for artificial insemination were identified as carriers. This paper describes the clinical signs in the cases observed so far, the development of a screening test for the mutation and the preliminary results of further screening.

# Materials and methods

### *Clinical syndrome*

Clinical signs were originally observed in four closely related red Brahman calves and have been described in detail by Thompson (1998). Using the recorded pedigrees of

the affected calves, closely related animals were traced and their owners were questioned about the occurrence of similar clinical signs in their calves.

When the mutation responsible for the condition had been identified and a screening test had been developed (as described below) it was possible to test calves that were reported to be showing signs of muscle weakness. The clinical signs and post-mortem findings in a homozygous affected calf are described.

***Screening test***

Blood samples were collected by venepuncture from 26 relatives (first, second or third generation offspring) of the known carrier bulls, 14 unrelated red Brahmans and eight cattle of other breeds, into Vacutainer tubes containing EDTA as anticoagulant. An aliquot of 100 $\mu$l of blood was transferred to FTA paper (Whatman Bioscience), allowed to air dry, and stored at room temperature until analysed. A 2 mm punch was removed from the stored sample, placed in a 200 $\mu$l PCR tube, and washed three times with 200 $\mu$l of FTA reagent (Whatman Bioscience) and twice with 200 $\mu$l of TRIS-EDTA buffer. The punches were allowed to air dry for one hour before being processed by PCR.

A specific primer pair was used to amplify a 211-base pair segment of exon 5 of the bovine *CHRNE* gene. The sequence of the forward primer was 5′-CCAACTCTCGGTTTCCTGGA-3′ and that of the reverse primer was 5′-GGGCCTGCGAACAAGTAAGT-3′. The 5′ end of the forward primer was labelled with 6-FAM (Applied Biosystems). The PCR was carried out on a GeneAmp 9700 PCR System (Applied Biosystems) in a total volume of 25 $\mu$l, containing 2 pmol of the forward and reverse primers, 200 $\mu$M of each dNTP, 1.5mM $MgCl_2$, 50 mM KCl, 15 mM Tris-HCl (pH 8.0) buffer and 0.1 U AmpliTaq Gold polymerase (Applied Biosystems). The parameters of the PCR were as follows: denaturation at 95°C for 10 minutes, followed by 33 cycles of 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds, followed by a final extension step at 72°C for 10 minutes.

The PCR product was diluted 10-fold in water and 1 $\mu$l was mixed with 10 $\mu$l of HiDi Formamide (Applied Biosystems) and 0.25 $\mu$l of Genescan size standard. The PCR fragments were sized by capillary electrophoresis on an ABI 310 Genetic Analyser (Applied Biosystems). Further analysis was carried out using STRand software (Board of Regents, University of California) on a personal computer.

For verification, 30 aliquots of the same blood samples were screened independently at the Institute for Human Genetics, University Hospital Bonn, Germany. The DNA was extracted from 100 $\mu$l of whole blood by lysing the erythrocytes with sodium chloride-EDTA, and then lysing the leucocytes with sodium hydroxide and heat. Exon 5 was amplified by PCR, and the fragments were separated by polyacrylamide-gel electrophoresis as described by Kraner et al. (2002). The results were confirmed by sequencing one of each different sized fragment using an ABI 377 Genetic Analyser (Applied Biosystems).

The screening test was also adapted for processing fresh or frozen semen. A 1:10 dilution of semen was made with a semen-processing diluent (Schulman et al., 2002). Aliquots of 100 $\mu$l of semen were placed on separate pieces of FTA paper and allowed to dry overnight. A 2 mm punch was removed from the stored FTA paper containing each semen sample, placed in a 200 $\mu$l PCR tube, and 200 $\mu$l of FTA purification reagent (Whatman Bioscience), 20 $\mu$l of 1M dithiothreitol (Sigma-Aldrich) and 5 $\mu$l of proteinase K (Roche Diagnostics) (20 mg/ml) were added to each tube. The tubes were incubated at 56°C for one hour in a GeneAmp 9700 PCR System (Schulman et al., 2002). The punch was again washed twice with 200 $\mu$l of FTA purification reagent and twice with 200 $\mu$l of TRIS-EDTA buffer. The punches were allowed to air dry for at least one hour, and then processed and analysed as described for the blood samples.

### *Further screening*

After the screening test had been validated, further testing was carried out. The 125 animals, other than unweaned calves, in the herd from which the original affected calves had come, were bled and tested, and 105 Brahman cattle attending a national show were also tested. Several farmers requested that some of their breeding cattle and stored semen should be tested. In addition, three Brahman herds reported having weak

calves; these calves, their dams and sires, and several other animals in these herds were tested. In an attempt to detect additional carriers among influential breeding animals, stored frozen semen from 68 Brahman bulls was tested; the selection of these samples depended upon the availability of stored semen and the willingness of the owners to have their animals tested.

## Results

### *Clinical syndrome*

One further affected calf was found, and confirmed to be homozygous for the 470del20 mutation. The owner reported that the heifer calf had been weak at birth and unable to rise. When helped to its feet it could stand and walk for 30 to 45 minutes before collapsing, and it could suck from its dam. The severity of its condition had fluctuated and then gradually deteriorated. However, it died at three months of age as a result of an abomasal and omasal volvulus, before it could be examined clinically. A post-mortem examination was carried out and the animal was found to be in fair body condition, without obvious muscle atrophy. It was markedly dehydrated and had a mild, acute to subacute bronchopneumonia, with some plant material in the bronchioli and moderate cerebral congestion. Its abomasum and omasum were twisted through 360˚ and distended, with foul-smelling, red-brown, watery contents. Histologically the muscles and peripheral nerves appeared normal. Its dam and sire were both tested, and confirmed to be carriers of the 470del20 mutation.

The owner of another herd, in which carrier animals were found, also reported having had several weak calves in previous years; they had apparently been normal at birth, but became progressively weaker after four to seven days of age. They had been destroyed after several weeks of gradual deterioration.

***Screening test***

Of the 26 related red Brahmans initially tested, eight were identified as heterozygous for the 470del20 mutation, and 18 were homozygous for the wild-type allele. All the 22 unrelated cattle were homozygous wild-type. Of the 30 samples tested at both laboratories, eight were carriers and 22 were homozygous wild-type. There was complete agreement between the laboratories in the results obtained. After computer analysis, the output of the PCR fragment sizing identified cattle homozygous for the wild-type allele by a single peak at 211 bp, heterozygotes by two peaks at 191 bp and 211 bp, and homozygous affected calves by a single peak at 191 bp.

***Further screening***

Of the 125 cattle tested in the herd in which the condition was originally detected, 15 (12%) were found to be carriers of the mutation; these nine cows and six bulls were all first, second, third or fourth generation descendants of one of the known carrier bulls. The screening of semen resulted in the detection of four carrier bulls, which had been widely used for artificial insemination, and three further carrier animals were detected by the screening of blood samples.

Testing animals in one of the three herds that had reported having weak calves, resulted in the detection of the affected three-month-old heifer calf described above. Testing the other two herds with weak calves showed that the mutation had not been the cause of the problem, although a single carrier was detected in one of the herds.

Of the 183 animals tested that were not selected because they were related to known carriers or because they were part of a herd reporting weak calves, three were found to be carriers.

# Discussion

The full range of clinical signs of congenital myasthenic syndrome in cattle is not yet known and requires further investigation. In the cases so far observed, the calves have either been weak at birth or become weak during the first month of life. In humans, the myasthenic symptoms appear from birth or early childhood, but some cases may develop only in later life (Engel and Ohno, 2002). The reason for the survival of patients homozygous for *CHRNE* mutations is thought to be the persistent expression of the *CHRNG* gene coding for the foetal type $\gamma$-subunit (Engel and Ohno, 2002); the AChR $\varepsilon$-subunit normally replaces the $\gamma$-subunit during the perinatal period.

The cases observed have survived for several months, although they required assistance to gain access to their mothers' teats. Because most Brahman cattle in South Africa are kept under extensive conditions, it is likely that many cases go unobserved and die as a result of starvation or predation. The abomasal volvulus in one affected calf is unlikely to have been related to the underlying myasthenic syndrome, because nAChR containing the $\varepsilon$-subunit occurs only at the neuromuscular junction of skeletal muscle and not in the gastrointestinal tract (Utkin et al., 2000).

Perinatal calf mortality is an important cause of economic loss in the beef cattle industry (Wikse et al., 1994). Although many important risk factors have been identified, numerous cases remain undiagnosed. The "dummy calf" syndrome, most commonly observed in Brahman cattle, is significantly affected by the sire (Kim et al., 1988), indicating a possible genetic component in its aetiology. Investigation into the possible involvement of the 470del20 mutation in cases of idiopathic perinatal calf mortality and the "dummy calf" syndrome is therefore warranted. Congenital myasthenic syndromes have also been found in association with arthrogryposis multiplex congenita in humans (Brownlow et al., 2001), and it may therefore be interesting to screen calves affected by arthrogryposis for the 470del20 mutation.

As in humans heterozygous for *CHRNE* mutations, carriers of the 470del20 mutation appear to be clinically unaffected, and some have been top performers at shows and in performance evaluations.

Although the prevalence of the mutation is unknown, the discovery of three carriers amongst 183 randomly selected animals suggests a prevalence of 1–2%. Theoretically, with unselected mating and in a population of unlimited size, this would result in approximately six affected calves per 100,000 conceived. However, within certain herds the prevalence of carriers can be much higher; in the herd described above it was 12%, despite the farmer having discontinued the use of the carrier bulls eight years previously. It was also shown that there were carriers amongst stud bulls being used for artificial insemination.

An examination of the recorded pedigrees, extending back for up to 12 generations, has revealed no ancestors common to all the known carriers. However, it is unlikely that the 470del20 mutation occurred independently more than once within the Brahman population. The bov*CHRNE* sequence adjacent to the 470del20 mutation does not show any obvious features of a mutation hot-spot. In human patients, more than 50 *CHRNE* mutations have been found, most of them only once. Only one human mutation was detected in patients from several different families, most of which were of Gypsy origin and therefore probably distantly related (Abicht et al., 1999). A more plausible explanation for the frequency and distribution of the 470del20 mutation in South African Brahmans, would be a founder effect resulting from imported ancestors. The mutation is therefore also likely to be present in Brahman populations elsewhere in the world.

The validation of this screening test provides a rapid, inexpensive and accurate method for identifying carriers of the bov*CHRNE* 470del20 mutation responsible for this congenital myasthenic syndrome. It can therefore be used to investigate the prevalence and the clinical and economic importance of the mutation, and to implement a screening programme if it should be deemed necessary, as it has been for generalised glycogenosis in Brahman cattle in Australia (Dennis et al., 2002).

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# Chapter 5

# The *CHRNE* 470del20 mutation causing congenital myasthenic syndrome in South African Brahman cattle: prevalence, origin, and association with performance traits

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# Abstract

Genotyping of the South African registered Brahman cattle population for the 470del20 mutation in the *CHRNE* gene causing congenital myasthenic syndrome (CMS), was carried out in 1,453 animals. Overall prevalence of carriers was 0.97% (95% confidence interval 0.50% to 1.68%). Carrier prevalence amongst breeding bulls in 2004 was 1.22% (95% confidence interval 0.65% to 2.15%), and had not changed significantly since 2000. Using segregation analysis, CMS genotype probabilities were calculated for all 612,219 animals in the pedigree, leading to the identification of two founder animals as the most likely original carriers. Pedigree analysis revealed no ancestors common to all known carriers, but that the mutation had been introduced at least twice into the South African Brahman population, probably via animals imported from the U.S.A.. The effects of CMS genotype probability on adjusted birth, 200 d, 400 d, and 600 d weights, as well as on EBVs for birth, 200 d, 400 d, and 600 d weights, and milk, were estimated, accounting for effects of sire. Heterozygosity for the *CHRNE* 470del20 mutation was associated with a 13.3 kg increase in adjusted 600 d weight ($P = 0.03$). Positive effects of CMS carrier status on all weight EBVs were found, but no effect on milk EBV. We conclude that CMS carriers have a weight advantage at 600 d, and possibly also at birth, 200 d, and 400 d. This may confer a selective advantage and tend to increase the frequency of the mutation.

# Introduction

Congenital myasthenic syndrome (CMS) in Brahman cattle is caused by homozygosity for a 20 base pair deletion (470del20) in the *CHRNE* gene, resulting in absence of the epsilon subunit of the nicotinic acetylcholine receptor at the neuromuscular junction (Kraner et al., 2002). This results in a non-functional adult-type acetylcholine receptor, causing progressive muscle weakness and mortality in young calves (Thompson, 1998; Thompson et al., 2003b).

A PCR-based DNA test for the mutation has been developed and used for screening of breeding animals (Thompson et al., 2003b). Screening of a limited number of animals has revealed that the estimated prevalence of carriers in the South African pedigree Brahman population was 0.67% (95% confidence interval: 0.17, 2.1%) (Thompson et al., 2003a). In the same report, a survey of 2,434 stored Australian Brahman DNA samples detected two carriers (0.08%), but none amongst 541 samples from foreign (mainly American) Brahmans being tested prior to import to Australia during 1995–2003. It is not known whether the mutation is present in Brahman populations elsewhere in the world, although it is thought to have been introduced into the South African population by imported ancestors (Thompson et al., 2003b).

Although heterozygotes appear phenotypically normal (Thompson et al., 2003b), it is not known whether carrier status is associated with traits that are used in the selection of breeding animals, and hence whether the frequency of the mutation is likely to increase or decrease due to selection on performance traits.

The objective of this study was to estimate more precisely the prevalence of carriers of the CMS mutation in the registered South African Brahman breeding population, to determine the origin of the mutation in the South African Brahman population by identifying common ancestors of known carriers, and to determine whether there is any association between CMS genotype and measured performance traits.

## Materials and Methods

Genotyping of Brahman cattle registered with the Brahman Cattle Breeders' Society of South Africa for the *CHRNE* 470del20 mutation was performed on a voluntary basis, together with compulsory DNA profiling for parentage verification, in accordance with breed society policy. DNA was extracted from tail hair roots, and exon 5 of *CHRNE* was amplified as previously described (Thompson et al., 2003b). DNA amplicons were size-separated by polyacrylamide gel electrophoresis and animals were classified as homozygous wild-type ("non-carrier") or heterozygous ("carrier"). Results up to August 2005 were included in the analysis. The sample consisted of 1,453 animals (1,102 males and 351 females), representing over 300 of the approximately 500 registered herds in South Africa. Of these, 210 animals had been purposively sampled, either by virtue of their relationship to known carriers, or during investigations of calf mortality.

The prevalence of carriers amongst bulls that sired at least one registered calf, and the prevalence of carriers amongst registered calves born, were calculated for each year from 2000 to 2004. Any animals which had been purposively sampled were not included in these calculations. Exact hypergeometric confidence intervals (≥95%) for the prevalences were obtained using StatCalc 1.1 (Krishnamoorthy, 2000). Numbers of sires and calves, and the number sampled each year are shown in Table 1.

The South African registered Brahman pedigree contained records of 310,320 males and 301,878 females, including 15,680 sires and 160,156 dams, tracing back to animals born in the early 1940s. Genotype probabilities for all animals in the pedigree were calculated using the Geneprob programme, which employs the segregation analysis algorithm of Kerr and Kinghorn (1996). The genotype probabilities were used in order to identify ancestors with relatively high probability of being heterozygous for the 470del20 mutation. Using Pedigree Viewer (Kinghorn, 1994), the entire ancestor tree for each known carrier was extracted and their common ancestors were identified.

Performance records (birth weight, 200 d weight, 400 d weight and/or 600 d weight), adjusted for age at weighing and age of dam, were available for 118,390 animals, descended from 4,583 sires. However, relatively few animals (5,130) had all four

weight records available, while the majority (67,314) had only one of the weights recorded. The number of animals and their sires for which each weight was available is shown under Results (Table 3). Contemporary groups were defined based on herd of origin, herd at time of weighing, calving year, sex, birth number (single/twin), birth type (natural/embryo transfer), breeder-defined management group, parity of dam (heifer/multiparous, for birth weight only), recipient dam breed (embryo transfer calves), and age (maximum age range in a group was 45 d for birth and weaning weights, and 60 d for 400 and 600 d weights).

To estimate the effect of CMS carrier status on birth, 200 d, 400 d, and 600 d weights, performance records were regressed on genotype probability, in a model that also accounted for contemporary group and sire. The sire effect was included to account for family effects possibly being confounded with genotype probabilities, through either selection or drift. Relationships amongst sires were accounted for by tracing back sires to their ancestors, up to a maximum of nine generations. The statistical model was

$$\mathbf{y} = \mathbf{b}.\boldsymbol{P}(\mathbf{het}) + \mathbf{sire} + \mathbf{cg} + \mathbf{e}, \quad (1)$$

where **y** is the weight performance record, **b** is the effect of carrier status on this performance, ***P*(het)** is the probability of being heterozygous for the 470del20 mutation, calculated using Geneprob, **sire** is a random effect with covariances proportional to their additive genetic relationships, **cg** is a fixed effect accounting for contemporary group, and **e** is the residual.

In addition, the effect of an animal's CMS carrier status on its EBV for birth weight, 200 d weight, 400 d weight, 600 d weight, and milk, was estimated using a sire model. BREEDPLAN EBVs based on BLUP (Henderson, 1984) were obtained from the Brahman Cattle Breeders' Society of South Africa. Only EBVs with an accuracy >75% were used, except for milk EBV, where a cut-off of 65% was used, because there were only 32 milk EBV accuracies >75%. There were 35,549 animals, descending from 1,896 sires, with one or more EBVs fulfilling these criteria. However, very few animals (270) had all five EBVs available, while the majority (29,860) had only one EBV available. The number of animals and their sires for which each EBV was available is shown under Results (Table 4). The statistical model was

$$\mathbf{y = b.P(het) + sire + e}, \quad (2)$$

where **y** is the bull's EBV, and **sire** was fitted as in Model 1. The residual variance was assumed homogeneous, which is partly justified through only using highly accurate EBVs. Analysis was done using residual maximum likelihood as implemented in ASReml (Gilmour et al., 2002).

# Results

### *Prevalence of CMS mutation*

**Table 1.** Prevalence of CHRNE 470del20 carriers amongst registered South African Brahman breeding bulls and registered calves, 2000–2004

| Year | Registered breeding bulls used | | | | Registered calves born | | | |
|---|---|---|---|---|---|---|---|---|
| | Total [1] | Number tested | CMS carrier prevalence (%) | 95% confidence interval [2] | Total | Number tested | CMS carrier prevalence (%) | 95% confidence interval [2] |
| 2000 | 1,515 | 201 | 1.00 | 0.13, 3.43 | 19,734 | 215 | 0.93 | 0.11, 3.30 |
| 2001 | 1,481 | 273 | 1.10 | 0.20, 2.97 | 19,072 | 202 | 0.50 | 0.01, 2.72 |
| 2002 | 1,433 | 370 | 2.43 | 1.26, 4.32 | 18,297 | 114 | 0.00 | 0.00, 2.59 |
| 2003 | 1,261 | 489 | 1.64 | 0.79, 2.85 | 14,426 | 56 | 0.00 | 0.00, 5.21 |
| 2004 | 1,069 | 574 | 1.22 | 0.65, 2.15 | 13,347 | 21 | 0.00 | 0.00, 13.3 |

[1] Bulls that sired at least one registered calf

[2] Exact hypergeometric confidence interval (actual confidence level varies between 95% and 96.8%).

Of the 1,243 non-purposively sampled animals genotyped, 12 (0.97%; 95% confidence interval 0.50% to 1.68%) were heterozygous for the 470del20 mutation. An additional four carrier bulls had previously been detected as a result of selective sampling of relatives of a known carrier. No animals were found to be homozygous for the mutation. Table 1 shows, for each year from 2000 through 2004, the prevalence of CMS carriers amongst bulls that sired at least one registered calf, and the prevalence of CMS carriers amongst calves registered. There were no significant differences in carrier prevalence between years, or between bulls used and calves born within each year.

***Pedigree analysis***

There was insufficient information for the segregation analysis algorithm of Geneprob to provide a stable estimation of base population allele frequency, therefore the frequency of the dominant, wild-type allele in the base population was set at 0.98. This was slightly lower than the raw observed frequency of 0.994 in genotyped animals, on the assumption that the mutant allele frequency had very slowly been decreasing over generations. The distribution of CMS carrier probabilities in the Brahman pedigree is shown in Table 2. For the 5,883 half-founder or founder animals in the pedigree (defined as foreign-born animals, with one or both parents unknown), segregation analysis calculated *P*(het) to be <0.1 in all but 13; of these, two were markedly higher (0.19 and 0.22). No single carrier founder could be identified for all known carriers. However, 36 animals were identified, each of which was a common ancestor to all but one of the known carriers. Amongst these 36 animals were the two founder animals with the highest *P*(het). One of the 36 was an imported American Brahman bull, while the others were all ancestors of animals or semen imported from the U.S.A.

**Table 2.** Distribution of *CHRNE* 470del20 carrier probabilities in the South African registered Brahman pedigree, calculated by segregation analysis

| *P*(het) [1] | Frequency | | | |
|---|---|---|---|---|
| | Animals with performance records [2] | Animals with accurate EBV [3] | Founders and half-founders [4] | Total |
| 0 to 0.01 | 54,364 | 22,688 | 1 | 123,478 |
| >0.01 to 0.05 | 54,067 | 10,677 | 5,809 | 444,957 |
| >0.05 to 0.1 | 4,104 | 949 | 60 | 25,294 |
| >0.1 to 0.2 | 2,944 | 634 | 12 | 9,688 |
| >0.2 to 0.3 | 1,770 | 358 | 1 | 5,760 |
| >0.3 to 0.4 | 178 | 29 | 0 | 569 |
| >0.4 to 0.5 | 844 | 186 | 0 | 2,187 |
| >0.5 to 0.6 | 103 | 20 | 0 | 253 |
| >0.6 to 0.99 | 5 | 3 | 0 | 17 |
| >0.99 | 11 | 5 | 0 | 16 |
| Total | 118,390 | 35,549 | 5,883 | 612,219 |

[1] Probability of being heterozygous for the 470del20 mutation.

[2] Animals with one or more of the following records available: birth weight, 200 d weight, 400 d weight, 600 d weight.

[3] Animals with one or more of the following available: birth weight EBV >75%, 200 d weight EBV >75%, 400 d weight EBV >75%, 600 d weight EBV >75%, milk EBV >65%.

[4] Defined as foreign-born, with one or both parents unknown.

***Effect on performance***

The effect of CMS carrier status on body weight, using own phenotype in a sire model, is shown in Table 3. Heterozygosity for the 470del20 mutation was associated with a 13.3 kg greater 600 d weight ($P$ = 0.03). No significant effects were detected for the other traits, although birth and 400 d weights also tended to be greater in carriers. Heritability estimates obtained from the sire model were 0.32 for birth weight, 0.27 for 200 d weight, 0.28 for 400 d weight, and 0.27 for 600 d weight.

**Table 3.** Effect of heterozygosity for the *CHRNE* 470del20 mutation on growth phenotypes in South African Brahmans, using a sire model

| Phenotype | Number of records | | *b* | SE(*b*) | 95% confidence interval | *P*-value |
|---|---|---|---|---|---|---|
| | animals | sires | | | | |
| Birth weight | 70,099 | 3,265 | 0.66 | 0.48 | -0.28, 1.60 | 0.17 |
| 200 d weight | 62,760 | 3,133 | -0.50 | 2.87 | -6.13, 5.13 | 0.86 |
| 400 d weight | 38,980 | 2,528 | 5.66 | 4.75 | -3.65, 14.97 | 0.23 |
| 600 d weight | 27,079 | 2,154 | 13.30 | 5.94 | 1.66, 24.94 | 0.03 |

The effect of CMS carrier status of bulls on EBVs for weight and milk using model 2, are shown in Table 4. Consistently positive effects of 470del20 heterozygosity on weight EBVs were estimated, but no effect was found on milk EBV. The effect on 600 d weight using EBV (+14.9 kg) was comparable to that using own phenotype (+13.3 kg).

**Table 4.** Effect of heterozygosity for the *CHRNE* 470del20 mutation on growth and milk EBVs in South African Brahmans, using a sire model

| EBV | Number of records | | *b* | SE(*b*) | 95% confidence interval | *P*-value |
|---|---|---|---|---|---|---|
| | animals | sires | | | | |
| Birth weight | 32,468 | 1,755 | 1.13 | 0.24 | 0.66, 1.60 | <0.001 |
| 200 d weight | 3,143 | 519 | 9.08 | 3.26 | 2.69, 15.47 | 0.005 |
| 400 d weight | 5,519 | 595 | 12.12 | 3.97 | 4.34, 19.90 | 0.002 |
| 600 d weight | 4,862 | 556 | 14.93 | 5.11 | 4.91, 24.95 | 0.004 |
| Milk | 349 | 99 | -1.55 | 1.86 | -5.20, 2.10 | 0.41 |

# Discussion

### *Prevalence of CMS mutation*

The 1.2% prevalence of carriers amongst 2004 breeding bulls was consistent with a previous estimate based on a smaller sample (Thompson et al., 2003a). The failure to detect any homozygous recessives was expected, since it is assumed that this genotype results in early mortality. There was insufficient evidence to show any recent change in carrier prevalence. Because of the low numbers of younger animals tested since 2000, it is difficult to say whether the apparent downward trend in the prevalence of carrier calves born is real or due to random sampling error. However, due to the 3–4 year lag period between birth and selection for breeding, it is possible that any downward trend in the frequency of carrier calves born has not yet resulted in significant change in the frequency of carriers amongst breeding bulls. For a lethal recessive mutation such as this, a decrease in carrier prevalence over successive generations is to be expected, due

to natural mortality of homozygous recessives. However, this decrease is slow: assuming random mating and no selective advantage or disadvantage of the heterozygote, a carrier prevalence of 1% is expected to decrease to 0.995% over one generation (Hedrick, 2000).

The animals were not sampled randomly, as participation in the testing programme was voluntary, nevertheless most samples submitted for the mandatory DNA profiling for parentage verification were also elected to be genotyped for CMS. It is possible that breeders who suspected the presence of the mutation in their herd and wished to avoid the risk of negative publicity, may have been unwilling to have their bulls genotyped, resulting in an underestimate of the true carrier prevalence. Another possible source of bias is the fact that carrier prevalence amongst breeding bulls and calves for each year was not measured during that year, but mainly between 2003 and 2005, thus only those animals that survived until then were sampled. If there had been a difference in survival between carriers and non-carriers, due to natural and/or artificial selection (i.e., differential genotype fitness), prevalence estimates may have been biased.

Nevertheless, the presence of the 470del20 mutation in the Brahman population at a frequency of this order of magnitude is unlikely to be economically significant for the breed as a whole. Assuming random mating, and that carrier prevalence amongst breeding cows is equal to that in bulls, a carrier prevalence of 1.22% (95% confidence interval 0.65% to 2.15%) in the breeding population will result in the birth of 0.004% homozygous recessive calves (95% confidence interval 0.001% to 0.012%). For the South African pedigree Brahman population of approximately 15,000 cows, this would mean only between 0.16 and 1.73 affected calves per year. However, carriers are not randomly distributed through the population, and the condition may become economically significant for individual breeders. In the herd in which the mutation was originally detected, 12% of animals were found to be carriers (Thompson et al., 2003b). Inadvertent use of a carrier bull on all cows in this herd would have resulted in 3% of the calves being affected with CMS. An increase in the frequency of the mutation might occur when the carrier animals have a selective advantage due to a favourable effect of the mutation on performance traits. The associations between carrier status and performance traits were estimated in this study and are discussed below.

### *Pedigree analysis*

It is unlikely that a similar size deletion has occurred more than once in the same region of the *CHRNE* gene, since it shows no obvious features of a mutation hotspot (Thompson et al., 2003b). Therefore, assuming that there are no errors in the pedigree, the absence of a single common ancestor leads to the conclusion that the 470del20 mutation was introduced at least twice into the South African Brahman population. This was most likely by the importation of carrier animals or semen from the U.S.A., because the most likely carrier founders, identified by segregation analysis, were American ancestors. However, to our knowledge, no disease similar to CMS has previously been described in American Brahmans or in any other breed of cattle. The Brahman breed was developed in the southern U.S.A. during the late 19th and early 20th centuries, mainly from the Ongole, Kankrej, Gir and Krishna Valley breeds from India (Porter, 1991). Whether the mutation event occurred in the U.S.A., or in one of the ancestral Indian breeds, would require further investigation.

### *Effect on performance*

Regression on genotype probability, rather than known genotype, allows the utilisation of records from non-genotyped animals, thus increasing the power of the analysis. This approach has been used in an animal model for QTL detection (Weller et al., 2003), producing similar estimates to a model utilising information from genotyped animals only. In retrospect, our assumption of a base population wild-type allele frequency of 0.98 may not have been accurate, because it was based on the assumption that the mutant allele frequency had slowly been decreasing over generations. A possible weight advantage in the heterozygote may instead have caused the frequency to remain stable or increase slightly. However, only animals born since 1984 had performance records, whereas the pedigree extended back to the 1940's. The assumed base population allele frequency would therefore have had only very little effect on the calculated genotype probabilities of the animals included in the analyses. Consequently, this is unlikely to have affected the results of the regression of performance on genotype probability. In addition, *P*(het) calculated using base population allele frequencies of 0.98 and 0.994 (or slightly greater) would be almost perfectly correlated, and would therefore yield similar effect estimates.

In the analysis of the effects of carrier status on phenotypes or EBV, genetic relationship between sires were taken into consideration to avoid overestimation of levels of significance (Kennedy et al., 1992). A sire model was used for the analysis as it is computationally much easier, and dams usually have only one or a few offspring. Sire model results were compared with those from an animal model for 400 d and 600 d weights (data not shown) and agreed closely, although the sire models tended to yield more conservative and less significant estimates. The heritability estimates obtained from the sire model for birth, 200 d, 400 d, and 600 d weights were within ranges reported elsewhere (Davis, 1993). We consider the sire model to be an acceptable alternative to the animal model, as selection is mainly amongst sires. Most of the potential confounding due to family will therefore be due to sire family, which is accounted for in the sire model.

It is interesting that the models of EBV showed consistently significant positive effects of CMS carrier status on weight, which increased with increasing age. Although the effect on 600 d weight EBV was similar to the effect on 600 d weight (own phenotype), it is unclear why the effects at lower ages differed between models. The two analyses used different subsets of animals, which may explain the difference in estimates between the two approaches. The EBV used in the analysis had an accuracy of >75% (65% for milk EBV), and consequently, the residual variance for the EBV was substantially lower than that of a single phenotype, which had a positive effect on the accuracy of the estimates. However, this was counterbalanced by the lower number of animals with EBV of high accuracy. The exclusion of animals with low accuracy EBV meant that animals of the poorer performing genotype were less likely to be included in the analyses, due to their having insufficient progeny. This could have resulted in underestimates of the effect of genotype on EBV.

An EBV is an estimate of the additive or transferable genetic merit for that trait compared to the breed average, incorporating the animal's own phenotype, adjusted for age at weighing and age of dam and compared within contemporary groups, as well as the performance of relatives. Because of inclusion of the latter, EBV of related animals are not regarded as independent observations, and regression models of EBV may therefore result in underestimation of standard errors. However, only using EBV of high accuracy, which are based mainly on progeny, will tend to minimise this, because correlations between EBV should be lower.

We conclude that there is strong evidence that CMS carriers have a greater 600 d weight, and possibly also have greater birth, 200 d, and 400 d weights. This effect may be due either to linkage or pleiotropy. The *CHRNE* gene has not been mapped on the USDA-MARC linkage map (USDA-ARS, 2005), but is located in a poorly mapped region of BTA 19 between X82261 at 19.4 cM and BMS2142 at 44.7 cM. The bovine growth hormone gene (*BGH*) is located on the same chromosome at 65.7 cM. Polymorphisms in *BGH* have been associated with variations in the plasma concentrations of GH and insulin-like growth factor I in various cattle breeds (Schlee et al., 1994), although not with growth itself. Known or suspected QTL for growth traits on BTA 19 include one for birth weight and ADG on feed at 67.4–98.4 cM (Taylor et al., 1998), and ones for preweaning ADG at 4.8–15.9 cM and ADG on feed at 52–52.7 cM (Kneeland et al., 2004). It is possible that linkage may exist between *CHRNE* and one of these, or other unknown QTL.

A weight advantage conferred by the carrier state may have resulted in a selective advantage for animals heterozygous for the CMS mutation, which would tend to increase the frequency of the mutant allele in the population. However, with the availability of a screening test for the mutation, many of the carriers still present in the population are likely now to be identified and excluded from breeding, and the prevalence is expected to drop considerably. Alternative strategies for the control of a recessive disease allele such as this, where there is differential fitness between genotypes, have recently been modelled and discussed (Thompson et al., 2006).

## Implications

The *CHRNE* 470del20 mutation causing congenital myasthenic syndrome is segregating in the South African Brahman cattle population at a low frequency, with approximately 1.2% of breeding bulls heterozygous for the allele. The allele frequency did not change markedly between 2000 and 2004. With the possible exception of individual herds, with a relatively high frequency of carrier cows, in which bulls of unknown CMS genotype are used, the mutation is unlikely to have a significant economic impact on the breed. However, there is evidence that carriers have greater 600 d weight, and possibly also greater birth, 200 d, and 400 d weights, which may

confer a selective advantage. Selection on these traits may tend to increase the frequency of the mutation. Nevertheless, continued genotyping of breeding animals should allow breeders to virtually eliminate the disease.

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# Chapter 6

# Changes in disease gene frequency over time with differential genotypic fitness and various control strategies

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# Abstract

A spreadsheet model was constructed to describe the change in allelic frequency over time for a lethal recessive mutation in an animal population. The model allowed for relative fitness to differ between genotypes, between sexes, and over time. Whereas a lethal recessive allele is naturally eliminated very slowly from a population, a small selective disadvantage of the heterozygote results in a large increase in the rate of elimination. With selective advantage of the heterozygote through linkage with a production trait or pleiotropy, the allele is never naturally eliminated, but tends towards a stable equilibrium frequency. The model was used to investigate various alternative control programmes, based on the detection of heterozygotes by genotyping and their exclusion from breeding. The programmes (genotyping males only, genotyping males and 50% of females, and genotyping all breeding animals) were modelled for various initial heterozygote frequencies, and results were described in terms of the number of generations, number of tests, and number of culls required to reduce the heterozygote frequency to a predefined level. The model can be used to compare the feasibility and cost of various control strategies and to illustrate clearly to breeders the expected outcomes, as well as the danger of prematurely terminating a control programme when there is a selective advantage of the heterozygote.

# Introduction

Several hundred single-locus defects have been described in domestic animals, many of which are lethal autosomal recessives (Nicholas, 2005). These conditions may be economically important, and there are several accounts of attempts to eradicate such mutations (Jolly, 1975; Robinson et al., 1993; Hogasen et al., 1997). Control programmes depend largely on the ability to detect heterozygotes, complete removal of which from the breeding population will ensure eradication of the mutant allele. For some mutations, the availability of specific PCR tests (Bilstrom et al., 1998; Dennis et al., 2000; Kraner et al., 2002) enables rapid and accurate detection of carriers. However, in practice it is often not possible to genotype all breeding animals.

In most autosomal recessive conditions, heterozygotes are phenotypically indistinguishable from normal homozygotes, and relative fitness of the two genotypes is assumed to be equal. However, if there is a difference in phenotype of the heterozygote, due either to an effect of the mutant allele itself (pleiotropy) or to close linkage with another gene affecting form or function, selection either for or against the heterozygote may occur, altering its relative fitness and therefore its change in frequency from one generation to the next.

When considering alternatives for the eradication or control of a genetic disease, it is important to be able to predict changes in gene frequency over time. This can be calculated using formulae to account for differences in relative fitness between genotypes and between sexes (Hartl and Clark, 1997; Hedrick, 2000). Alternatively, and more flexibly, it can be iteratively modelled from one generation to the next, allowing fitness also to vary over time and providing graphical depiction of changes. The objective of this paper is to describe the changes in frequency of a lethal autosomal recessive mutation over time in a spreadsheet model and to use it to predict the results of various control strategies.

## Materials and Methods

A single bi-allelic locus, with alleles *A* and *a*, was considered, with *A* representing the wild-type allele and *a* representing the allele carrying the mutation. The frequency of allele *A* was denoted by $p$ and that of allele *a* by $q = 1 - p$; the initial ratio of *AA* : *Aa* : *aa* in the base population at birth was therefore $p^2 : 2pq : q^2$, assuming Hardy-Weinberg equilibrium with no selection, new mutation, or migration. Henceforth, and for all subsequent generations, individuals underwent natural and/or artificial selection and survived to breeding with probabilities defined by the relative fitness of their particular genotype. The relative fitnesses of the *AA*, *Aa*, and *aa* genotypes were denoted by $w_{AA}$, $w_{Aa}$, and $w_{aa}$, respectively ($w_{AAm}$, $w_{Aam}$, and $w_{aam}$ for males, and $w_{AAf}$, $w_{Aaf}$, and $w_{aaf}$ for females). A selective advantage of the heterozygote over the wild-type homozygote existed if $w_{Aa} > w_{AA}$, and a selective disadvantage of the heterozygote existed if $w_{Aa} < w_{AA}$.

The frequency of each genotype at birth was thus multiplied by its relative fitness, producing a ratio *AA* : *Aa* : *aa* amongst adult males of $p^2w_{AAm} : 2pqw_{Aam} : q^2w_{aam}$, and amongst adult females of $p^2w_{AAf} : 2pqw_{Aaf} : q^2w_{aaf}$. Each value (i.e., product of genotypic frequency at birth and relative fitness) was standardised by dividing it by the sum of the values for the three genotypes (also known as the mean fitness value), to obtain breeding adult genotypic frequencies, e.g., the frequency of the *Aa* genotype amongst adult males after selection was $2pqw_{Aam}/(p^2w_{AAm} + 2pqw_{Aam} + q^2w_{aam})$. After selection, mating was assumed to be random. Offspring genotypic and allelic frequencies were then calculated for each subsequent generation, assuming Mendelian segregation.

The model further assumed infinite population size, non-overlapping generations, constant fitness over time for any specified genotype, and that no further mutations occurred in the same gene. For the control strategies, it was assumed that an accurate test existed to distinguish between heterozygotes and normal homozygotes.

The model was constructed using a commercial spreadsheet programme (Excel 2003, Microsoft Corporation, Redmond, WA). Inputs were initial allelic frequencies, relative fitnesses for each genotype/sex combination, numbers of males and females selected

for breeding, and for the control strategies, proportions of males and females genotyped. Outputs for each subsequent generation were genotypic and allelic frequencies at birth and after selection for breeding, and, for the control strategies, the expected number of tests and culls required to obtain the desired number of non-carrier breeding males and females, as well as cumulative totals.

***Base Scenarios***

Models were generated for different scenarios by varying the initial mutant allelic frequency $q$ (0.4, 0.1, and 0.01) and the relative fitness $w$ for each genotype in males and females. The scenarios and fitness ratios ($w_{AA} : w_{Aa} : w_{aa}$) considered were: lethal recessive, 1 : 1 : 0 (Scenario 1), lethal recessive with selective advantage of the heterozygote, 1 : 1.1 : 0 (Scenario 2) and 1 : 1.5 : 0 (Scenario 3), and lethal recessive with selective disadvantage of the heterozygote, 1 : 0.9 : 0 (Scenario 4) and 1 : 0.5 : 0 (Scenario 5). The results of each model were expressed in terms of the time required to reduce $q$ by 50% and by 90%, and the equilibrium frequency reached, if any.

***Control Strategies***

The model was then used to investigate strategies for the eradication of a lethal autosomal recessive mutation in a hypothetical animal population of 15,000 breeding females and 1,200 breeding males. It was assumed that an accurate genotype test was available and that every heterozygous animal tested was excluded (culled) from the breeding population. Thus, in each generation the required number of candidate males and/or females were tested to select the next breeding generation of 15,000 females and 1,200 males. The model was run using various starting values (0.4, 0.1, and 0.01) for the prevalence of heterozygotes in the base generation.

The control strategies were modelled by varying the relative fitnesses of each genotype in males and females. For example, a genotype that was culled was assigned a fitness of zero. The options considered for the eradication of the mutation were as follows:

- ***Genotyping of males only***: All breeding males were certified as non-carriers, but females were not tested, $w_{AAm} : w_{Aam} : w_{aam} = 1 : 0 : 0$ and $w_{AAf} : w_{Aaf} : w_{aaf} = 1 : 1 : 0$ (Strategy F).

- ***Genotyping of males and partial genotyping of females***: All breeding males but only half of the breeding females were certified as non-carriers, $w_{AAm} : w_{Aam} : w_{aam} = 1 : 0 : 0$ and $w_{AAf} : w_{Aaf} : w_{aaf} = 1 : 0.5 : 0$ (Strategy G).

- ***Genotyping of all breeding animals***: Breeding males and females were all certified to be non-carriers, $w_{AAm} : w_{Aam} : w_{aam} = w_{AAf} : w_{Aaf} : w_{aaf} = 1 : 0 : 0$ (Strategy H).

In addition, the scenario was considered of a lethal recessive with selective advantage of the heterozygote, $w_{AA} : w_{Aa} : w_{aa} = 1 : 1.5 : 0$, in which genotyping of breeding males only was performed (Strategy I). In this case, the effect of terminating the control programme after five generations was also investigated (Strategy J).

Two alternative endpoints for each strategy were used:

- Attainment of an arbitrary low prevalence (≤0.1%) of heterozygotes in the population.

- "Eradication", defined as being 95% certain that no carriers will be present amongst the 16,200 animals selected for breeding. This would be the case when the prevalence of carriers amongst animals available for selection is less than 0.0003%. This value was obtained using the binomial distribution:

$$P(0\ ,\ 16{,}200\ ,\ 0.000003) = \binom{16{,}200}{0} \times 0.000003^{0} \times (1 - 0.000003)^{16{,}200\ -\ 0} \approx 0.95$$

For each control strategy, the number of generations, cumulative number of tests, and cumulative number of culls required to achieve the above endpoints were calculated.

# Results

The number of generations required to reduce the frequency of the mutant allele by 50% and 90%, and the equilibrium frequency reached, if any, are shown in Table 1.

**Table 1.** Changes in allelic frequency over time with differing genotypic fitnesses and initial allelic frequencies

| Type | Relative fitnesses *AA* : *Aa* : *aa* | Initial frequency *q* of allele *a* | Generations required to reduce frequency by: | | Equilibrium frequency $\hat{q}$ of allele *a* |
|---|---|---|---|---|---|
| | | | 50% | 90% | |
| Lethal recessive | 1 : 1 : 0 (Scenario A) | 0.1 | 10 | 90 | 0 |
| | | 0.01 | 100 | 900 | 0 |
| Lethal recessive with selective advantage of heterozygote | 1 : 1.1 : 0 (Scenario B) | 0.4 | 4 | – [1] | 0.083 |
| | | 0.1 | – | – | 0.083 |
| | | 0.01 | – | – | 0.083 |
| | 1 : 1.5 : 0 (Scenario C) | 0.4 | – | – | 0.25 |
| | | 0.1 | – | – | 0.25 |
| | | 0.01 | – | – | 0.25 |
| Lethal recessive with selective disadvantage of heterozygote | 1 : 0.9 : 0 (Scenario D) | 0.1 | 5 | 18 | 0 |
| | | 0.01 | 7 | 22 | 0 |
| | 1 : 0.5 : 0 (Scenario E) | 0.1 | 1 | 4 | 0 |
| | | 0.01 | 1 | 4 | 0 |

[1] Not possible because equilibrium frequency is reached

The changes in allelic frequency over time are shown in Figure 1. When the heterozygote has no selective disadvantage compared to the wild-type homozygote (A), a lethal recessive allele is eliminated very slowly from a population. With selective advantage of the heterozygote (B and C), the frequency of *a* reaches a stable equilibrium, which is independent of the initial allelic frequency, but dependent on the relative fitness of the three genotypes. In such cases, the recessive allele will never be eliminated naturally from a population, but will persist at an expected equilibrium frequency $\hat{q} = (w_{Aa} - w_{AA}) / (2w_{Aa} - w_{aa} - w_{AA})$ (Hartl and Clark, 1997). In contrast, a small selective disadvantage of the heterozygote (e.g., D: $w_{Aa} = 0.9$) results in a large increase in the rate at which the allelic frequency is reduced. If the relative fitness of the heterozygote is further reduced (e.g., E: $w_{Aa} = 0.5$), the allelic frequency declines very rapidly, irrespective of the initial frequency.

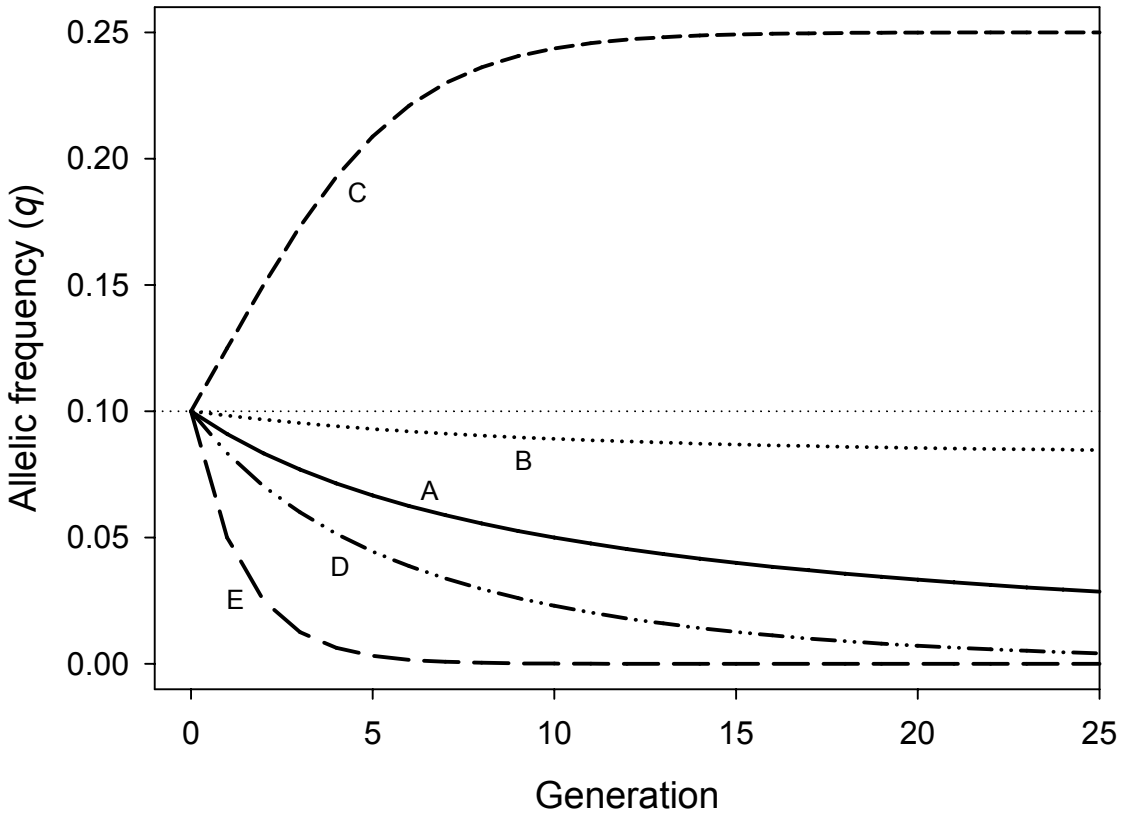


**Figure 1.** Changes in recessive allelic frequency over 25 generations, with different relative genotypic fitnesses. Relative genotypic fitnesses ($w_{AA}$ : $w_{Aa}$ : $w_{aa}$) are as follows: A = 1 : 1 : 0; B = 1 : 1.1 : 0; C = 1 : 1.5 : 0; D = 1 : 0.9 : 0; E = 1 : 0.5 : 0.

Tables 2 and 3 show the number of generations that would be required to reduce the prevalence of heterozygotes in our hypothetical population to below 0.1% and 0.0003% respectively, using various test and cull strategies, and for different initial heterozygote prevalences. For each strategy, the expected cumulative numbers of animals required to be genotyped and culled over the duration of the programme are also shown. For example, at an initial carrier prevalence of 10%, testing all males and culling all carriers (Strategy F) will reduce the carrier prevalence to <0.1% over seven generations, requiring 8,654 tests and the culling of 254 carrier males. Testing and culling females rather than males will achieve the same rate of reduction, but with a more than ten-fold increase in the number of tests and culls required (results not shown). Testing all males with partial (50%) implementation of testing of breeding females (Strategy G) will greatly reduce the time required to achieve the same objective, resulting in fewer males (176) being culled, but requires the testing and culling of a large number of females. If the carrier has a selective advantage, eradication is a far longer and more costly process (Strategy I). However, if all breeding animals are tested, the selective advantage is of no consequence, and it becomes equivalent to Strategy H, in which the mutant allele is eliminated from the population within one generation.

Figure 2 illustrates the expected changes in heterozygote prevalence over the first ten generations for each strategy, compared with no control programme (N), starting at a carrier frequency of 10%, with no heterozygote advantage (N, F, G, and H) and with selective advantage of the heterozygote (I). The mutant allele is completely eradicated from the population only in eradication strategy H (i.e., ensuring that no carriers are used for breeding). In all other strategies the carrier frequency is merely reduced. To illustrate the latter point, the effect of discontinuing a control programme when a heterozygote selective advantage exists is shown in Figure 2 (line J). In such a situation, the frequency of the mutant allele begins to increase again towards the equilibrium frequency $\hat{q}$.

**Table 2.** Characteristics of alternative eradication strategies to reduce carrier prevalence to below 0.1% for a lethal recessive mutation in a population of 15,000 females and 1,200 males, for different relative genotypic fitnesses ($w_{AA}$ : $w_{Aa}$ : $w_{aa}$) and initial carrier prevalences ($2pq$)

| Relative fitness *AA* : *Aa* : *aa* | Strategy | Initial carrier prevalence (%) | Required to reduce carrier prevalence to below 0.1% | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Generations | Tests | | | Culls | | |
| | | | | male | female | total | male | female | total |
| 1 : 1 : 0 | F Test and cull males | 40 | 9 | 12,114 | 0 | 12,114 | 1,314 | 0 | 1,314 |
| | | 10 | 7 | 8,654 | 0 | 8,654 | 254 | 0 | 254 |
| | | 1 | 4 | 4,823 | 0 | 4,823 | 23 | 0 | 23 |
| | G Test and cull males and 50% of females | 40 | 5 | 7,014 | 43,835 | 50,849 | 1,014 | 6,335 | 7,349 |
| | | 10 | 4 | 4,976 | 31,097 | 36,073 | 176 | 1,097 | 1,273 |
| | | 1 | 2 | 2,416 | 15,095 | 17,511 | 16 | 95 | 111 |
| | H Test and cull all males and females | 40 | 1 | 2,000 | 25,000 | 27,000 | 800 | 10,000 | 10,800 |
| | | 10 | 1 | 1,334 | 16,667 | 18,000 | 134 | 1,667 | 1,800 |
| | | 1 | 1 | 1,213 | 15,152 | 16,365 | 13 | 152 | 165 |
| 1 : 1.5 : 0 | I Test and cull males | 40 | 19 | 24,681 | 0 | 24,681 | 1,881 | 0 | 1,881 |
| | | 10 | 16 | 19,660 | 0 | 19,660 | 460 | 0 | 460 |
| | | 1 | 8 | 9,644 | 0 | 9,644 | 44 | 0 | 44 |

**Table 3.** Characteristics of alternative eradication strategies to reduce carrier prevalence to below 0.0003% for a lethal recessive mutation in a population of 15,000 females and 1,200 males, for different relative genotypic fitnesses ($w_{AA}$ : $w_{Aa}$ : $w_{aa}$) and initial carrier prevalences ($2pq$)

| Relative fitness *AA* : *Aa* : *aa* | Strategy | Initial carrier prevalence (%) | Required to reduce carrier prevalence to below 0.0003% | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Generations | Tests | | | Culls | | |
| | | | | male | female | total | male | female | total |
| 1 : 1 : 0 | F Test and cull males | 40 | 17 | 21,716 | 0 | 21,716 | 1,316 | 0 | 1,316 |
| | | 10 | 15 | 18,256 | 0 | 18,256 | 256 | 0 | 256 |
| | | 1 | 12 | 14,425 | 0 | 14,425 | 25 | 0 | 25 |
| | G Test and cull males and 50% of females | 40 | 9 | 11,815 | 73,840 | 85,655 | 1,015 | 6,340 | 7,355 |
| | | 10 | 8 | 9,777 | 61,101 | 70,878 | 177 | 1,101 | 1,278 |
| | | 1 | 6 | 7,217 | 45,101 | 52,318 | 17 | 101 | 118 |
| | H Test and cull all males and females | 40 | 1 | 2,000 | 25,000 | 27,000 | 800 | 10,000 | 10,800 |
| | | 10 | 1 | 1,334 | 16,667 | 18,000 | 134 | 1,667 | 1,800 |
| | | 1 | 1 | 1,213 | 15,152 | 16,365 | 13 | 152 | 165 |
| 1 : 1.5 : 0 | I Test and cull males | 40 | 39 | 48,685 | 0 | 48,685 | 1,885 | 0 | 1,885 |
| | | 10 | 36 | 43,664 | 0 | 43,664 | 464 | 0 | 464 |
| | | 1 | 28 | 33,648 | 0 | 33,648 | 48 | 0 | 48 |

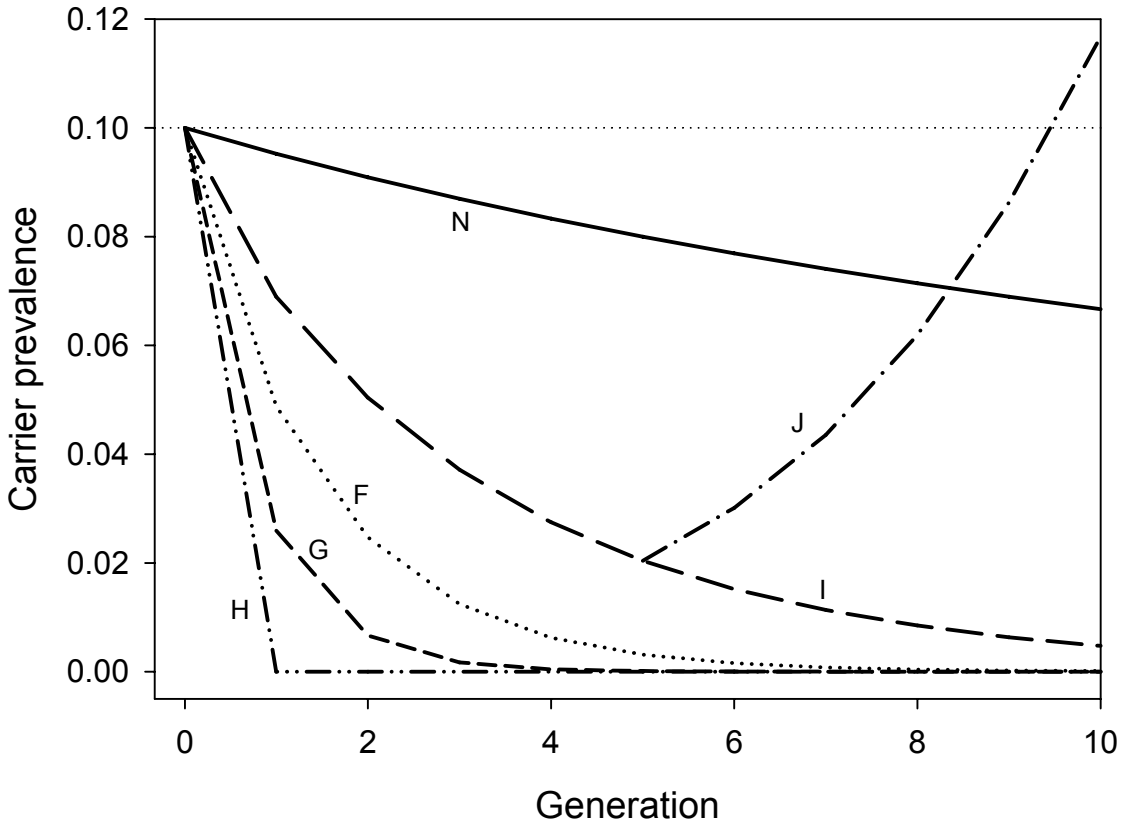


**Figure 2**. Changes in prevalence of carriers over ten generations using various strategies for the eradication of a lethal recessive allele. N = no control programme; F = testing males only and culling carriers; G = testing males and 50% of females and culling carriers; H = testing all breeding animals and culling carriers; I = testing of males only and culling carriers (selective advantage of heterozygote); J = as for I, but testing and culling ceases after five generations. Relative genotypic fitnesses ($w_{AA}$ : $w_{Aa}$ : $w_{aa}$) are 1 : 1 : 0 for N, F, G, and H, and 1 : 1.5 : 0 for I and J.

## Discussion

Our results show that the outcome of a programme to eradicate a lethal recessive from a population depends greatly on the fitness value of the heterozygote (Tables 2 and 3). This is in line with results in the literature (Lefort and Lauvergne, 1974; Hoeschele and Meinert, 1990).

***Differential Genotypic Fitness***

A selective advantage or disadvantage of the heterozygote over the wild-type homozygote can also be quantitatively expressed as $w_{Aa} - w_{AA} = s$, the selection coefficient (Hedrick, 2000). In this model, however, only the relative fitness values of the three genotypes were specified. This implies the implicit assumption that the average fitness value was sufficiently large to maintain a stable population size.

Examples of lethal recessive conditions in cattle where there is no apparent heterozygote advantage include $\alpha$-mannosidosis in Angus and Murray Greys in Australia and New Zealand (Jolly et al., 1974), generalised glycogenosis in Brahmans (Dennis et al., 2000), and myophosphorylase deficiency in Charolais (Bilstrom et al., 1998). In humans, the majority of inherited defects, and particularly autosomal recessive defects, are expressed early in life, before puberty (Costa et al., 1985), although there are several autosomal recessive disorders, e.g., some forms of parkinsonism (Lincoln et al., 2003), with a much later age of onset. The same is likely also to be true in animals, where a mutation causing a visible disease or defect, even if not lethal, will usually result in the animal not being selected for breeding. Thus, the fitness of the disease genotype will effectively be zero.

In some recessive disease conditions, the heterozygote has a selective advantage over the homozygous wild-type animal due to a pleiotropic effect or close linkage with genes affecting a trait under selection. Selection on that trait will therefore increase the fitness of the heterozygote relative to that of the homozygous wild-type animal. For example, Brown Swiss cows carrying the weaver gene have a greater milk yield (Hoeschele and Meinert, 1990), Brahman cattle carrying the *CHRNE* 470del20 mutation causing congenital myasthenic syndrome have increased 600 d weight (P. N. Thompson, unpublished data), and carriers of the halothane gene causing malignant hyperthermia in pigs show better feed efficiency, and greater carcass yield and carcass lean content (Leach et al., 1996). Selective advantage of the heterozygote may also be due to a pleiotropic effect of the recessive allele itself. In humans, carriers of sickle-cell anaemia (Aidoo et al., 2002) and cystic fibrosis (Gabriel et al., 1994; Schroeder et al., 1995) may have a selective advantage due to increased resistance to other diseases.

Selective disadvantage of the heterozygote may also occur. In chondrodysplasia of Dexter cattle, the heterozygote is easily recognised by shortness of the limbs, whereas the homozygote shows lethal chondrodysplasia (Harper et al., 1998). In these and other conditions in which the heterozygote is phenotypically different from the wild-type homozygote, the relative fitness of the heterozygote will depend on breeders' preferences for phenotypes and their awareness of the genetic defect. Selective disadvantage of the heterozygote may also occur due to close linkage with genes affecting production traits, or due to pleiotropy. An example of the latter is the finding that fresh meat quality is poorer in carriers of the halothane gene in pigs (Moelich et al., 2003). Carriers of the mutation responsible for bovine factor XI deficiency show a partial factor XI deficiency, which may negatively affect survival or productivity (Brush et al., 1987). In chondrodysplasia of Hereford and Angus cattle, heterozygotes show skeletal abnormalities, but to a much lesser degree than those seen in homozygous affected calves (Emmerson and Hazel, 1956). In the case of bovine leucocyte adhesion deficiency, a significant negative association of the mutant allele with milk protein yield has been reported (Powell et al., 1996); however, is not clear whether this is due to pleiotropy or close linkage. Another mechanism by which heterozygotes may be at a selective disadvantage is if the homozygous recessive genotype results in embryonic or foetal loss, resulting in the breeder selecting against the heterozygous dam. This occurs in inherited deficiency of uridine monophosphate synthase in Holstein cattle, where homozygous recessive embryos are lost at about day 40 of gestation (Shanks and Robinson, 1989). In general, recessive diseases with selective disadvantage of the heterozygote are likely to be less common than those with selective advantage of the heterozygote, because, as shown in the model, such mutations will decline in frequency relatively rapidly. A small decrease in performance will also be amplified by selection on that trait, resulting in a bigger decrease in relative fitness and a more rapid decline in frequency of the recessive allele. This also means that it is difficult for such a mutation to reach a high frequency in the population after initially occurring in a single animal. An exception would be if the mutation was present in an influential sire, in which case the allelic frequency would initially reach 0.25 amongst its progeny.

***Elimination of Recessive Alleles***

It is well known that a recessive lethal gene, with no heterozygote advantage or disadvantage, will naturally be eliminated very slowly from a population, because most copies of the recessive allele occur in heterozygotes. The frequency ($q_t$) of allele *a* after *t* generations can be calculated as $q_t = q_0 / (1 - tq_0)$ (Hedrick, 2000); by rearrangement, the time required to reduce the allelic frequency from $q_0$ to $q_t$ can be calculated as $t = (1 / q_t) - (1 / q_0)$. However, this is valid only when there is no heterozygote advantage. Various other equations can be used to calculate $q_t$, depending on whether the mutant allele is recessive, additive, or dominant (Hedrick, 2000). Our simulation model enabled studying the dynamics of the mutant allele using any desired combination of relative fitnesses, including differences in relative fitness between sexes and variation in relative fitnesses over time.

To prevent the occurrence of homozygote recessives, it is sufficient to test all parents from one sex, to ensure that no carriers are used for breeding. The model shows that the effect of genotyping all females only is the same as that of genotyping all males only, except that it requires more testing and culling. It therefore makes economic sense to test at least all parents of the sex with the smallest number of individuals (i.e., sires in most cases). However, if one wants to be sure of eradicating the mutant allele, rather than just preventing the occurrence of disease, then it is clear that both sexes need to be genotyped (Tables 2 and 3). This is particularly important when carriers have a selective advantage over homozygous wild type animals, because eradication is slower and a break in the testing programme may result in the frequency of the mutant allele increasing again. Partial testing of females, in addition to testing of males, increases the rate of elimination of the mutation. Although not considered in this model, it may be more cost-effective to target this testing to include only those females used in the production of future breeding males.

With a selective advantage of the heterozygote, the eventual equilibrium frequency $\hat{q}$ of the mutant allele can be predicted using $\hat{q} = (w_{Aa} - w_{AA}) / (2w_{Aa} - w_{aa} - w_{AA})$ (Hartl and Clark, 1997). As expected, our simulation model yields the same results for $\hat{q}$, with the advantage that it permits the relative fitnesses to differ between sexes. This

may be the case in sex-limited traits, and when there is a difference in the way the two sexes are tested and culled, as in strategies F, G, and I.

Another advantage of the simulation model described here is that estimates can be made of the costs of implementing a control programme on a generation by generation basis, and alternative control strategies can easily be evaluated. An example of a cost-benefit analysis was demonstrated by Jolly and Townsley (1980) for the bovine mannosidosis control programme. The cost of obtaining genotype information can be substantially reduced by using segregation analysis to calculate genotype probabilities and genotype probability indices for untested individuals, based on known genotypes of relatives (Kerr and Kinghorn, 1996). This, together with pedigree information, can then be used to select for further testing those individuals with the poorest genotype information, and which would contribute the most genotype information to the rest of the population (Kinghorn, 1999).

In our model we used non-overlapping generations, but it also provides good insight for situations with overlapping generations. Eradication programmes focus on identifying and eliminating carriers before animals have reached their reproductive age. The number of years for which individual animals are used for breeding (i.e., whether or not generations overlap), will not affect the change in gene frequency over generations (Moran, 1958; Jansen et al., 1984), but it will affect the translation from generations into time (i.e., years). It is possible to extend the model to account for overlapping generations, but this will not substantially change the conclusions.

An important part of the planning of a control programme is clear communication and presentation of the anticipated outcomes to the breeders, breed society, or other interested parties. Using a simulation model, projected changes in carrier and allelic frequencies over time can easily be demonstrated and compared between alternative control programmes. The potential danger of premature termination of a programme can also be demonstrated.

It is important to monitor the level of inbreeding when eliminating carriers of a mutant allele, particularly when the initial carrier frequency is high, when the population is small, and when the wild-type allele comes from a limited number of ancestral families (Sonesson et al., 2003). Elimination of carriers may lead to an undesired increase in

inbreeding, which, in turn, may lead to an undesired increase in the frequency of other genetic defects. An alternative to culling a carrier male would be to use it to produce a group of offspring and then to genotype and cull those that are carrying the mutant allele. Although more expensive, this would reduce the loss of genetic diversity.

## Implications

Recessive mutations causing disease are naturally eliminated very slowly from animal populations, because most copies of the mutant allele are hidden in apparently normal carriers. However, sometimes their performance may differ from that of non-carriers, resulting in either a selective disadvantage, with far more rapid elimination of the mutation, or a selective advantage, where the mutation is never eliminated, but reaches a stable equilibrium frequency. Our model can be used to predict the cost and time required to eradicate a recessive mutation under these conditions, using various strategies. To prevent disease due to homozygote recessives, it is sufficient to test all members of one sex (usually males) and to avoid breeding with carriers. But to be sure of eliminating the mutation from the population, it is necessary to test all breeding animals. If carriers have a selective advantage, termination of testing is expected to result in an increase in the frequency of the mutant allele.

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# Chapter 7

# General discussion

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# Introduction

This thesis describes the first clinical cases of congenital myasthenic syndrome (CMS) in cattle, the identification of the underlying mutation, and the investigation of its prevalence and potential impact in the South African Brahman cattle population. So far, this is the only naturally occurring condition known in cattle that involves an abnormality in the structure or function of the acetylcholine receptor (AChR). In this chapter, the most important findings of the preceding chapters are summarised and discussed, the impact of the CMS mutation in the South African Brahman population is examined in more detail, certain recommendations for the control of the condition are given, and gaps in our knowledge of the condition are highlighted for possible future research.

# Diagnosis of CMS in Brahman cattle

The diagnostic approach to the first calf presented was described in detail in Chapter 2. The most important clinical sign was the presence of muscle weakness (myasthenia) that was exacerbated by exercise but improved with rest, suggesting a defect in neuromuscular transmission. Other diseases in cattle in which impulse transmission at the somatic neuromuscular junction is impaired include $Ca^{2+}$ deficiency (milk fever), botulism and tick paralysis, in which release of acetylcholine (ACh) from the axon terminal is inhibited (Radostits et al., 2000). In addition, there are several pharmacological compounds, such as tubocurarine, succinylcholine and amino-glycoside antibiotics, that cause either competitive or non-competitive AChR block (Adams, 1982). After further investigation of the myasthenic calf, the most notable finding was the unequivocally positive response to edrophonium chloride (Tensilon) administration. Although, in humans, this is often regarded as diagnostic for myasthenia gravis, it may also be a finding in CMS, and occasionally in botulism, Lambert-Eaton myasthenic syndrome, Guillain-Barré syndrome, amyotrophic lateral sclerosis and drug-induced myasthenic syndromes (Oh and Cho, 1990). Myasthenia gravis is probably the best understood of all human auto-immune diseases (Sieb, 2005), and is also known to occur in dogs (Dewey et al., 1997) and cats (Joseph et al., 1988), but has never been described in cattle.

Repetitive peripheral nerve stimulation was performed on the calf, as an adjunct diagnostic tool, in order to detect neuromuscular transmission impairment. However, due to insufficient information available at the time, nerve stimulation was performed at a higher frequency (27 Hz) than is recommended. Nerve stimulation at low frequency (3 Hz) is the most effective at detecting neuromuscular transmission block in dogs (Waxenberger et al., 1992). Higher frequency stimulation may, in fact, cause a decremental response in the amplitude of successive compound muscle action potentials in normal dogs. In a comparison between distal (plantar and palmar interosseous) and proximal (cranial tibial) muscles in healthy dogs, Malik et al. (1989) found a progressive decremental response at high frequency (20 Hz) stimulation in distal muscles but a slight incremental response in proximal muscles. They suggested that the interosseous muscles may have a greater proportion of low efficiency synapses and that, if high frequency stimulation is performed, proximal limb muscles should be used. In our calf, a proximal limb muscle (the peroneus tertius) was used. In addition, the decremental response we observed was not progressive, as described with high frequency nerve stimulation in normal dogs, but more characteristic of the decrement seen in dogs and cats with congenital or acquired myasthenia gravis (Cuddon, 2002), i.e. an initial decrease in amplitude, whereafter it remains stable. However, the response to repetitive nerve stimulation in healthy cattle is unknown, therefore the clinical significance of our finding is difficult to assess. It did, however, provide supporting evidence to rule out botulism, which typically produces an incremental response at high frequency stimulation (Oh, 1977). Although the use of repetitive peripheral nerve stimulation is not absolutely necessary in order to make a diagnosis of CMS, if it is used in future cases it should preferably be done at low frequency.

Ultimately, however, it was the familial clustering of cases, consistent with a recessive mode of inheritance, which, together with the clinical signs and response to anticholinesterase treatment, resulted in a presumptive diagnosis of postsynaptic "congenital myasthenia gravis". This terminology, used in Chapter 2, was consistent with that in some of the veterinary literature describing analogous conditions in dogs and cats, e.g. Indrieri et al. (1983), and Miller et al. (1983). However, the term "myasthenia gravis" is perhaps misleading in that it implies an autoimmune pathophysiology. Therefore the term "congenital myasthenic syndrome" was used thereafter, consistent with the human medical literature and other, more recent, veterinary literature, e.g. Flagstad et al. (1989).

## Identification of the *CHRNE* 470del20 mutation

When a presumptive diagnosis of a new inherited disease has been made, one can search for descriptions of similar inherited diseases, with known molecular or genetic basis, in the same or different species. This can be done by consulting online databases such as Online Mendelian Inheritance in Man (OMIM, 2006), and its equivalent in animals, Online Mendelian Inheritance in Animals (Nicholas, 2006). In this way, a mutant gene known to cause a similar disease in another species may become a candidate gene to be investigated for involvement in the new inherited disease. If a description of a similar disease cannot be found, understanding of the putative underlying pathophysiological mechanisms may suggest the involvement of one or more specific genes. Cloning and sequencing of these genes may then lead to the discovery of the causative mutation in affected animals. This is the so-called "candidate gene" approach to disease gene identification (Fyfe, 2002), and is the approach of choice when one, or a small number of genes can be strongly implicated. Several inherited diseases of cattle have been characterised at the DNA level in this way, e.g. protoporphyria in Limousin and Blonde d'Aquitaine cattle (Jenkins et al., 1998), generalised glycogenosis in Brahmans (Dennis et al., 2000), and factor XI deficiency in Holsteins (Marron et al., 2004). Another example of the use of the candidate gene approach is in the identification of genes involved in resistance to bovine dermatophilosis (Maillard et al., 2002).

However, there are many conditions for which an analogous disease in another species is not known, or for which a candidate gene cannot be identified. In these cases the approach, although more resource-intensive, will be via a genome-wide scan for linked polymorphic DNA markers, followed by a positional cloning or "positional-candidate" approach (Beaudet et al., 1995). In this approach, the location of the disease gene is first determined as precisely as possible by linkage analysis, in a study comparing marker genotypes between affected and unaffected members within families. The mapped location on the chromosome is then used to clone the gene. For rare diseases, however, it may be difficult to obtain sufficient numbers of affected individuals and/or families. Although the genotyping of relatively large numbers of animals is expensive and time-consuming, the costs of genotyping are steadily decreasing, and this approach will become more feasible in the future. Some examples of the use of positional

cloning in cattle are the identification of the causative mutations for double muscling in Belgian Blue cattle (Grobet et al., 1997), and for chondrodysplastic dwarfism in Japanese brown cattle (Takeda et al., 2002). Positional cloning has also successfully been used to identify and sequence quantitative trait loci (QTL) in cattle (Grisart et al., 2002). For QTL mapping, the number of potential candidate genes is often very large, making the candidate gene approach uncertain and impractical.

In the case of this suspected CMS in Brahman calves, the existence of an analogous group of conditions in humans enabled us to pursue the candidate gene approach. Because the majority of CMS in humans are caused by mutations in the gene coding for the AChR ε-subunit (*CHRNE*), this was the candidate gene selected for further investigation. The fact that the cDNA sequence for the bovine AChR ε-subunit had previously been published (Takai et al., 1985), facilitated our determination of the genomic structure of *CHRNE*, and subsequent identification of the 470del20 mutation in exon 5 of the gene, as described in Chapter 3.

## Clinical manifestations of CMS in Brahman cattle and humans

The clinical signs of CMS in humans vary widely. Although most present during the first year of life, others may only be detected much later (Engel et al., 1996; Beeson et al., 1997; Zafeiriou et al., 2004). The clinical spectrum ranges from mild muscle weakness presenting late in life, to severe disability with life-threatening episodes (Zafeiriou et al., 2004). The onset of weakness in cases of CMS observed so far in Brahman calves varies from birth to four weeks of age, with subsequent deterioration in muscle strength over several weeks or months. This is comparable with the early-onset, severe forms of CMS reported in humans, but more severe than in most human patients with *CHRNE* truncating mutations.

The 470del20 mutation in *CHRNE* is predicted to result in the production of a presumably functionless polypeptide of 169 amino acids, in place of the normal AChR ε-subunit. However, mutations in the genes encoding the various AChR subunits may not necessarily be null mutations leading to the absence of the subunit, but may instead result in a kinetic abnormality of the AChR, either increasing or decreasing the

synaptic response to ACh (Engel and Sine, 2005). The exact position and nature of the mutation are therefore factors influencing the eventual clinical manifestation in the affected individual. However, there is also great variation in disease severity between individuals with the same mutation, presumably due to differences in compensatory mechanisms (Burke et al., 2004). The major compensatory mechanism, and the reason why the condition is not immediately postnatally lethal in patients with *CHRNE* null mutations, is likely to be the persistent expression of $\gamma$-AChR, providing phenotypic rescue (Engel et al., 1996; Croxen et al., 2001). Individuals with mutations in other AChR subunits may not survive, due to lack of a substituting subunit. Other compensatory mechanisms, if present, are unknown. There also appear to be species differences in compensatory capacity. Whereas mice with $\varepsilon$-subunit null mutations are severely affected (Witzemann et al., 1996), humans with similar mutations may be only mildly affected (Ohno et al., 1998; Engel et al., 1999; Middleton et al., 1999). The fact that calves homozygous for the *CHRNE* 470del20 mutation appear to show a more severe phenotype than is seen in most human CMS, may be due to more rapid postnatal down-regulation of *CHRNG* expression in cattle compared to humans. Postnatal expression of the gene coding for the AChR $\gamma$-subunit (*CHRNG*) should be studied in normal and affected cattle in order to determine whether this is the case, or whether there may be other compensatory mechanisms also playing a role.

The use of animal models such as this may therefore contribute to our understanding of human CMS, and a better understanding of the disease in cattle is desirable. However, due to the low number of cases of CMS seen thus far in Brahmans, several aspects of the disease in cattle remain uncertain, including the full range of its clinical manifestations, the electrophysiological characteristics, and the ultrastructural morphology of the neuromuscular junction. It was originally intended, as part of this study, to obtain further homozygous recessive calves in order to investigate the above aspects, as well as to investigate *CHRNE* and *CHRNG* expression in normal and affected calves. The condition was publicised by means of presentations at meetings of the South African Veterinary Association and of the Brahman Cattle Breeders' Society of South Africa (BCBSSA), direct communication with farmers and veterinarians, and articles in the BCBSSA journal and rural veterinary newsletter. As genotyping results became available, owners of known carrier animals were contacted in order to detect as early as possible the occurrence of any carrier-carrier matings, and then to genotype the calf produced. However, genotyping proceeded slower than anticipated, and the

mutation was found to be present at a low frequency in the population. Although the existence of several affected calves was determined, we did not succeed in obtaining further cases for investigation over a period of two years. For ethical reasons, the alternative of deliberate breeding between carriers in order to obtain homozygous recessive calves was initially rejected. However, in order to obtain a better understanding of CMS in Brahmans, this decision would have to be reconsidered. It would be easy, particularly using embryo transfer technology, to produce small numbers of homozygous recessive calves for further clinical and gene expression studies.

## Prevalence and origin of the *CHRNE* 470del20 mutation

Recessively inherited diseases are generally rare, because the reduced fitness of the recessive homozygote tends to slowly remove the mutant allele from the population. This was discussed in Chapter 6 for the case of the lethal recessive allele. However, some recessive diseases of cattle have, in certain populations, been found to be relatively common or to have increased over time, for example the weaver syndrome in Brown Swiss cattle (Hoeschele and Meinert, 1990) and bovine leukocyte adhesion deficiency (BLAD) in Holsteins (Shuster et al., 1992). The relatively high frequency of such mutations can be explained either by carriers having a selective advantage over non-carriers, or simply by the widespread use of one or more sires that coincidentally are carriers. The effect of a selective advantage of the heterozygote on the change in frequency over time, and on the results of various eradication strategies, were discussed in Chapter 6.

It is possible for a lethal recessive defect to occur in a livestock population at a fairly high prevalence and yet to escape detection. This may be due to several reasons (Healy and Dennis, 1993):

1. Neonatal mortality is fairly common, particularly in extensively reared populations, and diagnostic investigations are seldom done for isolated cases.
2. Few veterinary diagnostic laboratories possess the expertise for detailed investigation of a potentially vast range of genetic disorders.

3. Selection of animals is often based on performance data from adult progeny, and differences in neonatal mortality rates between offspring of sires may not necessarily be detected.

Many South African Brahman herds are kept on large farms, under extensive conditions, where predators are not uncommon. Some degree of perinatal calf mortality is expected to occur in beef cattle, even in well managed herds, and can be due to a variety of factors (Toombs et al., 1994). Occasional calf mortalities are therefore unlikely to raise suspicion of an inherited disease, unless it is noticed that a cluster of related calves are affected. In addition, the so-called "dummy calf" syndrome, first described in American Brahmans in the southern U.S.A. (Kim et al., 1988) is often encountered amongst South African Brahmans (P. N. Thompson, unpublished data), making it very likely that calves affected with CMS would be misdiagnosed as "dummy calves".

For the above reasons, a recessive mutation such as 470del20 that results in calf weakness and mortality has the potential to be present, even at relatively high levels, without being recognised, particularly in a beef cattle population. As described in Chapter 6, if heterozygotes have a selective advantage, the mutant allelic frequency may steadily increase to high levels before its presence is detected. In order to prevent this from occurring for other, as yet undescribed, recessive mutations, it is important that breeders keep good records of calf mortalities and their suspected causes. Thorough investigation of calf mortalities should be undertaken as far as possible (Wikse et al., 1994), with particular attention being paid to possible familial clustering.

Investigation into the prevalence of the CMS mutation in the South African Brahman population was therefore warranted, and was reported in Chapter 5. The mutant allelic frequency was nevertheless found to be low. It was estimated that, of the 1,069 bulls used for breeding in 2004, between 7 and 23 (0.7 to 2.2%) were carriers. The results presented in Chapter 5, Table 1, along with additional results for 1994 to 1999, are shown graphically in Figure 1. Also shown is the proportion of bulls used for breeding in each year, that were genotyped. Because the proportion tested prior to 1998 was very small, the estimate of the carrier prevalence was very uncertain. There was, therefore, insufficient evidence to conclude that there had been a decline in the prevalence of carriers in the breeding bull population since 1994.

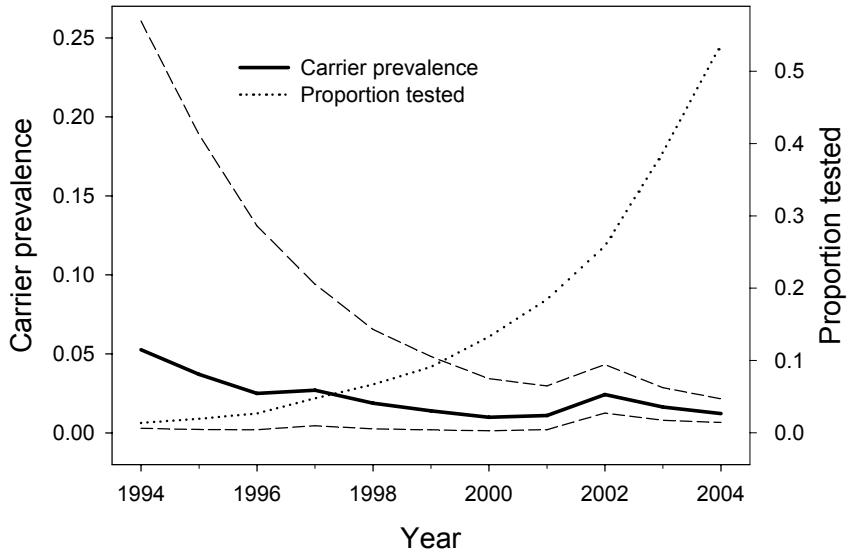


**Figure 1.** The prevalence of *CHRNE* 470del20 carriers amongst breeding South African Brahman bulls (solid line). The dashed lines show the 95% hypergeometric confidence limits for the prevalence. The dotted line shows the proportion of breeding bulls tested (right axis).

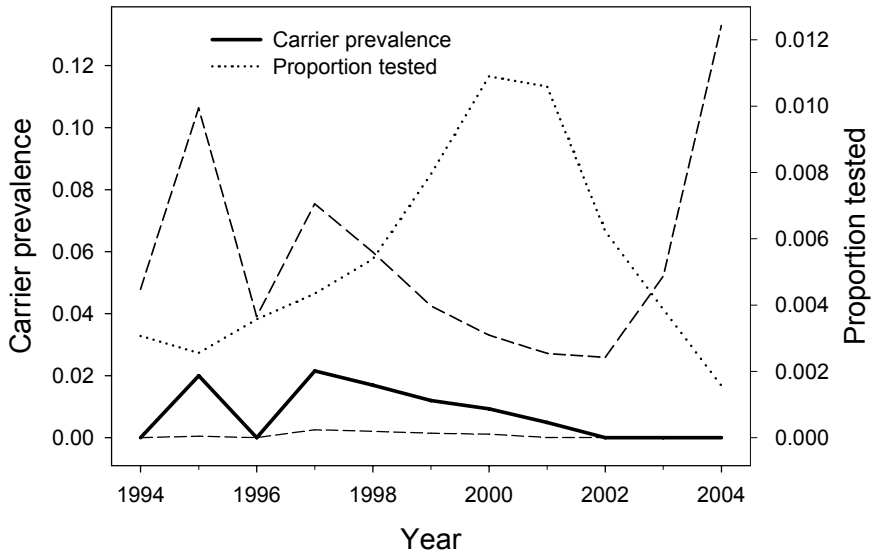


**Figure 2.** The prevalence of *CHRNE* 470del20 carriers amongst South African Brahman calves born (solid line). The dashed lines show the 95% hypergeometric confidence limits for the prevalence. The dotted line shows the proportion of calves tested (right axis).

Similarly, the results for calves born from 1994 to 2004 are shown in Figure 2. It was not possible to conclude that the carrier prevalence amongst calves born had decreased over this period. Very few animals born in 2003 and 2004 were tested, but the results for 2001 and 2002 calves show that the prevalence of carriers born in those years was probably less than 2.5–3%.

As discussed in Chapter 5, there were two potential sources of bias in the estimates of carrier prevalence amongst breeding bulls and amongst calves born during each year:

1. Genotyping was not random, but owners voluntarily submitted samples. For various reasons, some of which may have been related to the presence of the mutation, certain owners may have been either more or less likely to have had their animals tested.
2. Prevalence for each year was not measured during that year, but was based only on animals (or their semen) surviving at least until 2003, when testing was instituted. A selective advantage of carriers may therefore have resulted in slight overestimation of carrier prevalences prior to 2003.

It is, however, difficult to determine whether, and to what extent, these two factors may have affected the prevalence estimates.

Based on analysis of the pedigree, which included identification of common ancestors and calculation of genotype probabilities using segregation analysis, it was concluded in Chapter 5 that the 470del20 mutation was introduced at least twice into the South African Brahman population by the importation of animals or semen, probably from the American Brahman population. Two American Brahman bulls, born in the 1940's, were identified as the most likely founder carriers in the pedigree. However, their calculated probabilities of being heterozygous were fairly low (0.22 and 0.19), therefore it is difficult to implicate these animals with certainty. It is possible that many carriers existed amongst the more than 3,000 animals imported into South Africa, mainly from the U.S.A. Although CMS has never thus far been described in American Brahmans, the mutation is likely to be present in that population at a low frequency. As in South Africa, it could have remained undetected in the American Brahman population for the same reasons mentioned earlier. It would also be interesting to screen animals of the Ongole, Kankrej, Gir and Krishna Valley breeds in

India for the 470del20 mutation. These are the main ancestors of the Brahman breed, and still currently exist in India, mainly as dual-purpose animals on small-scale farms. If the mutation event occurred in one of the ancestral breeds, rather than during the development of the Brahman in the U.S.A., then it most likely occurred in one of these four breeds, and may still be present in modern-day populations.

The CMS mutation has also been found in Australian Brahmans, albeit in only two of more than 2,000 animals genotyped (Thompson et al., 2003). Animals intended for importation into Australia are now required by the Australian Brahman Breeders' Association to be genotyped and certified free of the CMS mutation. Because of this, a commercial genetics laboratory in the U.S.A. is planning to offer testing for the CMS mutation. It is unknown whether the mutation occurs in Brahman cattle in any other countries. In addition, it is possible that the CMS mutation may occur in other breeds derived from the Brahman, some of which are popular in the U.S.A., South Africa, Australia, and elsewhere. In humans, numerous mutations, involving several genes, but most commonly *CHRNE*, are known to cause CMS (Hantai et al., 2004). It is therefore possible that other mutations in the same gene may exist in cattle which, in the homozygous state, may result in CMS.

It is interesting that in a related species, the Asian water buffalo (*Bubalus bubalis*), a clinical syndrome identical to CMS in Brahmans has been observed, in closely related calves in Brazil (A. S. Borges, State University of São Paulo, Botucatú, Brazil, personal communication), although DNA was not obtained for genotyping. However, it is unlikely that the same mutation is involved, because *Bubalus* is the most divergent genus within the tribe Bovini and is estimated to have diverged from *Bos* between two and five million years ago (Ritz et al., 2000). After ruling out the other possible differential diagnoses mentioned earlier, the recommended approach to detecting the causative mutation in a suspected new CMS, whether in cattle or in other related species, would be first to sequence the *CHRNE* gene, since this is the most likely one to be involved. If no mutation is found, genes coding for other components of the neuromuscular junction could be targeted.

## Effect of 470del20 in the heterozygote

Whether a mutation results in a dominant or recessive phenotype depends on the effect of the resultant alteration of the gene product, almost always a protein, on the stability of the relevant biological system (Beaudet et al., 1995). In general, most loss-of-function alleles are recessive, because half the amount of gene product is usually sufficient to maintain the normal physiological process. Loss-of-function mutations at loci encoding enzymes, for example, usually result in recessive phenotypes, since a large reduction in enzyme activity is usually necessary before a significant change in flux occurs. In the case of most congenital myasthenic syndromes, including the one described in this thesis, the presence of only one functional allele has no apparent clinical effect on the heterozygous animal. To our knowledge, there are no reports of any clinical abnormalities in humans or animals that are heterozygous for *CHRNE* null mutations. Interestingly however, Flagstad (1993) found that carriers of a mutation causing CMS in recessive homozygote dogs could be distinguished from wild-type homozygotes by differing responses to repetitive nerve stimulation, although clinically both genotypes were normal. Since a mutation may have different effects in different phenotypes, the question arose as to whether the presence of a single copy of the 470del20 mutation has any measurable effect on the performance of carrier animals. This, in turn, may result in a possible selective advantage or disadvantage that may influence the mutant allelic frequency in the future.

The results of the sire models showed that carriers of the 470del20 mutation may have had a selective advantage over non-carriers, due to better growth performance. The models of adjusted 600 d weight and of 600 d weight EBV both yielded similar estimates of a positive effect in carriers. For earlier weights, however, the models of own phenotype and of EBV differed. Possible reasons for this were discussed in Chapter 5. Nevertheless, it is interesting that the models of all four weight EBV consistently gave positive estimates, that increased with increasing age (Chapter 5, Table 4). This positive effect of CMS carrier status on growth may be due to either pleiotropy or linkage. The presence in carrier animals of only one functional copy of *CHRNE* possibly results in a slight reduction in AChR density at the neuromuscular junction, although to our knowledge this has not been shown, and is perhaps unlikely. If it does, however, any reduction in muscle strength would, if anything, result in

reduced feed intake and poorer growth. It is also unlikely that the abnormal polypeptide, resulting from the mutant *CHRNE* allele, would itself promote growth. The most likely explanation is, therefore, that linkage exists between the *CHRNE* locus and another gene on BTA 19 that influences growth. The known or suspected QTLs on this chromosome are listed in Chapter 5. More precise knowledge of the location of *CHRNE* on BTA 19 would be useful in the selection of candidate QTL for further investigation.

## Impact of CMS in the Brahman breed

At its present low frequency (approximately 1.2% prevalence of carriers amongst breeding bulls), the *CHRNE* 470del20 mutation is unlikely to have a significant economic effect in the South African Brahman population, with the birth of only one carrier calf expected per year. However, under certain conditions, its impact may be somewhat greater:

1. There will be individual herds in which the prevalence of carrier cows is higher, and in which the use of a carrier bull will result in economic losses.
2. The fact that an individual bull is found to be a carrier, may result in significant economic loss to the owner, particularly if it is a top performing animal and sought-after sire. Economic loss in this situation, when the reputation of the breeder is at stake, is difficult to quantify.
3. It is possible that the frequency of the mutation may increase rapidly due to the widespread use of one or more carrier sires. At the herd level, the exclusive use of a single carrier bull on a herd of non-carrier cows will increase the mutant allelic frequency to 25%, and the carrier prevalence to 50%, within one generation.
4. It is possible that the prevalence of CMS carriers has been underestimated, due to owners suspecting the presence of the mutation in their herds and therefore being unwilling to have their animals tested. However, any such underestimate is likely to be fairly small, and is unlikely to change our assessment of the impact of the mutation.

Of interest to breeders, is whether the frequency of a disease-causing mutation is likely to increase or to decrease in the future, and whether the implementation of control measures needs to be considered. There are several factors that may tend to reduce the frequency of the CMS mutation in the South African Brahman population over successive generations:

1. Early mortality of homozygous recessive calves. This probably always occurs before puberty, thus preventing such animals from breeding and transmitting the mutation. However, because the mutant allelic frequency is already low, the effect of this on further reducing the frequency will be very small.
2. The culling of cows that fail to raise a calf. This is applied to varying degrees by many farmers, in order to select for fertility and mothering ability. Carrier cows that produce a calf affected with CMS may be culled for this reason, even though the presence of a genetic defect may not be suspected. However, most carrier cows will not produce affected calves, because they will most commonly be mated to non-carrier bulls, and will therefore never be detected in this way.
3. The culling of cows and/or bulls that have produced a diseased calf, and are therefore known or suspected by the farmer to carry a genetic defect. For the same reason given above, most carriers will not be detected in this way.
4. The culling of animals genotyped and shown to carry the mutation. It is clear that this is the only measure that will be effective in reducing the mutant allelic frequency.
5. Random genetic drift.

On the other hand, the factors that may tend to increase the frequency of the mutation over time are:

1. Selective advantage of carriers due to better growth and growth EBV. Estimated breeding values, including those for 200 d, 400 d and 600 d weights, are widely used amongst Brahman breeders for selection of breeding animals, particularly bulls.
2. Chance presence of the CMS mutation in a particularly influential sire.
3. Random genetic drift.

The effect of a selective advantage of the heterozygote on changes in allelic frequency over generations was investigated in some detail in Chapter 6, using spreadsheet models. However, it is difficult to calculate the relative fitness of carrier animals compared to non-carriers, because this will depend on several factors, including the criteria used for selection and the selection intensity. These will vary greatly between breeders. Nevertheless, for illustration, let us assume that selection is done purely on own performance for 600 d weight. The overall mean 600 d weight was 391 kg and CMS carriers were on average 13.3 kg, (3.4%) heavier. We could assume, for example, that the fitness of the carrier relative to the non-carrier is 1.034 : 1, and that the initial mutant allelic frequency is 0.61% (i.e., carrier prevalence of 1.22%). The model in Chapter 6 can then be used to predict the change in allelic frequency or carrier prevalence over future generations. Predictions of carrier prevalence for various relative genotype fitnesses are shown in Table 1, assuming that no control measures are instituted. Median generation interval in the Brahman pedigree was six years.

**Table 1.** Predictions of future prevalence of CMS carriers (%) amongst Brahman cattle, for an initial carrier prevalence of 1.22%, various relative fitnesses of carriers, and no genotyping or culling done

| Generation | Fitness of CMS carrier relative to non-carrier ($w_{Aa}$ : $w_{AA}$) | | | | |
|---|---|---|---|---|---|
| | 1 : 1 | 1.034 : 1 | 1.1 : 1 | 1.25 : 1 | 1.5 : 1 |
| 0 | 1.22 | 1.22 | 1.22 | 1.22 | 1.22 |
| 5 | 1.18 | 1.40 | 1.88 | 3.46 | 7.98 |
| 10 | 1.14 | 1.58 | 2.84 | 8.72 | 29.6 |
| 25 | 1.06 | 2.26 | 7.68 | 30.4 | 50.0 |
| ∞ | 0 | 6.38 | 16.7 | 33.3 | 50.0 |

## Control measures

The ability to control or eradicate a recessive mutation in a population depends largely on the ability to detect carriers, which are usually phenotypically indistinguishable from wild-type homozygotes. In the past, carrier detection was sometimes done by progeny testing involving sire-daughter matings, or matings of bulls with homozygous recessive females (Johnson, 1980; Healy et al., 1987a). However, the cost and time involved precluded its widespread use. Biochemical assays have been used to screen for carriers of mutations in genes coding for catalytic enzymes, because heterozygotes produce smaller amounts of these enzymes. Such tests have successfully been applied for carrier detection, e.g. for generalised glycogenosis (Pompe's disease) in Brahmans (Healy et al., 1987b) and for $\alpha$-mannosidosis in Angus and Murray Greys (Jolly, 1975). Enzyme activity assays have the advantage that they can be applied where there is molecular heterogeneity, i.e. the same biochemical defect caused by more than one mutation, for example in generalised glycogenosis in Brahmans and Shorthorns (Dennis et al., 2000). They have a number of limitations, however, such as variation in enzyme activity, overlap between genotypes, and incorrect results in cases of haemopoietic chimaerism (Healy et al., 1994; Healy, 1996). More recently, DNA tests for the causative mutations have become available for several inherited diseases, enabling accurate detection of heterozygotes. For CMS in Brahman cattle, the identification of the underlying mutation, described in Chapter 3, and the development of a screening test for it, described in Chapter 4, were crucial steps in the effort to control the disease. However, there are various ways in which the test can be applied, not all of which will ultimately prove to be cost-effective.

To completely prevent the occurrence of calves affected with CMS, it is sufficient to prevent matings between carriers. The use of sires that are tested and certified free of the mutation will achieve this. Using the control strategy model in Chapter 6, assuming a breeding population of 13,017 cows and 1,069 bulls (2004 figures), testing and culling only carrier breeding bulls is expected to result in the prevalence of carriers dropping below 0.1% after four generations (24 years). This strategy would require the testing of 4,300 bulls, and the expected culling of 25 carriers. In these estimates, any possible selective advantage of the carriers has not been taken into account. The number of carriers to be culled will increase when there is a selective advantage of

carrier cows. Complete eradication of the mutation using this strategy, according to the criteria used in Chapter 6, is expected to take 12 generations, requiring 12,855 tests and 27 culls. Alternatively, immediate eradication of the CMS mutation from the population could be achieved by genotyping all breeding males and females, and culling the carriers. This is expected to require the testing of 14,260 animals and the culling of 174 animals (13 bulls and 161 cows). Complete control of the disease, and elimination of any associated economic losses, will be achieved with both the above strategies. Although it will take longer, the former strategy (testing of bulls only) will be cheaper and easier to implement, and is therefore the option of choice.

It is unlikely, however, that voluntary testing of animals for CMS, as is currently BCBSSA policy, will result in the testing of all breeding bulls. Such an objective would require that testing of breeding bulls is made a prerequisite for registration. The question then arises as to whether it would be necessary to test all breeding bulls, in order to ensure a reduction in the frequency, and eventual elimination, of the mutant allele. In Table 2, predictions of mutant allelic frequencies are made for two control strategies, using the same relative fitness values as in Table 1. Strategy X is the genotyping of all breeding bulls, and strategy Y is the partial (in this example, 50%) genotyping of breeding bulls. In both strategies, all CMS carriers that are detected are prevented from breeding. It is further assumed that any selective advantage of carriers also applies to cows, although in reality this may not occur to the same extent as in bulls.

From the results in Table 2, it is clear that even the testing of only half of the breeding bulls is likely to achieve a satisfactory reduction in the frequency of the CMS mutation, even if there is a selective advantage of carriers amongst both bulls and cows. However, if the selective advantage is too great, for example if the relative fitness of the carrier is 1.5, then partial testing of bulls may be insufficient to reduce the frequency. Using the same model, it can be shown that only if the relative fitness of carrier cows is >2.025 will the testing of all breeding males be insufficient to achieve a long-term reduction in the mutant allele frequency. This is, however, unlikely to be the case in the Brahman population. The selective advantage amongst CMS carriers is likely to be far less for cows, because it is the bulls that are subjected to the greatest selection pressure. Therefore, in reality, the prevalence of carriers is likely to decline faster than predicted in Table 2.

**Table 2.** Predictions of future prevalence of CMS carriers (%) amongst Brahman cattle, for an initial carrier prevalence of 1.22%, various relative fitnesses of carriers, and either complete (X) or partial (Y) culling of carrier bulls

| Generation | Strategy | Fitness of CMS carrier relative to non-carrier ($w_{Aa}$ : $w_{AA}$) | | | | |
|---|---|---|---|---|---|---|
| | | 1 : 1 | 1.034 : 1 | 1.1 : 1 | 1.25 : 1 | 1.5 : 1 |
| 0 | | 1.22 | 1.22 | 1.22 | 1.22 | 1.22 |
| 5 | X [1] | 0.04 | 0.04 | 0.06 | 0.12 | 0.28 |
| | Y [2] | 0.28 | 0.34 | 0.46 | 0.86 | 2.08 |
| 10 | X | 0.002 | 0.002 | 0.004 | 0.01 | 0.06 |
| | Y | 0.06 | 0.1 | 0.18 | 0.62 | 3.44 |
| 25 | X | <0.0001 | <0.0001 | <0.0001 | 0.0001 | 0.001 |
| | Y | 0.001 | 0.002 | 0.02 | 0.22 | 10.5 |
| ∞ | X | 0 | 0 | 0 | 0 | 0 |
| | Y | 0 | 0 | 0 | 0 | 18.2 |

[1] Genotyping all breeding bulls and culling CMS carriers

[2] Genotyping 50% of breeding bulls and culling CMS carriers

It can be concluded that, in order to at least ensure that the frequency of the CMS mutation does not increase in the future, genotyping of all breeding bulls is probably not necessary. Continuation of the current voluntary testing programme is likely to achieve adequate control of the disease, with a long-term reduction in the mutant allelic frequency. However, progress will be markedly slower than that achieved by the testing of all bulls.

The models used above assume that all bulls contribute equally to the gene pool of the following generation. Of course, this is not the case, and maximum benefit could therefore be obtained from a limited number of tests by ensuring that at least the most widely used bulls are tested. This should include show animals and bulls used for artificial insemination. The current BCBSSA requirement is that all bulls entered for the annual National Sale be tested for the CMS mutation. However, this represents only a fraction of the bulls sold annually, and this requirement should be extended to include bulls and semen at all sales.

In light of the results presented in this thesis, in particular the finding that CMS carriers may have a selective advantage, it is argued that control measures against CMS are necessary. Although the incidence of a few affected calves may be economically acceptable, from an ethical point of view, the suffering of animals should be avoided. The image and reputation of the breed society, and of the breed as a whole, are also at stake. However, the objectives of such a programme need to be defined, as does the time frame involved, and the means of monitoring its success. The measures should be acceptable to the majority of members of BCBSSA, and should be accompanied by an adequate educational programme.

Our recommendation is that genotyping of sires for the CMS mutation be made a requirement for the registration of their offspring, for the following reasons:

1. A quicker, and more predictable, reduction in mutant allelic frequency would be achieved than with partial, voluntary testing of animals.
2. The effect of any selective advantage of carrier bulls, no matter how great, would be eliminated.
3. Accurate monitoring of the prevalence of CMS carriers, and therefore of the success of the control programme, would be achieved.
4. Even if CMS carriers are not required to be excluded from registration or culled, public knowledge of their status would ensure that they are used less.
5. A carrier bull of exceptional genetic merit could still be used for breeding, provided that his offspring, approximately half of which will be carriers, are genotyped.
6. The once-off cost of genotyping a breeding bull is a very small fraction of the value of the bull, therefore cost should not be a constraint.

## Scope for future research

Certain aspects of CMS in Brahman calves remain unclear. Although we assume that affected calves survive no longer than a few months, it is possible that a wider variation in the onset and severity of clinical signs may occur. Therefore, there is a need to investigate more clinical cases of CMS. When further calves affected with CMS are examined, low frequency repetitive nerve stimulation should be done in order to confirm that a decremental response occurs, consistent with descriptions for other species. It would be interesting then to determine whether carriers can be distinguished from non-carriers on the basis of their response to repetitive nerve stimulation, as has been described in dogs. This also raises the issue that the electrophysiology of normal bovine muscle is poorly known and that there is scope for research in this field. Other aspects of CMS that still require investigation are the ultrastructure of the neuromuscular junction and the postnatal expression of *CHRNE* and *CHRNG*. Such studies would probably require the intentional breeding of homozygous recessive calves. Better understanding of all these aspects of CMS in cattle will help determine its suitability as an animal model that may ultimately contribute to a better understanding of the disease in humans.

The clinical signs in calves with CMS show similiarities to those seen in "dummy calves", and it is possible that CMS may play a role in some cases of this syndrome. Research is required into the epidemiology and aetiology of dummy calf syndrome in South African Brahmans. This may include testing for the *CHRNE* 470del20 mutation, and possibly also for other mutations in the same gene.

Determination of the presence and frequency of the *CHRNE* 470del20 mutation in other Brahman populations around the world, as well as in other cattle breeds derived from the Brahman, is something that requires further investigation. Genotyping of animals of the various *Bos indicus* cattle breeds in India may provide clues to the origin of the CMS mutation in Brahmans. Knowledge of the genomic structure of the bovine *CHRNE* gene now also enables us to detect other mutations in the same gene, which may produce a similar disorder but will not be detected by our screening test. There is also a need to further investigate the effect of CMS heterozygosity on growth.

More precise knowledge of the location of *CHRNE* on BTA 19 and identification of the linked QTL, may add to our understanding of the genetics of growth in cattle.

Aspects of the investigation of CMS in Brahmans that are described in this thesis will also be relevant for other genetic defects, both in cattle and in other species. Although the candidate gene approach used here will not be applicable in all situations, there undoubtedly exist further, as yet undescribed, genetic conditions in animals that either are analogous to known conditions in humans, or in which a candidate gene or genes can be identified. Experience gained with CMS will help in the future investigation of such conditions. Our investigation of the effect of the mutation, and of its impact in the population, is an example of how further information can be generated which will assist in deciding on a course of action to be taken. The mere fact that a genetic defect has been identified in an animal population, does not necessarily mean that it should be eradicated at all costs. Our spreadsheet model can easily be adapted to predict changes in allele frequency for genetic diseases with various modes of inheritance, in any animal population, and also to compare alternative control strategies. Close collaboration with the relevant breed society is highly desirable, firstly in order to ensure the co-operation of breeders and the acquisition of sufficient, reliable data, and ultimately to ensure effective dissemination of results.

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# Summary

Many inherited defects have been described in cattle, the majority of which are known or suspected to be inherited in an autosomal recessive manner. However, in most of these conditions the causative mutation has not yet been described. This thesis describes a previously unknown recessively inherited disease in Brahman cattle, congenital myasthenic syndrome (CMS), from its first clinical presentation, through the elucidation of its molecular basis and description of the underlying mutation, the development of a screening test for the mutation, the investigation of the epidemiology of the disease and its impact in the population, to the evaluation of options for its control or eradication.

The presentation and clinical investigation of the initial clinical case are described in Chapter 2. A five-week-old Brahman heifer calf was presented with a two week history of progressive muscular weakness that was exacerbated by exercise but improved with rest. The fact that three other half-siblings, a male and two females, showed similar clinical signs suggested that this was an inherited disease. Their dams, as well as their common sire and common maternal grandsire, were all apparently normal, which was consistent with an autosomal recessive mode of inheritance. The most striking finding during clinical investigation was the dramatic, but transient response to the intravenous administration of edrophonium chloride (Tensilon). Several other diagnostic tests were performed in order to rule out other possible differential diagnoses. Although a definitive diagnosis could not be made at that stage, on the basis of the clinical presentation, the familial clustering, and the response to edrophonium administration, a presumptive diagnosis of postsynaptic congenital myasthenia gravis, or congenital myasthenic syndrome, was made.

Congenital myasthenic syndromes are a heterogeneous group of disorders of neuromuscular transmission at the skeletal muscle motor endplates. They are most commonly caused by a deficiency or a kinetic abnormality of the acetylcholine receptors (AChR) on the postsynaptic membrane, due to homozygosity (or compound

heterozygosity) for a mutation in one of the genes coding for the five AChR subunits. In humans, the most common subunit affected is the epsilon subunit, which replaces the gamma subunit as foetal AChR is replaced by adult AChR during the perinatal period. The reason that patients without a functional $\varepsilon$-subunit are able to survive, at least temporarily, is thought to be due to persistent expression of the foetal $\gamma$-subunit encoded by the *CHRNG* gene. The gene encoding the $\varepsilon$-subunit (*CHRNE*) was therefore the candidate gene chosen in a search for the underlying mutation in the calf. This is described in Chapter 3. Using the published bov*CHRNE* cDNA sequence, and by comparison with the human genomic *CHRNE* sequence, the genomic structure of bov*CHRNE* was determined and primers were designed for the exons. This led to the discovery of a homozygous 20 base pair deletion (470del20) within the coding sequence of exon 5. The mutation was predicted to result in a premature stop codon and to produce a completely non-functional receptor subunit, thus explaining the impairment of neuromuscular transmission. Several relatives of the affected calves, including their common sire and maternal grandsire, were found to be carriers of the 470del20 mutation. An interesting question arose from the fact that calves homozygous for this mutation showed a more severe phenotype than is seen in most human CMS patients, namely whether there is a more rapid and complete postnatal downregulation of *CHRNG* expression in cattle than in man, or whether there may be other compensatory mechanisms involved. Comparative studies including animal models such as this one may therefore contribute to our understanding of complex genotype-phenotype relationships.

Chapter 4 describes the development and validation of the PCR screening test for the 470del20 mutation, and the initial screening results from a limited sample of animals. Several further carrier animals were detected, including influential breeding sires. In the herd from which the original affected calves came, 12% of the cows were found to be carriers. Economic losses can be expected in such high prevalence herds if a carrier bull is used for breeding. A clearer picture of the clinical manifestation of CMS in Brahmans also emerges in this chapter. Muscle weakness may be present at birth, but more commonly calves are initially normal, with clinical signs beginning within the first month of age. The weakness is gradually progressive, although calves remain alert and able to suck. All cases observed thus far have been euthanased at between two and five months of age. However, affected calves in the field are unlikely to survive unassisted beyond 1 to 2 months. Nevertheless, because only very few cases have been

seen, it is possible that a greater variation in the clinical manifestation of the condition may exist.

In order to obtain a more accurate estimate of the frequency of the mutation in the South African Brahman population, voluntary genotyping of animals was performed in conjunction with the Brahman Cattle Breeders' Society of South Africa's programme of mandatory DNA profiling. Chapter 5 describes the results of the testing of 1,453 animals from 2003 to 2005. The prevalence of carriers amongst breeding bulls used in 2004 was estimated to be 1.2% (95% confidence interval 0.7% to 2.2%), with insufficient evidence to show any recent change in prevalence. Pedigree analysis revealed no single carrier founder in the South African Brahman pedigree, but that the most likely ancestral carriers were animals or semen imported from the U.S.A.

Although carriers of the 470del20 mutation are clinically normal, Chapter 5 presents evidence that they have a greater 600 day weight, and possibly also greater birth, 200 day and 400 day weights. This was determined by regressions of both performance data and estimated breeding values on genotype probability using a sire model. This effect of carrier status on weight may be due to linkage between *CHRNE* and another gene on the same chromosome (BTA 19) that influences growth, or possibly due to some pleiotropic effect of the mutant allele. It is therefore possible that carriers may have a selective advantage over non-carriers and that inadvertent selection for the mutation could occur.

The question then arises as to what control measures, if any, should be recommended. When deciding between alternative strategies for the control or eradication of an undesirable mutation in a population, it is important to be able to predict changes in mutation frequency over time. A spreadsheet model was therefore developed for this purpose and is presented in Chapter 6. It allows for relative fitness to differ between genotypes, between sexes, and over time, and can therefore be used to model mutation frequency where there is heterozygote advantage or disadvantage, and/or when various control measures are implemented. Although a lethal recessive allele such as 470del20 should naturally be very slowly eliminated from the population, when there is a selective advantage of the carrier, the mutant allele is never naturally eliminated, but will tend towards a stable equilibrium frequency. In order to prevent the occurrence of calves affected with CMS, it would be sufficient to prevent matings between carriers,

by ensuring that all breeding bulls are tested and certified to be non-carriers. Alternatively, rapid eradication of the mutation from the population can be achieved by genotyping all breeding males and females and preventing all carriers from breeding. These, and examples of other genotyping and culling strategies, were modelled for various carrier frequencies and relative genotype fitnesses. The results were presented in terms of the number of generations, number of tests, and number of culls required in order to reduce the carrier frequency to a predefined level. The model can therefore be used to compare the expected outcomes and costs of alternative control strategies and to illustrate these to breeders.

Due to the low frequency of the 470del20 mutation in the South African Brahman population, its economic impact on the breed in general is likely to be small. This, however, may not be true in individual herds with a relatively high prevalence of carriers, in which a carrier bull is inadvertently used. The danger exists, too, that the frequency of the mutant allele may increase in the future, either slowly due to a selective advantage of carriers, or potentially rapidly due to the widespread use of a popular bull that happens to be a carrier. We therefore recommend that measures be taken to reduce the frequency of the mutation. Continued voluntary CMS genotyping of breeding bulls is likely to achieve adequate control of the disease, with a long-term reduction in the mutant allelic frequency, provided that carriers are excluded from breeding, or at least that test results are made public. If only partial testing of bulls is done, at least the most widely used breeding animals should be included. However, progress will be slower than will be achieved by mandatory testing of all breeding bulls. For the reasons mentioned above, in order to ensure a rapid reduction in the frequency of the mutation, as well as to enhance the reputation of the breed and the breed society, our recommendation is that CMS genotyping of all sires be made a requirement for the registration of their offspring.

# Samenvatting

Vele erfelijke afwijkingen bij vee zijn beschreven en het is bekend of verwacht dat het grootste deel hiervan op een autosomale recessieve manier overgedragen wordt. Echter, bij de meeste van deze aandoeningen is de causatieve mutatie nog niet beschreven. Dit proefschrift beschrijft een eerder onbekende recessief overgedragen ziekte bij het Brahman rund, congenitaal myastheen syndroom (CMS), vanaf de eerste klinische presentatie, via de opheldering van de moleculaire basis en de beschrijving van de onderliggende mutatie, de ontwikkeling van een screening test voor de mutatie, het onderzoek van de epidemiologie van de ziekte en haar impact op de populatie, tot en met de evaluatie van mogelijkheden voor controle of uitroeiing.

De presentatie en het klinische onderzoek van de eerste klinische casus worden beschreven in Hoofdstuk 2. Een vijf weken oude Brahman vaarskalf werd gepresenteerd met een twee weken durende progressieve spierzwakte die verergerde door lichaamsbeweging, maar minder werd met rust. Het feit dat een halfbroer en twee halfzussen vergelijkbare klinische symptomen vertoonden suggereerde dat dit een erfelijke ziekte was. De moeders, evenals de gemeenschappelijke vader en gemeenschappelijke grootvader van moeder's kant, waren allen ogenschijnlijk normaal, wat consistent was met een autosomale recessieve manier van overerving. De meest opvallende vondst gedurende het klinische onderzoek was de dramatische, maar geleidelijke reactie op de intraveneuze toediening van edrophonium chloride (Tensilon). Verscheidene andere diagnostische tests werden uitgevoerd om andere mogelijke differentiaal diagnoses uit te sluiten. Hoewel een definitieve diagnose niet gesteld kon worden in dat stadium, op basis van de klinische presentatie, de familiaire clustering, en de reactie op de toediening van edrophonium, werd een voorbarige diagnose van postsynaptische aangeboren myasthenia gravis, of congenitaal myastheen syndroom, gesteld.

Congenitaal myastheen syndromen zijn een heterogene groep van afwijkingen van neuromusculaire transmissie aan de musculaire motorische eindplaten van het skelet.

Ze worden gewoonlijk veroorzaakt door een gebrek of een kinetische abnormaliteit van de acetylcholinereceptoren (AChR) op het postsynaptische membraan, als gevolg van een mutatie in één van de genen die coderen voor de vijf AChR subeenheden. Bij mensen wordt de epsilon-subeenheid, het vaakst aangetast; deze epsilon-subeenheid vervangt de gamma subeenheid wanneer feutaal AChR vervangen wordt door volwassen AChR gedurende de perinatale periode. Men denkt de reden dat patiënten zonder een functionele $\varepsilon$-subeenheid in staat zijn te overleven, op zijn minst tijdelijk, de constante uiting van de feutale $\gamma$-subeenheid is, gecodeerd in het *CHRNG* gen. Het gen dat de $\varepsilon$-subeenheid (*CHRNE*) codeert is daarom gekozen als kandidaatgen in een zoektocht naar de onderliggende mutatie in het kalf. Dit wordt beschreven in Hoofdstuk 3. Gebruikmakend van de gepubliceerde bov*CHRNE* cDNA sequentie, en door vergelijking met de humane *CHRNE* sequentie, is de genoomstructuur van bov*CHRNE* bepaald en zijn primers ontworpen. Dit leidde tot de ontdekking van een homozygote 20 basepaar deletie (470del20) binnen de coderingssequentie van exon 5. De mutatie was voorspeld te resulteren in een prematuur stop-codon en te leiden tot een volledig niet-functionele receptor subeenheid, om aldus de schade aan neuromusculaire transmissie te verklaren. Verschillende familieleden van de getroffen kalveren, inclusief de gemeenschappelijke vader en de grootvader van moeder's kant, bleken drager te zijn van de 470del20 mutatie. Een interessante vraag komt op uit de observatie dat kalveren die homozygoot zijn voor deze mutatie ernstigere verschijnselen in fenotype laten zien vergeleken met de meeste humane CMS patiënten. Men kan zich afvragen of er een snellere en volledigere postnatale neerwaartse regulatie van *CHRNG* expressie is bij runderen vergeleken met de mens, of dat er andere compenserende mechanismen in het spel zijn. Vergelijkende studies met diermodellen, zoals deze studie, kunnen derhalve bijdragen aan een beter begrip van complexe genotype-fenotype relaties.

In Hoofdstuk 4 wordt de ontwikkeling en validatie beschreven van een PCR screeningstest voor de 470del20 mutatie, en de eerste screeningsresultaten van een relatief kleine steekproef onder Brahman runderen. Verschillende dragers werden hierbij gevonden, waaronder belangrijke fokstieren. In de kudde waar de oorspronkelijke gevallen vandaan kwamen bleek 12% van de runderen drager te zijn. Dergelijke hoge prevalenties kunnen economische gevolgen hebben als de dragerstier voor fokdoeleinden wordt ingezet. In dit hoofdstuk ontstaat ook een duidelijker beeld van de klinische manifestatie van CMS in Brahman runderen. Spierzwakte kan al

voorkomen bij geboorte, maar meestal zijn de kalveren aanvankelijk ogenschijnlijk normaal en beginnen de eerste symptomen een maand na geboorte. De verzwakking is progressief, maar de meeste kalveren blijven alert en zijn in staat te zogen. Alle tot dusver gevonden gevallen moesten worden ge-eutanaseerd tussen twee en vijf maanden na geboorte. Normaal gesproken is het onwaarschijnlijk dat de overleving langer dan 1 of 2 maanden is. Omdat echter maar een gering aantal gevallen is bestudeerd is het mogelijk dat er een grotere variatie in klinische manifestatie bestaat.

Teneinde een betere schatting te krijgen van de frequentie van de mutatie in de Zuid-Afrikaanse Brahman populatie, werd vrijwillige genotypering uitgevoerd van dieren in samenwerking met de Brahman fokkers vereniging van Zuid Afrika en haar verplichte programma van DNA-typering. In Hoofdstuk 5 worden de resultaten beschreven van het testen van 1453 dieren tussen 2003 en 2005. De prevalentie van dragers onder fokstieren die gebruikt zijn in 2004 werd geschat op 1,2% (95% betrouwbaarheidsinterval: 0,7% - 2,2%). Er was onvoldoende bewijs om mogelijke recente veranderingen in die prevalentie te kunnen vaststellen. Stamboomanalyse gaf aan dat er geen unieke dragende voorvader is in de Zuid Afrikaanse Brahman stamboom, maar dat zeer waarschijnlijk een drager of sperma van dragers ingevoerd werd uit de Verenigde Staten.

Alhoewel dragers van de 470del20 mutatie klinisch normaal zijn, geeft Hoofdstuk 5 aanwijzingen dat deze dieren een hoger 600-dagen gewicht hebben, en mogelijk ook een verhoogd geboorte gewicht en gewicht op 200 en 400 dagen. Dit werd vastgesteld door regressie van zowel data over de prestaties als de geschatte fokwaarde (EBV) op genotypewaarschijnlijkheid. Dit effect van dragerschap op gewicht kan het gevolg zijn van een link tussen *CHRNE* en een ander gen op hetzelfde chromosoom (BTA 19) dat groei beïnvloedt, of mogelijk het gevolg van een pleiotroop effect van het gemuteerde allel. Het is door dit gewichtseffect mogelijk dat dragers een selectievoordeel hebben boven niet-dragers en dat onbedoelde selectie in het voordeel van de mutatie kan optreden.

De vraag die dan op komt is welke controlemaatregelen aangeraden moeten worden, als het al nodig is om in te grijpen. Bij het afwegen van verschillende strategiën voor beheersing of eradicatie van een ongewenste mutatie in een populatie, is het van belang om veranderingen in de mutatie prevalentie in de tijd te kunnen voorspellen.

Een eenvoudig populatiemodel dat gemaakt is voor dit doel wordt gepresenteerd in Hoofdstuk 6. In het model is variatie mogelijk in relatieve fitness tussen genotypen, tussen geslachten en in de tijd; het model kan gebruikt worden om de frequentie van de mutatie te vervolgen onder verschillende condities, zoals een heterozygoot voordeel of nadeel, en inde aanwezigheid van verschillende controlemaatregelen. Alhoewel een lethaal recessief allel zoals 470del20 op natuurlijke wijze langzaam uit een populatie zou moeten verdwijnen is dat niet het geval als er een selectief voordeel is voor dragers. In dat geval zal de prevalentie naar een stabiel evenwicht groeien. Om kalveren met CMS te voorkomen is het dan afdoende om het fokken met twee dragers te voorkomen. Dit kan gebeuren door te zorgen dat alle fokstieren getest zijn en gecerticeerd niet-drager. Een snelle eradicatie van de mutatie kan bereikt worden door het genotyperen van alle dieren die voor de fok worden gebruikt. Deze en verschillende andere voorbeelden van genotypering en verwijderingsmaatregelen werden gemodelleerd voor verschillende dragerprevalenties en relatieve genotype fitness. De resultaten zijn gepresenteerd in termen van het aantal generaties, het aantal uit te voeren tests en het aantal te verwijderen dieren die nodig zijn om de dragerfrequentie te verlagen naar een van te voren vastgesteld niveau. Het model kan derhalve gebruikt worden om de verwachte uitkomsten en kosten van beheersmaatregelen te illustreren.

Dankzij de lage prevalentie van de 470del20 mutatie in de Zuid-Afrikaanse Brahman populatie is de economische impact op het ras waarschijnlijk klein. Dit hoeft echter niet te gelden voor individuele kuddes met een relatief hoge prevalentie waar onbewust een dragende stier is gebruikt. Ook bestaat het gevaar dat in de toekomst, traag door een selectief voordeel van dragers, of snel door wijde inzet van een populaire stier, de prevalentie toeneemt. We raden daarom aan om maatregelen te nemen die de prevalentie reduceren. Voortgezette vrijwillige CMS genotypering van fokstieren geeft waarschijnlijk een afdoende beheersing, vooropgesteld dat dragende stieren inderdaad niet voor de fok worden gebruikt, of dat minimaal de testresultaten openbaar worden gemaakt. Als selectief getest wordt op stieren dan zouden de vaakst gebruikte stieren in ieder geval moeten worden getest. Voortgang is dan echter langzamer dan met verplicht testen van alle fokstieren. Zoals boven beargumenteerd is het onze aanbeveling, om snelle reductie in de prevalentie te bewerkstelligen, dat CMS genotypering van alle stieren verplicht wordt voor de registratie van hun nakomelingen.

# Related publications

### *Journal articles*

Sieb, J. P., S. Kraner, P. N. Thompson, and O. K. Steinlein. 2003. Congenital myasthenic syndrome in cattle due to homozygosity for a truncating mutation in the acetylcholine receptor (AChR) epsilon-subunit gene. Annals of the New York Academy of Sciences 998:125–127.

### *Conference presentations*

Thompson, P. N. 1998. Suspected congenital myasthenia gravis in Brahman calves. XXth World Association for Buiatrics Congress, Sydney, Australia, 6–10 July.

Sieb, J. P., S. Kraner, P. N. Thompson, and O. K. Steinlein. 2002. Congenital myasthenic syndrome in cattle due to homozygosity for a truncating mutation in the acetylcholine receptor (AChR) epsilon-subunit gene. Xth International Conference on Myasthenia Gravis and Related Disorders, Key Biscayne, Florida, U.S.A., 29 May – 1 June.

Thompson, P. N. 2004. Congenital myasthenic syndrome (CMS) and generalised glycogenosis (Pompe's disease) in South African Brahman cattle. South African Veterinary and Paraveterinary Congress, Cape Town, South Africa, 27–30 July.

### *Conference posters*

Thompson, P. N., O. K. Steinlein, E. van Dyk, S. Kraner, C. K. Harper, A. J. Guthrie, A. Nel, and E. Bell. 2002. Screening test for a congenital myasthenic syndrome in cattle. Second Annual Conference of the Southern African Society for Veterinary Epidemiology and Preventive Medicine, Onderstepoort, South Africa, 22–23 July.

Thompson, P. N., O. K. Steinlein, E. van Dyk, S. Kraner, C. K. Harper, A. J. Guthrie, A. Nel, and E. Bell. 2002. Screening test for a congenital myasthenic syndrome in cattle. 18th Faculty Day, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, 19 September.

Kraner, S., J. P. Sieb, P. N. Thompson, and O. K. Steinlein. 2002. Congenital myasthenia in Brahman calves caused by homozygosity for a *CHRNE* truncating mutation. 13th Annual Meeting of the German Society of Human Genetics, Leipzig, Germany, 29 September – 2 October.

Thompson, P. N., J. A. Dennis, K. N. Mercieca, C. K. Harper, and A. J. Guthrie. 2003. Screening for two genetic diseases affecting calf survival in South African and Australian Brahman cattle. 19th Faculty Day, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, 25 September 2003.

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***Popular articles***

Thompson, P. N. 2003. Testing for genetic diseases in Brahmans. South African Brahman Journal 23(3):14.

Thompson, P. N. 2003. Weakness in Brahman calves: possible genetic causes. Livestock Health and Production Review (Livestock Health and Production Group of the South African Veterinary Association) 6:6–7.

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# Curriculum vitae

Peter Thompson was born on 31 May 1966 in Johannesburg, South Africa. He completed his schooling at Westville Boys' High School, Durban, in 1983, and his B.V.Sc. at the University of Pretoria in 1990. He then completed an internship programme in large animal medicine at the New York State College of Veterinary Medicine, Cornell University and spent some time in mixed animal practice in the United Kingdom and South Africa, before joining the Faculty of Veterinary Science, University of Pretoria at Onderstepoort in November 1992. He worked as Senior Lecturer in the Department of Medicine and in the Production Animal Clinic of the Onderstepoort Veterinary Academic Hospital. He obtained the M.Med.Vet.(Med) degree and registration as specialist in bovine medicine in 1999. Since 2000 he has been Senior Lecturer and since 2006, Associate Professor, in the Section of Epidemiology, Department of Production Animal Studies, where he is involved with teaching and research in veterinary epidemiology and production animal medicine.