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Manifestation of neuronal ceroid lipofuscinosis in Australian Merino sheep: observations on altered behaviour and growth

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Abstract

Neuronal ceroid lipofuscinoses (NCL) is an inherited neurodegenerative disorder in children. Presently there is no effective treatment and the disorder is lethal. NCL occur in a variety of non-human species including sheep, which are recognised as valuable large animal models for NCL. This experiment investigated the progressive postural, behavioural and liveweight changes in NCL-affected lambs, to establish practical, non-invasive biomarkers of disease progression for future preclinical trials in a *CLN6* Merino sheep model. A flock of eight lambs at pasture was studied, with the observer blind to the disorder status. Three genotypes were compared: homozygous affected NCL; $n = 4$), clinically normal heterozygous (Carrier; $n = 2$) and homozygous normal (non-carrier control (Normal); $n = 2$). Direct observation during daylight and continuous accelerometer measurements over 72 h were used to quantify lamb posture and behaviour in 11 sessions between 26–60 weeks of age, conducted at 3–5 week intervals. There was a Genotype (G) $\times$ Age (A) interaction ($P = 0.001$) for liveweight of the lambs in the experiment, with NCL, Carrier and Normal lambs gaining 11.8, 16.5 and 23.4 kg, respectively, between 26 and 60 weeks of age. G $\times$ A interactions were also found for walking behaviour (means for NCL, Carrier and Normal genotype groups at 26 and 60 weeks, were 1.7 and 7.9%, 3.3 and 3.1%, and 2.5 and 1.9% of observations, $P = 0.008$) and a composite variable of key behaviours identified in the principal components analysis ($P < 0.001$), with mean values for NCL lambs increasing three-fold compared to non-affected lambs as age increased. Similarly, NCL lambs became less responsive to visual and auditory stimuli as they aged. Mean responsiveness scores (out of 3) to visual stimuli for the NCL, Carrier and Normal genotypes at 26 and 60 weeks of age were 2.7 and 1.4, 2.8 and 2.9, and 3.0 and 3.0, respectively (G $\times$ A, $P < 0.001$). Changes in response to auditory stimuli were similar to visual stimuli. NCL lambs took more ($P = 0.015$) steps per 24 h than Carrier and Normal genotype lambs, but there was no G $\times$ A interaction. At 26

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Additional information regarding Neuronal Ceroid Lipofuscinosis (NCL)

For detailed reading about NCL, see the special issues of *Biochimica et Biophysica Acta*, volumes 1762 (special issue 10; October 2006) and 1832 (issue 11; November 2013).

For information regarding Batten Disease, the following URL may be of interest to readers: <http://www.ucl.ac.uk/ncl/treatment.shtml> [accessed 24 April 2014]

and 60 weeks of age respectively, NCL lambs took 2724 and 4121 steps per 24 h, compared to Carrier (1708 and 3105 steps) and Normal genotype lambs (2109 and 3506 steps). NCL lambs also performed less ($P = 0.018$) grazing behaviour than Carrier and Normal genotype lambs (66.5, 72.3 and 72.5% of observations for NCL, Carrier and Normal lambs, respectively). A number of behavioural changes identified in the experiment could form the basis for a protocol for monitoring and evaluation of disease progression.

Keywords

Behavioural indicators; Neurodegenerative disease; Sheep; Genetics; Animal model

1. Introduction

Neuronal ceroid lipofuscinoses (NCL), also known as Batten Disease, are a group of inherited neurodegenerative disorders characterised by the accumulation of autofluorescent storage bodies, mostly within neurons and retinal cells that lead to progressive brain and retinal atrophy (Mole et al., 2011; Anderson et al., 2013). NCL are mostly recessively inherited, and in humans NCL represent the most common neurodegenerative disorder in children (Schulz et al., 2013). Historically, four main types of NCL were recognised based on the time of onset of disease: infantile, late infantile, juvenile and adult (Zeman, 1976). More recently, different variants of the disease have been classified based on at least 13 disease-causing genes, *CLN1-8,10-14* (Williams and Mole, 2012; Warrier et al., 2013). Although these variants share similarities, they can differ in the age of onset, severity and rate of progression of clinical signs and pathology, and composition and ultrastructure of storage material (Mole et al., 2011). Presently, several underlying pathological mechanisms for these diseases are under discussion (Palmer et al., 2013) and several therapeutic approaches are being evaluated in clinical trials, but there is no effective treatment available and all forms of NCL are lethal (Mole et al., 2011).

Mutations responsible for NCL have also been identified in non-human animals, and a number of animal models have been investigated to facilitate research into the disease mechanisms and for development and evaluation of therapeutic strategies for NCL (see review by Bond et al., 2013). While small animal models such as mouse models are very useful in studies on NCL, mice have a less complex brain, and do not demonstrate the severe brain atrophy, intense neuronal loss and retinal degeneration characteristics observed in large animals such as with sheep, or human NCL (Frugier et al., 2008). Therefore, the use of large animal (such as sheep) models is increasing, since the NCL clinical signs more closely resemble those of humans and life expectancy is longer in sheep than mice (Tammen et al., 2006). Sheep breeds affected by NCL have included the South Hampshire, Swedish Landrace, Rambouillet, Merino and Borderdale (Jolly et al., 1980; Järplid and Haltia 1993; Woods et al., 1994; Cook et al., 2002; Jolly et al., 2002). Two different mutations in the *CLN6* gene are responsible for NCL in Merino and South Hampshire sheep (Tammen et al., 2006), NCL in Swedish Landrace is caused by a mutation in the *CTSD/CLN10* gene (Tyynelä et al., 2000), whereas NCL in Borderdale sheep is caused by a mutation in the

CLN5 gene (Frugier et al., 2008). These ovine diseases represent models for human variants caused by mutations in the corresponding human genes.

The onset of clinical signs of NCL in the *CLN6* Merino sheep model used in this study is reported to occur from 7 to 10 months of age (Tammen et al., 2001, 2006; Cook et al., 2002). Clinical signs include behavioural change, motor deficit, seizure and visual impairment. Reported behavioural changes include reduced awareness of the environment, decreased response to auditory stimuli, startle response to visual and auditory stimuli, aimless walking and walking in circles (Cook et al., 2002; Tammen et al., 2006). The flocking behaviour also appears to diminish and NCL-affected sheep may be found isolated from their group. Motor disturbances observed in NCL-affected sheep include the dragging of feet, stumbling, tremors and at late stage of disease, seizures (Tammen et al., 2006). Profound visual impairment occurs from about 19 months of age, with death by 27 months of age (Cook et al., 2002). The present experiment is the first known controlled experiment investigating the temporal changes to the behavioural time budget and responsiveness of Merino sheep with the homozygous, heterozygous and non-affected genotypes. Previous studies by Tammen et al. (2001, 2006) and Cook et al. (2002) were genetic or prospective clinical-based trials.

The objectives of this experiment were to analyse the progressive postural, behavioural and liveweight changes in NCL-affected Merino sheep compared to heterozygous carriers and homozygous normal sheep, and to establish behavioural indicators that may enable earlier identification of neurological changes in NCL-affected sheep. The findings of the present experiment may assist in measuring the effectiveness of therapeutic trials in sheep, which could potentially assist with trialling therapies for human late infantile NCL.

2. Methods

The experiment was conducted at the University of Sydney Farms, Camden NSW Australia, under approval from the University of Sydney Animal Ethics Committee, and in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. While the lambs were cared for using standard sheep husbandry management procedures, an additional requirement of the Animal Ethics approval was that all sheep in the flock were inspected daily. This was performed by stockpeople experienced in the management of NCL-affected sheep observing all sheep at least once daily from a short distance. In addition, the sheep were brought into a yard at least monthly for closer inspection, weighing and individual health checks. The experiment commenced in mid-March and concluded in late November, 2011. As described later, the disease status of all lambs born in this flock was identified using a direct DNA test. Thus all NCL-affected animals were known to the main researchers and stockpeople before onset of clinical signs, which were expected to commence after about 7 months of age. The AEC protocol required that NCL-affected animals were euthanised before severe mental retardation occurred. In extreme cases in the research flock, this had occurred at about 2 years of age, but lambs identified as NCL-affected were nevertheless closely monitored from 6 months of age for any signs of behavioural change, such as standing quietly in the paddock, diminished fear response if the human approached or loss of herding instinct. Similarly, if seizures were

observed or if the state of health deteriorated (for example, it was noticed that the individual had lost weight, was not feeding, drinking or showed signs of physical discomfort, distress or suffering), they were euthanised. The approved method of euthanasia was via barbiturate over dose through intravenous injection of Sodium Pentobarbitone.

2.1. Subjects and experimental procedures

An experimental Merino *CLN6* research flock was maintained at University of Sydney Farms, Camden, which was based on affected and carrier sheep that were sourced from two medium-wool Merino flocks in northern New South Wales (Tammen et al., 2001; Cook et al., 2002). Clinically normal carrier ewes and carrier rams were mated to produce homozygous affected, heterozygous and homozygous normal lambs in an expected ratio of 1:2:1. Thirty-six lambs born during the 2010 lamb-drop were ear tagged for individual identification and subsequently tested for genotype using a direct DNA test for the disease-causing c.184C>T mutation in ovine *CLN6* (Tammen et al., 2006). Four female lambs homozygous for the c.184C>T mutation (i.e. NCL-affected) were selected for the present experiment, and a sub-flock of eight female lambs was formed consisting of the four NCL-affected lambs, two heterozygous (Carrier) and two non-affected (Normal) lambs. The eight lambs had been born over a 2-week period in September-October 2010. Birth weight of the four NCL-affected lambs were 1.7, 2.6, 3.75 and 3.73 kg, while it was 4.15 and 4.44 kg for the Carrier and 4.2 and 6.0 kg for the Normal lambs. The lambs were weaned from their dams at 23.9 ($\pm$ std dev 0.57) weeks of age. The eight experimental lambs were maintained as a single group, alternately grazed in two adjacent paddocks measuring 800 and 840 m², respectively, to manage pasture availability and worm burdens. Observation of lamb behaviour commenced 2 weeks after weaning and the experiment proceeded for 8 months. During this time, behaviour observations were conducted in 11 observation weeks, at approximately 3–5 week intervals. All behaviour observations were recorded while lambs were located in the 800 m² paddock; the lambs had been in the paddock for at least 4 days prior to commencement of the respective observation week.

On Monday morning in observation weeks the lambs were brought to a nearby yard by two experimenters. Each lamb's ear tag number was recorded and a number between 1 and 8 was sprayed on the sides of the lambs using scourable spray-mark (Leader Stock Marker), for visual identification in the paddock. The respective identifying numbers were allotted at random at the commencement of the experiment, so that the experimenter who conducted the direct visual observations would be blind to the genotype status. A commercially available 3-axis accelerometer (IceTag® Sensor, ver 1.008 (FW 2.008); IceRobotics Ltd, Edinburgh, Scotland, UK) was attached to the left, rear leg using 50 mm wide cloth tape, just above the metatarso-phalangeal joint. The IceTag Sensors weighed 95 g and measured 65 × 60 × 30 mm. The cloth tape used to attach the sensor weighed about 15 g. The use of IceTag Sensors to measure activity in Merino sheep has previously been reported by Fisher et al. (2010). After the IceTag Sensors had been attached to all subjects, the group of lambs was returned to the paddock. Direct behaviour observations occurred on Thursday in the observation week, and on Friday morning the lambs were brought back to the yards, where the IceTag Sensors were removed and the lambs were individually weighed.

2.2. Direct observation of lamb posture and behaviour

The direct visual observations involved recording the posture and behaviour of each lamb in each of the 11 observation weeks. All direct observations were conducted by a single experimenter seated in an elevated position about 5 m outside the fence line of the paddock, and with a clear view of all lambs. Binoculars were used when necessary for closer inspection of the lambs. Each observation day commenced at 09:00 h and was divided into six, 1-h periods. All observations were conducted during daylight hours. The starting times for the six observation sessions per day were: 09:00, 10:15, 11:30, 13:00, 14:15 and 15:30 h. That is, three observation sessions commenced in the morning and three in the afternoon, each with about a 10 min break from the end of one session to the start of the next, with the exception of a 30 min lunch break between observation sessions 3 and 4. On average, the start of observation session 1 was 2.5 h after sunrise (range 2 to 3.5 h), while the completion of observations occurred about 1.5 h before sunset (range 0.5 to 3 h). The instantaneous sampling technique (Martin and Bateson, 2007) was used to record the posture and behaviour of each lamb at 3 min intervals within each 1-h observation period, based on a catalogue of two postures (stand and lie) and nine behaviours (graze, walk {but not grazing while walking}, drink, ruminate, urinate/defecate, groom, stationary but alert, idle and other {including sham-grazing}). After each 1-h observation period, the experimenter (visual stimulus) stood and walked (~10 m) to the entry gate of the paddock, opened the gate, entered the paddock then closed the gate behind her, and quietly walked towards the centre of the paddock. Whilst walking, the experimenter noted on a clip-board the level of reaction of each lamb to her presence in the paddock. Level of reaction was scored on an ordinal scale from 1 to 3, and defined as:

1. 'nil to minimal reaction', in which the sheep did not alter its behaviour, and / or did not orient towards the stimulus
2. 'oriented towards the stimulus briefly, before resuming another behaviour', and
3. 'immediate, prolonged vigilance towards the stimulus', that is remained vigilant and did not resume other behaviours for at least 1 min.

The same 3-point rating scale was used to score the level of behavioural reaction of lambs to an accompanying auditory stimulus. The auditory stimulus was included to supplement the visual stimulus (moving human) and was applied as the experimenter approached the centre of the paddock, and while the experimenter was facing the lambs. Different sounds were used in each of the six test situations per day in an effort to avoid habituation (viz. calling out, whistling, rustling a plastic bag, clapping hands, banging sticks together or hitting a small drum). The test was completed in less than 2 min, and at the conclusion of the test the experimenter left the paddock via the gate. At least 10 min elapsed before the next 1-h observation period commenced.

2.3. Indirect measurement of lamb behaviour, via activity sensors

As described above, an IceTag Sensor was fitted to each lamb at the start of observation weeks. Once activated, the IceTag Sensor continuously recorded the number of steps taken, number of lying bouts, and proportion of time standing and lying. After retrieving the devices from the lambs, the raw data were downloaded from the on-board memory to a

desktop computer and analysed by IceManager 2010 software (IceRobotics Ltd., Edinburgh). IceTag Sensor data were collated for a 72 h period commencing at midnight on the day after attachment of the sensors to the lambs (i.e. Tuesday), and presented on an average daily basis, that is per 24 h. Variables obtained from the IceTag sensor data set have been appended with the prefix 'IceTag_'. For example, the proportion of 24 h that lambs stood is referred to as IceTag_Stand%/24h. The direct visual observations therefore, occurred within the third 24-h period of IceTag Sensor data collection.

2.4. Statistical analysis

Data were analysed using Residual Maximum Likelihood (REML) in GenStat (Release 16.1), with genotype and lamb age (in weeks) specified as fixed effects, and lamb identity (ear tag number) specified as the random effect in the model. An interaction between lamb genotype and age was also included in the model, but removed when found to be non-significant. Wald *F*-tests were used to assess statistical significance, and the default Kenward and Rogers method implemented in GenStat was used to calculate the denominator degrees of freedom for these tests. Traits considered in the analyses were Liveweight and Posture and Behaviour variables. Postures and Behaviours recorded via direct observation of the lambs were expressed as the proportion of direct observations (scans) in which the individual animal occupied the following postures or performed the following behaviours: Stand, Graze, Walk, Ruminant, Alert or Idle. It should be noted that Lie and Other were not analysed, since Stand + Lie = 100, Graze + Walk + Ruminant + Alert + Idle + Other = 100. The response to Visual stimulus and the subsequent Auditory stimulus were analysed as the mean of six tests per day per lamb. Postures and Behaviours recorded indirectly via the IceTag Sensors were expressed as the proportion of time (24 h) in the standing posture (IceTag_Stand%/24h), the number of steps taken per 24 h (IceTag_Steps/24h) and the number of lying bouts per 24 h (IceTag_Lying Bouts/24h). IceTag_Lie%/24h was not analysed, since IceTag_Stand%/24h + IceTag_Lie%/24h = 100. When significant differences involving genotype were observed, either in the form of an interaction, or as a main effect, between-genotype comparisons were undertaken using a least significant difference (LSD) approach. The assumptions of normal distribution of data and variance homogeneity were checked by inspection of residual plots.

To determine if particular posture and behaviour traits could collectively provide a better separation between the genotypic groups, the following 11 traits were analysed using principal components analysis using GenStat: Stand, Graze, Walk, Ruminant, Alert, Idle, Mean response to Visual stimulus, Mean response to Auditory stimulus, IceTag_Stand%/24h, IceTag_Steps/24h, and IceTag_Lying Bouts/24h. The first principal component, a linear weighted combination of the above 11 traits that explained the largest amount of variation, was then considered as an additional trait for analysis using the same REML procedure as noted above.

During direct behaviour observations when the lambs were about 38 weeks of age (observation week 4), the observer reported that one lamb may have had a 'seizure' in the paddock. Thereafter, the lamb was observed to return to 'normal' activity. The lamb, which was from the NCL-affected group, was examined next morning by a veterinarian and the

decision was made to euthanize the lamb by intravenous barbiturate. Thereafter in the statistical analysis, the lamb was treated as a missing value. Thus, in observation weeks 1 to 4, there were 4, 2 and 2 lambs, respectively in the NCL, Carrier and Normal Genotype groups. Thereafter, following the removal of one NCL lamb, there were 3, 2 and 2 lambs per treatment group, respectively.

Results

A key objective of the research was to study differences due to Genotype, particularly as the subjects aged. As such, the results presented only relate to Genotype, or Genotype $\times$ Age interactions.

3.1. Effects on growth

NCL-affected lambs on average weighed 20.1 kg at 26 weeks of age while Carrier and Normal weighed 25.2 and 26.2 kg, respectively. Average liveweight gains between 26 and 60 weeks of age were 11.8, 16.5 and 23.5 kg, respectively, and there was a Genotype $\times$ Age interaction on liveweight the lambs ($F = 3.0$; $df = 20,43$; $P = 0.001$).

3.2. Effects on lamb posture and behaviour

3.2.1. Posture—Standing was the main posture of lambs, recorded via both the direct and indirect recording methods. The pooled mean ($\pm$ std dev) proportion of observations that lambs stood during the direct observations was 90.6% (± 7.57) of the 6 daylight hours of observations per session, whereas the IceTag Sensor data estimated that lambs stood for an average of 59.8% of the 72-h measurement periods. While there was no effect of Genotype on the proportion of direct observations in which the lambs occupied the standing posture, there was an effect of Age ($P < 0.001$) with lambs standing for 80% of observations at 26 weeks and 100% of observations at 60 weeks ($F = 15.3$, $df = 10, 63$). There was no interaction between Genotype and Age. The IceTag Sensor data for time spent standing (3-day average) indicated no effects of Genotype or Age, although there was a weak Genotype $\times$ Age interaction ($F = 1.75$, $df = 20,43$; $P = 0.062$). As shown in Fig. 1, the occurrence of standing posture measured by the IceTag sensors was somewhat erratic over the course of the experiment, although normal lambs consistently spent less time standing. The occurrence of lying bouts identified by the IceTag Sensor algorithms was not different among the main effects and there was no Genotype $\times$ Age interaction.

3.2.2. Behaviour—Grazing was the predominant behaviour recorded for lambs during direct behaviour observations, recorded for 71% of observations (pooled average across lambs and observation sessions). Of the remaining catalogued behaviours, the proportion of observations (pooled averages) in which the behaviours were recorded were: 'idle' (14.8%), 'ruminate' (7.1%), 'walk' (3.4%), 'alert' (2.7%) and 'other' (1.1%).

The change in the time budget for grazing over the course of the experiment is presented in Fig. 2. There was a significant effect of Genotype, with NCL-affected lambs spending less time grazing than Carrier or Normal group lambs (66.5, 73.2 and 75.2% of observations, respectively; $F = 11.53$, $df = 2,4$; $P = 0.018$). There was no Genotype $\times$ Age interaction for

grazing behaviour. Differences in the predicted values for the genotype groups are presented in Fig. 2 with constant difference across ages. Differences in the occurrence of rumination ($F = 11.17$, $df = 10,64$; $P < 0.001$), alert ($F = 2.02$, $df = 10,64$; $P = 0.046$) and idle ($F = 34.07$, $df = 10,64$; $P < 0.001$) behaviours were found due to age, but there were no effects of Genotype and no Genotype $\times$ Age interactions.

The occurrence of walking behaviour recorded during direct observations is presented in Fig. 3a. There was a significant Genotype $\times$ Age interaction ($F = 2.39$, $df = 20,44$; $P = 0.008$) for walking behaviour, although the occurrence of the behaviour was somewhat erratic when plotted over time, with average values for the Genotype $\times$ Age combinations ranging from 0.4 to 8.0% of observations (Fig. 3a). Nevertheless, there was a trend for NCL-affected lambs to be recorded walking more often than the other genotypes. Lambs in the Carrier group tended to walk more than lambs in the Normal group, but the difference was not significant.

The mean number of steps per 24 h period recorded via the IceTag Sensors for all lambs over the 11 observation sessions was 2,940 steps (range 1,375–5,817). While there was a significant Genotype $\times$ Age interaction in walking behaviour, there was no interaction in the number of steps per 24 h period recorded via the IceTag Sensors (Fig. 3b). However, there was a significant effect of Genotype, with NCL-affected animals taking significantly more steps than Carrier or Normal group lambs (3427, 2412 and 2813 steps for NCL, Carrier and Normal, respectively, $F = 13.11$; $df = 2,4$; $P = 0.015$; Fig. 3b).

3.2.3. Responses to visual and auditory stimuli—The mean response scores to visual stimuli for the NCL-affected, Carrier and Normal genotypes were 2.7, 2.8 and 3.0 at 26 weeks of age and 1.4, 2.9 and 3.0 at 60 weeks of age, respectively. There were significant Genotype $\times$ Age interaction effects on the level of response by lambs to the visual ($F = 3.51$, $df = 20,43$; $P < 0.001$) and auditory ($F = 4.31$, $df = 20,43$; $P < 0.001$) stimuli. While the responsiveness of lambs in the Carrier and Normal genotypes remained constant over the course of the experiment, NCL-affected lambs showed a progressive reduction in responsiveness. The responses to auditory stimuli were similar to those recorded in response to the visual stimuli (Fig. 4).

3.3. Principal Components Analysis

The first principal component (PC_1) explained 39% of the variation in the 11 traits. A reasonable interpretation of PC_1 is a contrast between ambulation (+) vs. grazing/eating and responsiveness (–), with animals with positive scores for PC_1 being more ambulatory than sedentary (i.e. idle) and less vigilant, that is, less sensitive to visual and auditory stimuli, and spending less time grazing/eating (Table 1). The latter description therefore represents the profile of an NCL-affected lamb. Conversely, the reverse conclusion can be stated for a Normal (and to some extent a Carrier) genotype group lamb. A further 21% of the variation was explained by the second principal component (PC_2), but there was no obvious behavioural interpretation to this component. After analysing PC_1 as a trait in REML, there was a significant Genotype $\times$ Age interaction ($F = 4.65$, $df = 20,43$; $P < 0.001$). While the scores increased with increasing age for all genotypes, this was far more rapid for NCL-

affected lambs (Fig. 5). Virtually no difference was found between Carrier and Normal genotype lambs for this composite trait.

4. Discussion

The main objective of this experiment was to investigate some practical, non-invasive approaches to facilitate accurate monitoring and evaluation of the progression of NCL in affected Merino lambs. We recorded posture, behaviour and liveweight data, and quantified changes to the variables as the lambs aged. Our approach utilised data collected via a range of relatively simple techniques, including close and distant inspection, and remote monitoring via accelerometers. In addition, a more complex assessment involving analysis of a combination of the variables was performed using principal components analysis. In the future, frequent monitoring of a relevant indicator(s) could occur using automatic recording technology, to facilitate the early detection of NCL symptoms as well as assess disease progression in therapeutical trials. As expected for a recessive disease, the present experiment found no differences between lambs that were heterozygous (Carrier) compared to non-carrier (Normal) lambs for any relevant variables. This discussion therefore will mainly focus on temporal differences between NCL-affected compared to non-affected (Carrier and Normal genotype) lambs. Two limitations of the present experiment concern the small number of NCL-affected, Carrier and Normal individuals involved, and that the lambs were managed as a single flock. High between-animal variation may be exacerbated by small sample size, while studying a single group presents the risk that individuals do not behave in an independent manner. Nevertheless, these limitations may be partly offset by the frequent and detailed observations recorded, and that control animals were included in the sub-flock.

At the commencement of the experiment the lambs were 26 weeks old. While Swedish landrace sheep with *CTSD/CLN10* disease show clinical signs at birth (Järplid and Haltia, 1993) and Woods et al. (1994) reported that one Rambouillet ewe (with an unassigned variant of NCL) showed behavioural signs of NCL at 17 weeks of age, observations on Merinos with *CLN6* disease by Tammen et al. (2001) and Cook et al. (2002) indicated that the earliest clinical signs of NCL (*viz.* mild behavioural changes) occurred after about 30–34 weeks of age. South Hampshire sheep with a different variant of *CLN6* disease and Borderdale sheep with *CLN5* disease are reported to have a somewhat later onset of disease (Bond et al., 2013). Our observational data support the previous findings for Merinos, and indeed one NCL-affected lamb in our experiment was observed ‘having a seizure’ in the paddock at 38 weeks of age. Furthermore, it is relevant that the average liveweight of NCL-affected lambs was lower at the commencement of the experiment than the non-affected lambs, and the growth of the NCL-affected lambs remained poorer throughout the experiment. Storage material and histopathological changes can be observed at birth in the brain of preclinical South Hampshire and Merino sheep with *CLN6* disease, which may result in accrued effects on growth or behaviour from an early age that has not been previously noted (Oswald et al., 2005). Although effects on behaviour due to the homozygous NCL genotype were not expected prior to about 30–34 weeks of age, the statistical analysis identified two variables that differed due to the NCL-affected genotype (*viz.* less grazing/eating behaviour and more IceTag Sensor steps per 24 h), and for which

there was no Genotype $\times$ Age interaction. One interpretation of these results might be that some behavioural effects of NCL in Merino lambs were indeed manifest earlier than 30–34 weeks. NCL-affected lambs for example, performed less grazing/eating behaviour than the non-affected lambs.

Grazing is a major component of the time budget of sheep on pasture (Gonyou, 1983), with time spent grazing per day influenced by a number of factors including season, temperature, diurnal patterns and pasture availability (Arnold, 1960, 1962). The potential variation in grazing time due to these factors suggests that grazing time should not be relied on as a sole predictor of NCL disease onset in lambs. In the present experiment about 70% of behaviour was recorded as grazing, which is very similar to the average of 68% of sheep's diurnal activity reported by Arnold (1960, 1962) for Border Leicester $\times$ Merinos (8.2 h grazing time averaged over 12 h of daylight), Penning et al. (1993) for Scottish Halfbred ewes (10 h eating time over 24 h period, based on measurements of jaw movements) and Sibbald et al. (2006) for Scottish Blackface sheep (79.8% of point sample observations in a test situation). While grazing behaviour and liveweight gain are likely related via feed intake, it is not possible to draw conclusions regarding these relationships in the present experiment, particularly due to the small number of animals involved. Furthermore, Cook et al. (2002) reported that NCL-affected lambs performed sham-eating, although the behaviour occurred in animals that were older than 12 months. Sham eating, which may be considered an abnormal behaviour in which the animal stands with its head down and seems to graze without ingesting food, may be difficult to detect by casual observation. However, the experienced observer in the present experiment was able to differentiate between grazing and sham grazing/eating, which was recorded as 'Other' behaviour. The finding therefore, that 'real' grazing behaviour was reduced in NCL-affected lambs, may only be partly related to the occurrence of sham grazing/eating since the occurrence of the latter behaviour was quite rare and would not account for the full extent of reduced grazing behaviour, compared to the non-affected lambs. Woods et al. (1994) reported changes in eating behaviour involving the consumption of less feed in NCL-affected Rambouillet sheep, but the sheep were older than 12 months at the time of observation. Ingestion of food is clearly important for animal survival, and it is likely that the neurological changes associated with NCL adversely impact on the animal's ability to find and ingest feed. The aetiology of growth deficiency in lysosomal storage diseases such as NCL is unknown, however Woloszynek et al. (2007) found significantly reduced adiposity and lower body weight in infantile NCL mice, despite normal or greater caloric intake. Reduced liveweight gain determined by regular weighing, may therefore be a useful indicator of the onset of NCL in sheep.

In the Camden research facility, close inspection of lambs maintained for NCL research occurs in association with normal husbandry practice: For example, humans enter the paddock at least weekly to perform a 'close' inspection of the sheep, and less frequently, the sheep are mustered between paddocks or brought to the yards for physical inspection including weighing and/or various other husbandry procedures such as vaccination, drenching, shearing, etc. Such inspection events assist with the detection of NCL disease signs by identifying lambs that are not behaving 'normally'. In the present experiment, these 'inspection events' were observed. For example, when the experimenter entered the paddock and generated noise, the observer recorded that normal lambs typically flocked together,

Furthermore, using the principal components analysis enabled all the traits measured to be combined into a single measure, allowing the profile of an NCL-affected versus the other genotype groups to be compared. Our interpretation of this measure for NCL-affected animals describes a lamb with increased ambulation, reduced grazing/eating and lower vigilance (responsiveness to visual and auditory stimuli). Conversely, Normal (and to some extent Carrier) genotype lambs were the opposite description.

In conclusion, over the course of 11 observation weeks from 26–60 weeks of age, there were quantifiable changes in ambulation, grazing and vigilance behaviours that could be useful for differentiation between NCL-affected compared to heterozygous carriers and normal Merino lambs. Monitoring and evaluation of disease progress of NCL in this animal model is clearly important, and the findings of the research provide the basis for a non-invasive way of determining the effectiveness of future therapeutic trials by observing the presence or absence of behavioural signs at progressive stages of NCL disease.

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Highlights

- NCL (Batten Disease) is a fatal neurodegenerative disorder in children
- Lambs are used as animal models for research into NCL disease mechanisms
- Non-invasive determinants of NCL progress are needed to evaluate future therapies
- We investigated some non-invasive biomarkers as indicators of disease progression
- Ambulation, grazing and vigilance were relevant for monitoring NCL disease progress

Fig. 1. Time standing per day measured indirectly via IceTag Sensors during 72 h in observation weeks. The values shown are model-based predicted means $\pm$ standard errors.

Fig. 2. Grazing behaviour measured via direct observation. The figure depicts an effect of genotype. The values shown are model-based predicted means $\pm$ standard errors.

a.

Fig. 3.

a. Walking behaviour, not associated with grazing, measured via direct observation (Genotype $\times$ Age interaction). The values shown are model-based predicted means $\pm$ standard errors.

b. Number of steps taken per 24 h period measured indirectly via IceTag Sensors during 72 h in observation weeks. There was an effect of genotype. The values shown are model-based predicted means $\pm$ standard errors.

Fig. 4.

Response of lambs to the visual stimulus of a human entering, and walking toward the centre of, the lambs' paddock, plotted over the course of the experiment (Genotype $\times$ Age interaction). Values shown are predicted means standard $\pm$ errors.

Fig. 5. Predicted mean values $\pm$ standard errors of the PC₁ scores for the three genotypes over the course of the experiment (Genotype $\times$ Age interaction).

Table 1

Loadings (latent vectors) on the separate variables for principal components 1 and 2.

Variable	Loadings for PC ₁	Loadings for PC ₂
Stand	0.151	0.592
Graze	−0.414	0.429
Walk	0.353	0.094
Ruminate	−0.137	−0.598
Alert	−0.047	−0.092
Idle	0.427	−0.272
Mean response to Noise stimulus	−0.492	−0.059
Mean response to Visual stimulus	−0.485	0.115
IceTag_Stand%/24h	0.295	−0.035
IceTag_Steps/24h	0.373	0.086
IceTag_Lying_Bouts/24h	0.059	0.266