Vocal behaviour as an indicator of lamb vigour

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Certification

CERTIFICATION OF DISSERTATION

I certify that the ideas, experimental work, results, analyses and substance reported in this dissertation are entirely my own effort, except where otherwise acknowledged. I also certify that this work is original and has not been previously submitted for any other degree or qualification.

14th April 2015

Signature of Candidate

Date

Abstract

The viability and survival of the neonate lamb relies on its ability to communicate and maintain a strong attachment with its dam. To date there has been little concise information available about the role of the lamb's behaviour, and in particular the importance of acoustic cues, in this relationship as greater attention has been focused on maternal attributes important in facilitating the maternal-young bond. In human and rodent neonates, acoustic features of the distress vocalisation are used as indices of neurological deficit and integrity both at birth and in infant acoustic cry analysis.

The aim of this thesis was to investigate potential behavioural indicators of lamb vigour, with a particular focus on vocal behaviour, within the first 12 hours of life. Such measures could provide valuable information for development of reproductive breeding objectives, and provide clarity regarding the role of the lamb in failed maternal-young interactions.

Delayed vocalisation initiation in response to a separation stimulus was found to be associated with poor vigour-related behaviour reflecting the capacity of the lamb to reunite and follow the dam over 12 hours postpartum. Vocalisation delay was also associated with risk factors related to poor lamb survival including longer parturition duration, male sex, first parity, heavier birth weight and sire-related conformational attributes likely to result in a more difficult birth. Blood assay markers reflecting fetal distress including poor blood oxygenation, and elevated plasma glucose and lactate levels sampled at birth were also demonstrated to be correlated with vocalisation latency. These associations were concluded to reflect impacts on the lamb's neurological system rather than genetic influences because of evidence provided by within-litter comparisons, and to demonstrate neuroregenerative processes over a 12 hour measurement period.

An analysis of lamb distress signals modelled on acoustic cry analysis of the human neonate was also undertaken to compare vocalisation characteristics of lambs with delayed responses to those with rapid responses indicating vigour. Signal features of delayed response lambs were more likely to demonstrate acoustic parameters reflecting glottal instability, lower amplitude and reduced repetition rate. These lambs were more likely to emit inefficient or inappropriate signals in the context of isolation. A

significantly higher fundamental frequency, an indicator of pathology in the human infant, was not clearly demonstrated to be associated with compromised lambs in this study. It was also found in a two-choice test, where sheep dams were required to demonstrate a preference for signals of their own co-twins, that ewes preferred acoustic signals of lambs correlated with rapid vocalisation response, higher pitch and greater signal stability.

The results indicate that delayed vocalisation responsiveness and other acoustic measures are associated with fetal compromise in the neonate lamb, as shown in the human and rodent models. It was concluded that delayed vocal initiation is a marker for poor postnatal outcome characterised by diminished responsiveness to a distress condition. This research has important implications for understanding failed maternal-young relationships and the consequences for survival in mammalian neonates.

This thesis is dedicated to my mother

To whom I owe the many attributes which made this thesis possible, and who revived and nourished many hundreds of abandoned lambs during her lifetime.

"If the infant fails to cry vigorously….it forfeits its life, the omission being considered an evil omen." Basden (1921), *Among the Ibos of Nigeria*.

Acknowledgements

Firstly I would like to acknowledge the input and support of my supervisors Geoff Hinch and Ali Small who were always ready to provide a sounding board and valuable advice as well as putting in the extra hours during my trials. To Geoff I owe much gratitude for his initial faith to "take me on", for such patient guidance and the encouragement to go in the direction in which I was interested. Geoff also deserves credit for initiating lamb bleat measurements, which became the focus of this thesis, as a behavioural measure in the Sheep CRC INF lambing project. Ali deserves especial credit for facilitating my trials at CSIRO and organising all things logistical so capably.

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Chapter 1

Introduction

Loss of newborn lamb life is of concern in terms of both reproductive wastage and animal welfare. However despite advances in genetic, reproductive and nutritional management in Australia, lamb mortality based on average loss in commercial flocks has consistently remained between 20 and 30% of lambs born per annum across Australian flocks (Fowler, 2007; Hinch & Brien, 2014).

Many interdependent factors are associated with successful survival of the lamb, especially in the early days postpartum, and the body of literature dealing with the many aspects of neonate lamb survival, vigour, physiology and behaviour is considerable. The most complete summary and schematic representation of the complex of variables affecting lamb survival has been provided by Nowak and Poindron (2006) and Nowak, Porter, Blache, and Dwyer (2008), as shown in Figure 1.1. A recent comprehensive review of research pertaining to factors associated with lamb mortality and survival in Australia has also provided by Hinch and Brien (2014). Such information offers a means by which to understand the key physiological and behavioural risk factors affecting viability of the neonate in the first 24 hours postpartum and in turn identify potential management strategies and practices which may contribute to the improvement of lamb survival rates.

The aim of this thesis is to examine means by which lamb survival could be improved by concentrating on the behaviour of the lamb in the first 12 hours of life and identifying traits correlated with lamb vigour and viability. Initial investigation was focused on understanding the major causal factors associated with lamb loss and to continue on from prior research which had identified differences in lamb vigour scores associated with within-breed sire variation (Hergenhan, Hinch, & Ferguson, 2014). The investigation developed into an application of acoustic cry research (an indicator of vigour in the human and rodent infant) to the lamb neonate which, as far as is known, has not been attempted previously.

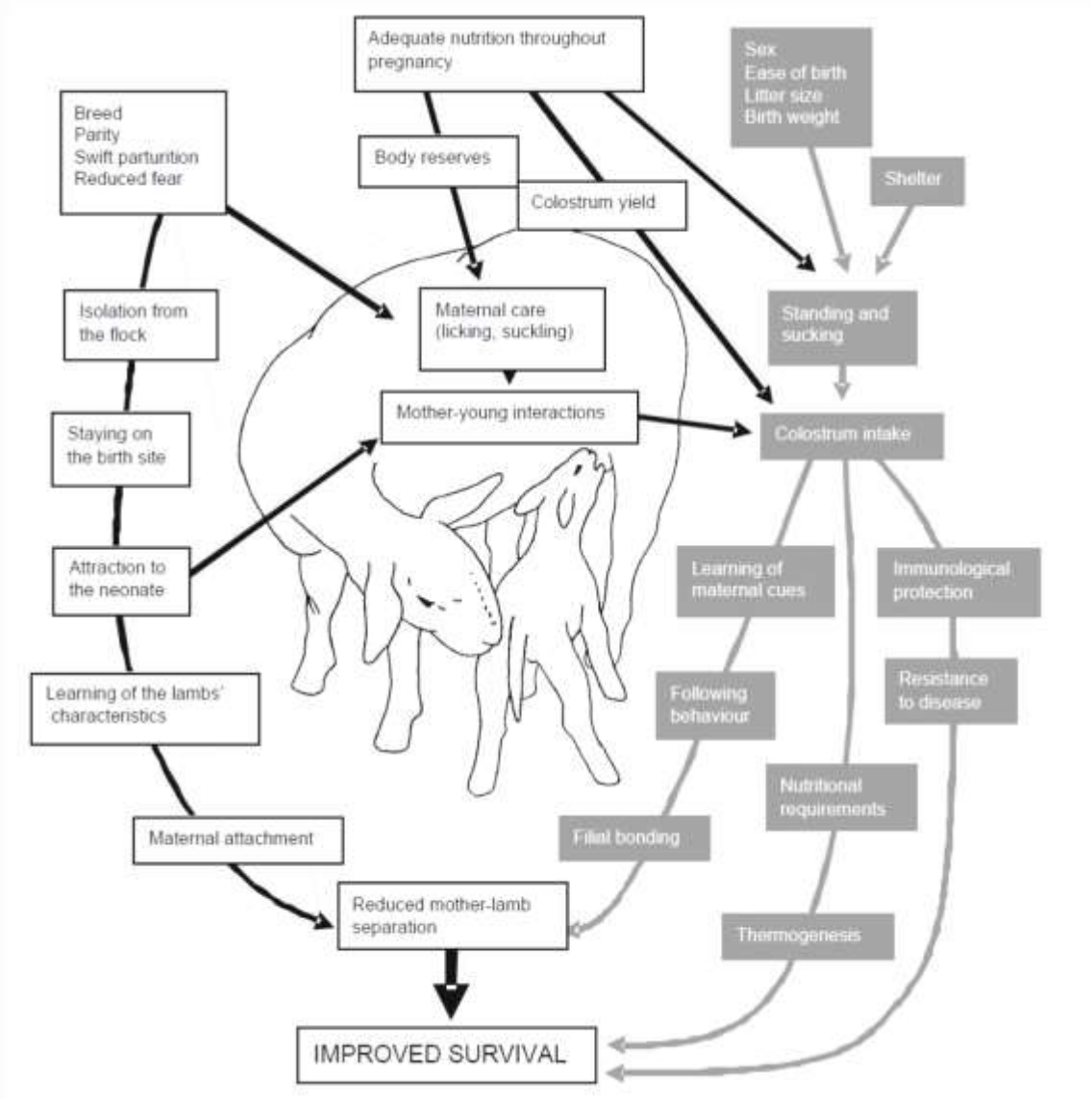


Figure 1.1: Behavioural and environmental factors influencing lamb survival. White boxes relate to maternal factors, and grey boxes offspring factors. Source: Nowak et al. (2008).

The following literature review in Chapter 2 provides a brief overview of risk factors impacting on lamb survival; the current means by which such risks are evaluated; and in particular investigates how other traits, such as vocalisation behaviour, might be used to measure lamb viability. A translational approach assessing relevant literature from the field of human neonate medical research and other mammalian models, in particular the rodent, is a focus of the review; and areas where information may be applicable to the *Ovis* neonate are highlighted.

The subsequent experimental chapters investigate potential indicators of lamb vigour identified in the review. Chapter 3 examines the effects of parity, sex, litter size and sire on vigour-related parameters including vocalisation; and Chapter 4 seeks to confirm those findings, which suggest that vocalisation responsiveness may be associated with fetal distress-related impacts in the neonate lamb. Chapter 5 then applies the human model of acoustic cry analysis to lamb distress signals to test whether other acoustic parameters indicating neurological deficit are paralleled in the lamb.

Finally the effect of vocal signal quality on maternal response is examined in Chapter 6 in an attempt to clarify the role of acoustic cues in failed maternal-young interactions. Chapter 7 discusses the results and conclusions of the experiments, and highlights areas where this thesis may contribute to the body of scientific research currently relevant to neurobehavioural assessment of the lamb and other mammals.

Chapter 2

Literature review

2.1 Lamb survival – the key risks

Mortality rates in the newborn lamb are greatest in the first 3 days of life during which time 80-90% of lamb deaths are estimated to occur indicating that events occurring in this period are the most critical to survival (Dutra, Quintans, & Banchero, 2007). As well as experiencing the challenging process of birth, the neonate is at its most vulnerable and must adapt to the transition from the ewe's uterine environment to an often hostile external environment (Nowak, Porter, Levy, Orgeur, & Schaal, 2000; Sawalha, Conington, Brotherstone, & Villanueva, 2007). A number of factors are reported to influence the chance of survival at this stage; although the two main contributors are commonly agreed to be dystocia (abnormal or difficult labour often resulting in hypoxic injury to the fetal central nervous system; and the starvation/mismothering/exposure complex (Alexander, 1984; Haughey, 1973; Haughey, 1973b).

Based on extensive autopsy studies, Haughey (1973; 1973b; 1980) reported that up to two-thirds of lamb deaths may occur as a direct result of birth trauma or ongoing central nervous system (CNS) effects which subsequently increase the risk of starvation, mismothering and exposure. Other authors including Barlow et al. (1987); Duff, McCutcheon, and McDonald (1982), Holst, Fogarty, and Stanley (2002), Dutra et al. (2007) and Smith (1977) have reported either high rates of mortality associated with cranial or spinal meningeal lesions based on autopsy of dead lambs or more overt signs of dystocia. Alexander (1984) suggested that core loss occurring in the majority of flocks was most likely to be associated with birth related factors and birth weight, but was less convinced of the incidence and role of meningeal haemorrhages in lamb loss. A detailed description of specific neurological and physiological effects of varying degrees of birth trauma, perinatal asphyxia and resulting cerebral oedema (hypoxic-ischemic encephalopathy) associated with high lamb mortality rate is provided by Dutra et al. (2007) implicating birth as the predominant causal factor of loss within the first 24 hours postpartum.

The key risks associated with dystocia and associated intrapartum asphyxia and hypoxic-ischemic encephalopathy have been reported to be high birth weight (Alexander, Stevens, & Bradley, 1990; Dickinson, Hancock, Hovell, Taylor, & Wiener, 1962; Holst et al., 2002; Speijers, Carson, Dawson, Irwin, & Gordon, 2010); male sex (Dwyer, Lawrence, Brown, & Simm, 1996; Smith, 1977); multiple litter size (Dutra & Banchero, 2011; Everett-Hincks, Dodds, & Kerslake, 2007); longer parturition duration (Dutra & Banchero, 2011; Dwyer et al., 1996), gestation length (Dwyer et al., 1996); breed (Dwyer & Bünger, 2011; Grommers, Elving, & Van Eldik, 1985; Knight, Lynch, Hall, & Hockey, 1988; Speijers et al., 2010) and nutrition (Holst et al., 2002) – factors which have also been linked to lamb mortality without an obvious link to the effects of dystocia. Conformational differences associated with shoulder size, muscle:bone ratios, neck musculature, forelimb length and brain maturity (Dutra & Banchero, 2011; Speijers et al., 2010) may also affect parturition duration and asphyxia risk.

From the literature cited above it would seem evident that a significant number of lambs born each year to flocks of varying breed, phenotypic type, nutritional status and litter size may be at risk of CNS impact associated with dystocia or fetal distress during the birth event which could impinge upon postpartum viability. As indicated by the sequelae of events reported by Dutra et al. (2007), not only may lambs be at risk of dying during or shortly after birth, but also death resulting from the cascade of events in the following days associated with impaired maternal-young interaction, thermogenesis, and milk intake.

2.2 Traditional indicators of birth related effects and lamb vigour

Survival probability and viability of the lamb are often equated in the literature with *lamb vigour*, a term cited in reference to traits and behaviours associated with enhanced survival (Alexander et al., 1983; Hergenhan, 2011; Stevens, Alexander, & Lynch, 1982). Such traits include subjectively assessed alertness or activity (Alexander et al., 1990; Owens, Bindon, Edey, & Piper, 1985); measured latencies to perform milestone behaviours such as standing and suckling (Dwyer, 2003; Dwyer et al., 1996; Matheson et al., 2011; Nowak, 1990b) or test arena performance based on lamb discrimination of their dam and problem solving skill (Bickell et al., 2009; Nowak, Poindron, Le Neindre, &

Putu, 1987; Pfister, Davidson, Panter, Cheney, & Molyneux, 2006). These behavioural indices measuring the general condition of the lamb, also reflect CNS function, with retardation of these behaviours being consistent with neurological damage associated with birth difficulty or neurotoxins (Dutra et al., 2007; Dwyer et al., 1996, Pfister et al., 2006). Measurement of these indices may also involve a significant level of vigilance and input which may not be practical in a commercial setting.

To investigate and evaluate the incidence and mechanisms of lamb mortality associated with fetal distress and CNS injury impacting upon postnatal survival, studies have also been conducted in flocks utilising the autopsy procedure outlined by Holst (2004) and Haughey (1973) in Australia (Cloete & Scholtz, 1998; Geenty et al., 2014; Holst et al., 2002), or lambing records of observed cases of dystocia (Smith, 1977). However, autopsy procedure can only be performed on lambs that have died and thus may represent incomplete data, as bodies are often predated upon (Holst et al., 2002; Rowley, 1970). Accurate observation of parturition duration, presentation or lambing ease may also assist to detect lambs which have suffered dystocia associated with obvious clinical signs such as oedema but may not identify subclinical cases of fetal distress (Dutra & Banchero, 2011; Dutra et al., 2007; Haughey, 1973). Such intensive monitoring processes as described above also require resources which are impractical to implement in extensive, or even intensive, production systems.

Where parturition can be monitored over 24 hours, such as in a research setting, lamb viability has been assessed by utilisation of a modified APGAR score, initially proposed for human neonates by Apgar (1953), measuring standing activity, pulse rate, ear/head movement, skin colour including meconium staining and respiration (Dutra & Banchero, 2011). Measurement of blood metabolites associated with hypoxia such as lactate, blood pH, blood gases and acid-base variables (Barlow et al., 1987; Dutra & Banchero, 2011) have also been evaluated. Hypoxic impacts on the CNS, resulting in impaired or compensatory thermostatic regulation (anapyrexia) may also be indicated by lower rectal temperatures (Branco, Gargaglioni, & Barros, 2006; Steiner, Rocha, & Branco, 2002; Trujillo-Ortega, Motra-Rojas, Juarez, Villanueva-Garcia, & al, 2011).

Many of the above measures are difficult and impractical to assess in commercial production systems, and studies seeking to find more practical measurements of neonate

vigour and survival probability are required. Comparison of markers indicating viability deficit in the newborn of other species may reveal useful information, especially in the human, porcine and rodent neonate models where a large volume of neonate research has been conducted. In particular the APGAR score, which has been applied to a number of nonhuman models to assess viability in the neonate, includes assessment of vocalisation parameters indicative of neurological functioning which have rarely been applied to animal neonates with the exception of rodents.

Understanding the relevance of all the parameters utilised in the human APGAR score, and exploration of human neonate medical literature, reveals that vocalisation characteristics have proven to be a valuable and sensitive marker for neurological deficit in both the human and rodent newborn. The following section outlines the common features and current status of human and nonhuman acoustic research and focuses on literature relevant to analysis of infant distress vocalisation and neurobehavioural assessment of the neonate. Further to the comparative research approach which has been applied to the human and rodent neonate, this examination may provide novel and valuable insight concerning vocal behaviour and its potential to be a useful indicator of viability in the neonate lamb.

2.3 Cry analysis as an indicator of neurobehavioural development in the neonate

2.3.1 Acoustic analysis as an indicator of health and pathology

While many of the underlying mechanisms of vocalisation formation and purpose are yet to be completely understood in nonhuman animals, the study of language development and associated vocalisation pathology in humans is relatively advanced. Investigation of vocalisation disorder in humans, including the fields of speech pathology and acoustic voice analysis, is based on diagnosis of neurologic dysfunction and vocal tract disorder in adults and children. The effects on human speech, language development and voice acoustics associated with CNS disorder are well recognised, clinically predictable, and have been reported to have potentially greater sensitivity than other neuropsychological assessments based on behaviour (Vogel, 2010). Neurological dysfunction of speech can manifest as a range of cognitive and motor speech disorders including categories of pathology such as *dysarthria* (disorder related to nervous system control of vocal tract movement), *apraxia* (where speech deficits are a result of impaired brain planning and programming) and *aphasia* (where there is a cognitive deficit manifest as lack of speech including that resulting from brain injury or stroke) (Duffy, 2013). Long-term consequences of even mild CNS damage at birth resulting from perinatal asphyxia of the human infant include deficits in receptive vocabulary and language (Low et al., 1988; Van Handel, Swaab, De Vries, & Jongmans, 2007). Age and gender differences are normally attributed to non-neurological effects such a hormonal disturbance, physiological effects or metabolic disturbances (Duffy, 2013).

Acoustic analysis has rarely been applied as a clinical marker for disease detection and neurological health status in nonhuman mammals. Apart from the increasing use of rodent ultrasonic vocalisation analysis in medical research (Branchi, Santucci, & Alleva, 2001; Zeskind et al., 2011) which will be detailed in a following section of this review, studies of animal condition using acoustic analysis have primarily focused on vocal behaviour as a communication of identify or welfare state, in particular those of animals utilised in intensive livestock production systems (Manteuffel, Puppe, & Schön, 2004; Weary, 1995). Such examples include pain and stress related vocalisation assessment in pigs (Manteuffel & Schön, 2002; Marx, Horn, Thielebein, Knubel, & von Borell, 2003; Moura et al., 2008; Weary, Braithwaite, & Fraser, 1998), feeding and deprivation related

vocalisations in poultry (Zimmerman, Koene, & van Hooff, 2000a, 2000b), and stress vocalisations in cattle and horses (Ikeda & Ishii, 2008; Jahns, 2008; Pond, Darre, Scheifele, & Browning, 2010; Watts, 1999; Watts, 2000, 2001). While bioacoustic studies are required to initially describe, classify and decode the range of signals for any one species, there is considerable potential in this area to use acoustic analysis as a marker for health disorders as has been employed in the human and rodent models.

2.3.2 Development of acoustic analysis in nonhumans

Development of the spectrograph and its application to speech analysis in the 1940s led to great advancement in human voice analysis (Joos, 1948; Koenig, Dunn, & Lacy, 1946; Potter & Peterson, 1948) although perceptual training and use of auditory investigation remains valuable (Vogel, Maruff, Snyder, & Mundt, 2009). However the availability of this technology enabled ornithologists to develop new areas of research including environmental monitoring associated with recording, classifying and analysing birdsong (Catchpole & Slater, 1995). Further to the body of avian research, there is now a growing body of bioacoustic research describing vocalisation characteristics of mammals including marine mammals, primates, deer, elephants, canids, rodents, bats, pigs and others (Boughman & Moss, 2003; Ehret, 1980; Kiley, 1972; Lingle, Wyman, Kotrba, Teichroeb, & Romanow, 2012; Reby & McComb, 2003; Simmons, Popper, & Fay, 2003).

The disciplines of human voice analysis, avian and more recently mammalian bioacoustics have further progressed with the availability of sophisticated digital signal processing software packages accompanied by technical advances in portability and sensitivity of recording devices (Blumstein et al., 2011). Digitised acoustic recordings can be translated to mathematical algorithms describing various features of the signal which can then be applied in objective sound analysis. Automated systems are now being developed to enable more standardised classification of a range of vocalisation parameters in human (Hariharan, Chee, & Yaacob, 2012; Hariharan, Sindhu, & Yaacob, 2012) and more recently nonhuman acoustic research (Clemins, 2005; Clemins & Johnson, 2006; Schön, Puppe, & Manteuffel, 2004).

One of the few areas of bioacoustic research exploring disease associated vocalisation characteristics has been based on the development of automated health detection systems for detection of cough in pigs (Exadaktylos, Silva, Aerts, Taylor, & Berckmans, 2008; Moshou et al., 2001; Van Hirtum & Berckmans, 2003). Evidence of pathology indicated by acoustic analysis has also been demonstrated in veterinary clinic treated animals by Riede (2000) and Riede, Herzel, Hammerschmidt, Brunnberg, and Tembrock (2001) who reported spectral anomalies including biphonation associated with cranio-cerebellar damage in a cat, and higher harmonics-to-noise ratio (HNR) in dogs, purported to be associated with laryngeal hypertension related to excessive barking or surgical nerve stimulation.

While there is much further clarification required in the field of nonhuman vocalisation research, evidence from data collected to date continues to demonstrate that human speech and mammalian vocalisation are founded on essentially similar vocal production systems (Fitch, 2006). Therefore, knowledge of human voice and speech acoustics, phonetics and physiology can continue to be constructively applied in acoustic studies of other mammals, and additionally provide valuable insight in the study of species evolution.

2.3.3 Production of sound and signal analysis

Production of sound by the mammalian vocal tract has been described as a *source and filter* model (Taylor & Reby, 2010) where a signal created by laryngeal restriction (the *source*) of outgoing lung airflow is modified by movement of structures such as the lips, teeth, nasal and pharyngeal cavities and structures of the vocal track (the *filter*) (Figure 2.1). The moveable components of the vocal tract which can be used to alter sound are also called *articulators*. The vibrating *vocal folds* (cords) of the larynx and the changing opening (*glottis*), alternate the pulse of airflow originating in the lungs, creating changes in pressure and resulting sound waves (Figure 2.2). The *ventricular folds* or 'false vocal folds' are fleshy structures above the vocal folds which do not normally take part in vocalisation. Air from the lungs forces the mobile folds apart and air flow builds up between them. Two forces (marked as P in Figure 2.2) pull the folds back to their central

position: the natural elastic qualities of the folds themselves and the local decrease in pressure as the air streams through the open glottis (the Bernoulli principal).

The sound produced is composed of a number of components – the rate of variation in air pressure and sound wave transmission within a second (*frequency*, measured in Hz), the height of the sound wave (*amplitude*, measured as dBs) and concentrations of enhanced frequencies into energy bands or overtones (*formant frequencies*, Hz).

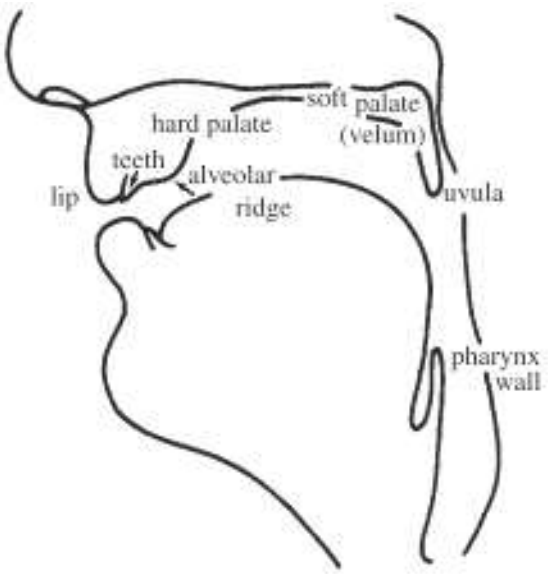


Figure 2.1: Moveable components (articulators) of the upper surface of the vocal tract. Source: Ladefoged (1993).

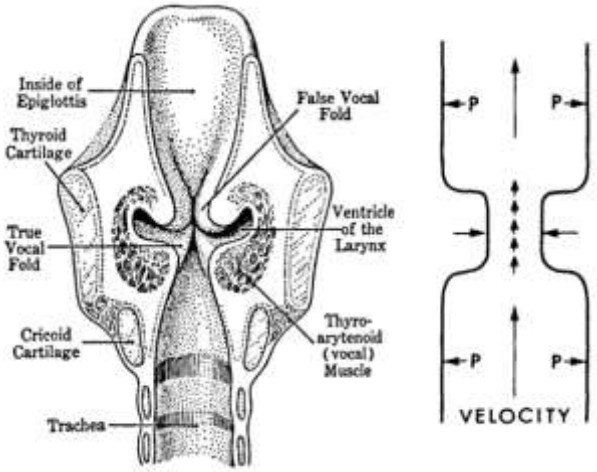


Figure 2.2: Cross section of the larynx showing the arrangement of the vocal folds which can be changed in length and tension by movements of the arytenoid and thyroid cartilages and contraction of the thyro-arytenoid muscles. The glottis is the gap between the vocal folds.
Source: http://www.phon.ucl.ac.uk/courses/

The *fundamental frequency (F*0) of a sound is usually equated with *pitch* (an auditory property enabling the listener to scale the *F*0 of a sound) which is dependent on the rate of vibration and functioning of the vocal cords. The overall loudness or *intensity* of a sound is proportional to the average amplitude value or size of air pressure variation and can be dependent on lung size, vocal tract muscle function and mouth characteristics (Taylor & Reby, 2010; Titze, 1994). Modification of sound by the vocal tract, particularly the length, shape and openness of the mouth aperture and its structures, affects resonance and *formant frequencies* (usually reported as *F1-F5*), the arrangement and values of which are responsible for the characteristic sounds of vowels (Ladefoged, 1993). Numerous studies have applied the source-filter theory to nonhuman bioacoustic studies in order to determine physical attributes of the caller (Briefer & McElligott, 2011; de la Torre, Briefer, Reader, & McElligott, 2015; Taylor & Reby, 2010), however as acoustic parameters can reflect both source and filter related qualities the information provided may be less easily clarified (Sanvito, Galimberti, & Miller, 2007). In fact many studies have shown that *F*0 appears to be a poor indicator of body or larynx size (Reby & McComb, 2003; Riede & Titze, 2008; Sanvito et al., 2007; Teichroeb, Riede, Kotrba, & Lingle, 2013). Analytical frameworks based on bird song measurement may also not be suitable for mammalian acoustic parameters as the syrinx of birds is located at the bottom

of the trachea and resonance properties of the upper vocal tract in mammals are quite different (Fitch & Hauser, 2003).

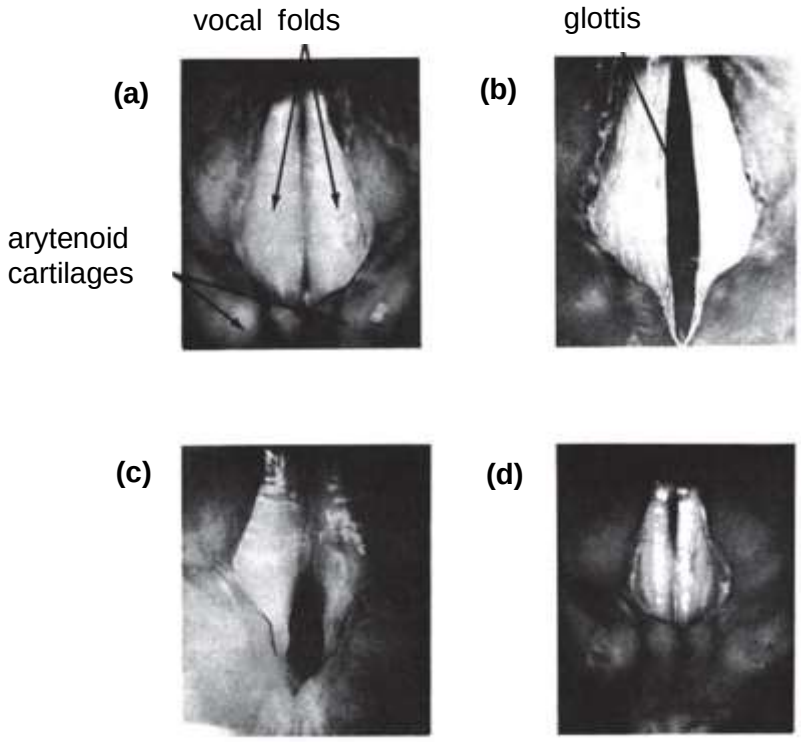


Figure 2.3: Four different states of the glottis.
(a) Touched or nearly touched – folds vibrating, sounded produced are voiced including all vowel and consonant sounds such as */ "a" / "i" / "m" / "n" / "b"/.*
(b) Wide apart – folds not vibrating, sounds produced are voiceless including / "*h" / "p" / "s" / "f" /.*
(c) Apart at one end - folds vibrating, sounds produced are murmured "*aha*" .
(d) Incomplete closure – a low pitched sound, vocal folds vibrating.
Source: Ladefoged (1993).

Most normal mammalian vocalisation can be described as periodic because of the regularity of the vibration of the vocal cords, although there is some deviation around this (Fitch & Hauser, 2003). These sounds create spectrographic signal components based on non-random changes in frequency which are linear and can be analysed by Fourier analysis. In human speech the activation of the vocal cords gives rise to what is termed a *voiced* signal, which can be clearly visualised with harmonic structure and glottal pulsing on a spectrogram. *Voiceless* phonations, evoked by the passage of air past unengaged or unclosed vocal cords (Figure 2.3), such as the sounds "*sssssss"* and "*fffffff"* are created by obstruction of air by articulators and are characterised by a nonlinear pattern on a spectrogram. Nasal sounds can also be made by closure of the mouth in conjunction with positioning of other upper vocal tract structures such as the velum. Mammals have large

surface areas of soft tissue within the upper vocal tract (including the nasal areas) which in the state of mouth closure acts as a resonance chamber (Fitch, 2000; Sanvito et al., 2007). Nasal sounds generally have lower energy concentration and lower formants. Humans frequently make signals with both nasal and oral components, and sounds with both voiced and voiceless components (Ladefoged, 1993).

Spectrographic analysis of the human voice is an additional aid to perceptual evaluation of voice change and pathology, and development of detailed standardised analysis procedures for voice analysis (as opposed to speech analysis which includes vocal tract effects) have been documented by Vogel et al. (2009) and Titze (1995). These methods involve measurement of speech and non speech utterances to assess acoustic parameters. A mid-section segment from the spectrogram of a sustained vowel test utterance, held for 2 seconds and voiced at comfortable pitch and loudness, is the recommended analysis protocol to assess glottal functioning and voice stability (Titze, 1995). For analysis purposes relevant to this dissertation, it should be noted that only open-mouthed signals can produce vowel like sounds (Ladefoged, 1993). Pathological voice measures indicative of poor neurological or associated physiological processes include perturbation, fluctuation, variability, jitter, shimmer, tremor, vibrato, flutter, roughness, breathiness and hoarseness (for a description of these terms refer to Table 2.1 and Titze, 1995). Interestingly *flutter,* which signifies a phonation of both amplitude and frequency modulation in the 8-12 Hz range, is also termed bleat because the fluctuation of the sound is associated with a lamb (Titze, 1995).

In nonhuman signal analysis the spectrographic structure of sounds with clear harmonics and formants, much the same as a voiced signal in human speech, are typically referred to as *tonal* (Lingle, Rendall, & Pellis, 2007; Riede et al., 2001). However nomenclature is often confused between acoustic parameters and phonetic descriptions so varying types of signal including closed-mouthed vocalisations appear to have been described as tonal, although the majority of signals referred to by this term are those where the vocal folds are engaged and usually associated with sound emission via the oral aperture. *Atonal* signals have been described as those relating to a *noisy* spectrographic structure of irregular energy without clear harmonics (Lingle et al., 2007; Riede et al., 2001). In mammalian bioacoustic studies, signals which do not fit a linear pattern have been also been described generally as nonlinear phenonoma, associated with deterministic chaos in

the dynamics of the vocal source (Fitch, Neubauer, & Herzel, 2002; Wilden, Herzel, Peters, & Tembrock, 1998) and, as noted by Wilden, considerable disparity in human and mammalian acoustic terms has promoted a lack of standardisation. Nonlinear phenonoma may refer to biphonation, subharmonics or other characteristics associated with uneven vocal fold functioning in human speech, which can be indicative of pathology or deliberate voice change such as *vocal fry* in humans (Titze, 1995). Many such features are also evident in nonhuman signals which may indicate either pathology (Riede, Tembrock, Herzel, & Brunnberg, 1997) or specific context-related signal features (Wilden et al., 1998) related to vocal tract manipulation. Because the documentation of the repertoire of signals of most species is only in its infancy, a great deal of further detailed investigation is necessary.

Few bioacoustic studies have identified nasal or non-laryngeal components in mammalian signals, or whether the signal vocalisation was made with the mouth open or closed. Closed-mouth signals or signal components will result in low frequency bands or nasal formants on the spectrogram. The study by Kiley (1972) applies phonetic terms to ungulate vocalisations such as *filtration* (whether the mouth is open or not) and includes useful and detailed descriptions of a number of other acoustic parameters. Those which have also noted nasal vocalisations include studies of primates (Hauser, 1992), horses (Yeon, 2012), elephants (Stoeger et al., 2012b), elephant seals (Sanvito et al., 2007), dogs and pigs (Fitch, 2000; Fitch, 2006) as well as ungulate mothers and young (Lenhardt, 1977; Lingle et al., 2007; Sèbe, Duboscq, Aubin, Ligout, & Poindron, 2010; Siebert, Langbein, Schön, Tuchscherer, & Puppe, 2011; Volodin, Lapshina, Volodina, Frey, & Soldatova, 2011). There appears to be a great need for improved observations and descriptions of these types of vocalisations to better understand spectral results and accurately interpret signal parameters (Sanvito et al., 2007). Acoustic signals gathered remotely, without the aid of visual interpretation of vocal tract opening or activity, may lead to deficits in understanding vocal tract positioning correlated to spectral data. Volodin et al. (2011) compared the production of oral and nasal vocalisations in young gazelles with supporting video data, describing commencement of oral calls with a nasal component.

For the purpose of this dissertation, definitions of signal type will adhere to phonetic nomenclature where tonal signals refer to those where the mouth aperture is open (also

described as oral calls by Volodin et al. (2011)0 and the vocal folds engaged so that the glottis is vibrating (i.e. a voiced phonation). A nasal signal refers to that which is emitted entirely through the nasal passages (Richardson, Jacobson, Muncy, & Perkins, 1983; Sanvito et al., 2007; Volodin et al., 2011), usually while the vocal folds are engaged and the velum lowered. The resultant filtering effects and resonance may depend on multiple resonators related to the morphology of the emitter, and therefore signal analysis is more complex (Sanvito et al., 2007). Nasal signals have also been described as low frequency vocalisations (de la Torre et al., 2015; Sèbe et al., 2010), close contact calls (Lingle et al., 2012) and mouth-closed calls (Sèbe et al., 2010). For reference, a general list of key acoustic terms and definitions related to bioacoustics, voice and cry research is shown in Table 2.1.

Table 2.1: Terms used in human and nonhuman acoustic analysis.

Terms	Definition
Common bioacoustic terms:	
Biphonation:	Phonation with two independent pitches. Shown as two nonparallel lines in a spectrogram.
Broadband:	A non tonal signal, often including nasal resonance. Energy is relatively evenly distributed across frequencies, as opposed to tonal signals where energy is concentrated into bands of fundamental and harmonics. Eg click, screech, growl or hiss.
Chaos:	Qualitative description of the behaviour of a dynamical system that is non random but aperiodic. Can refer to laryngeal noise, irregular pulses, arrhythmic cries or any other irregularity.
Distress vocalisation:	Vocal signal emitted when isolated or distressed - typically a tonal, oral signal of high amplitude.
Formant:	Group of overtones corresponding to resonating frequency in the airway above the glottis, accentuating certain harmonics in a phonation/vocalisation.
Frequency:	Number of cycles per second, and dependant on rate of variation in air pressure.
Fundamental frequency (*F*0):	The rate at which vocal folds vibrate, and corresponding to the perceivable pitch of the sound. Calculated as the reciprocal of the period (Hz). The rate of vibration depends on the length, thickness, and tension of the vocal cords, and thus is different for child, adult male and female speech.
Harmonics	Multiples of the *F*0, also termed overtones. Dependant on oral cavity traits and reshaping - lower harmonics become louder when the mouth cavity is longer, higher harmonics become louder when the cavity is shorter (human phonetic text - Silverman, 2006).
Source-filter theory:	A model of airflow dynamics where air from the lungs causes the vocal folds to vibrate (the sound source), which then may be altered by resonance features and manipulation of the upper vocal tract (filtered).
Subharmonics:	Frequencies below the *F*0 of an oscillator in a ratio of 1/n, also termed double harmonic break and a range of other dysphonic terms.
Tonal:	A signal with clear harmonic structure and constant periodicity or vocal fold vibration. Energy is concentrated into bands of *F*0 and harmonics.
USV:	Ultrasonic vocalisation produced by rodents, *F*0 usually above 22 kHz (adults) and up to 100 kHz (infants).
Velum:	Soft moveable part of the palate at the back of the throat.
Vocalisation:	Sound or signal made by an animal. Equivalent to an utterance but not necessarily a phonation.
Common human voice/cry analysis terms:	
Amplitude:	Intensity of sound, heard as loudness (dB).
Aperiodic:	Absence of periodic oscillations. Generally, any deviation from periodicity.
Aphasia:	Inability to produce an utterance.
Aphonia:	Inability to set the vocal folds in motion.
Duration:	Time from onset to offset of a single expiration utterance/vocalisation (ms).
Dysphasia:	Abnormal sequence of utterances.
Dysphonation:	Abnormal phonation such as caused by noisy or inharmonic vibration of vocal folds.
Gliding:	A sudden change of pitch of >600 Hz in 0.1 s. Assessed in infant cry analysis.

Terms	Definition
Glottis:	Airspace between the vocal folds.
Glottal instability:	Inability to maintain an acoustically stable laryngeal configuration or function.
Harmonic to Noise ratio (HNR):	An indication of the overall periodicity of the voice signal which quantifies the ratio between the periodic (harmonic part) and aperiodic (noise) components. Used as a measure of hoarseness and vocal fold function. Threshold for human pathology <20 (a sustained vowel signal should have a higher harmonic component).
Hyperphonation:	Cry segments of very high *F*0 (>1000 Hz).
Intensity:	Amount of acoustic energy in a sound, proportional to amplitude.
Jitter:	Cycle to cycle variation in the *F*0 of a signal. Measured on sustained vowel phonation. Threshold for human pathology >1.040%.
Latency:	Time from application of known stimulus to onset of the first cry utterance/vocalisation.
Melody type:	Structure of utterance based on change of *F*0 levels within a signal of more than 10% including falling, rising, falling-rising or flat (*F*0 does not change by more than 10% in one utterance).
Nasal	A sound where the soft palate is lowered so that there is no velic closure and air travels out through the nasal passage. Eg "*m*" in "*me*".
Noise	Signal segments without periodicity. Noise in a signal can be composed of a wide range of frequencies, depending on context. Overall term for an acoustic event with a chaotic or random structure, as opposed to a tonal signal.
Perturbation	Constant disturbance in a cyclic variable (amplitude and *F*0) and an indicator of poor vocal fold function. Measured by jitter, shimmer and HNR.
Phonation	Process of creating sound by vocal fold vibration. An utterance may not be a phonation.
Pulsed phonation	Phonation where temporal gaps of non-voiced signal are perceived. Acoustically perceived when signal segments fall below 70 Hz where formant energy fades before excitation of a new glottal pulse.
Shimmer	Cycle to cycle variation in the amplitude of a signal. Threshold for human pathology >3.810%.
Utterance	Term commonly used in cry analysis for a sound (vocalisation) made by the infant.
Vibrato	Rapid fluctuations of *F*0 within a 4-7 Hz range.
Voiced phonation	Vibrations of vocal folds occur during articulation. Eg "*me*".
Voiceless phonation	Articulation without vocal fold vibration. Eg only the "*s* " in "*see*" .

Reference material sourced from LaGasse, Neal, and Lester (2005), Ladefoged (1993), Michelsson (1971), Silverman (2006), Titze (1995), and Wilden et al. (1998).

2.3.4 Neurological control of vocalisation

Much of what is known about human and nonhuman initiation and control of signal production has in fact been based on experiments conducted on animals including monkeys, cats, dogs and rodents. Chemical and mechanical stimulation or elimination of the midbrain, brainstem and paraquandral grey (PAG) areas of the brain have provided evidence for distress signal origin (Jürgens, 2009; MacLean, 1985; Newman, 2007). This research has suggested that distinct areas of the brain contribute to different mammalian vocalisation features and that CNS control involves midbrain, brainstem, limbic system and motor cortex areas. At a direct and elementary level, damage to the cranial nerves 9-12 of the vagal complex and nucleus ambiguous in the lower brainstem may result in changes to fundamental frequency (*F*0) because of loss of control of glottal closure and muscles of the larynx. Likewise formant frequencies may be impacted upon due to loss of control of upper vocal tract components or articulators (Lester & Boukydis, 1992) (see Section 2.2.3).

Control of higher brainstem functionality is evident from the systematic lesioning experiments reported by Jürgens (2009) who proposed a model differentiating between the initiation of vocalisation and learning of vocal patterns consisting of 2 hierarchical pathways (Figure 2.4). This model describes a pathway whereby the initiation of, or readiness to, vocalise appears to be controlled by the anterior limbic cortex and hypothalamus which are activated on receival of stimuli. Depending on the affective state of the organism (for example hunger or pain in the neonate), transmission passes to the PAG which in turn is a relay station for processing of messages to the reticular formation of the lower brainstem. A second pathway responsible for voluntary and learned vocal patterns, such as species specific vocal behaviour, passes from the motor cortex to the reticular formation and then feeds back via 2 loops to the basal ganglia or the cerebellum. Evidence supporting a commonality of neural pathways has been observed in all animals studied so far, including humans with mutism (Jürgens, 2009).

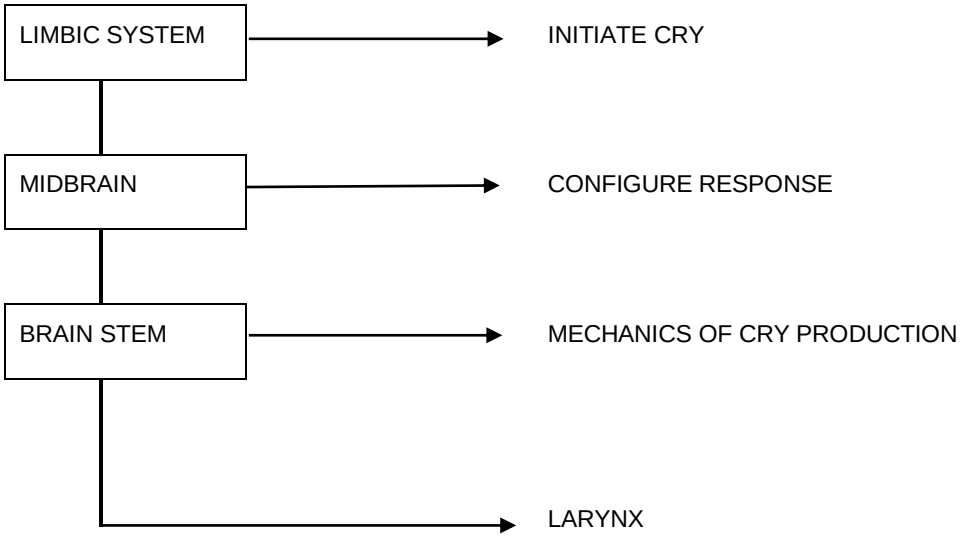


Figure 2.4: Hierarchical model of neonatal cry. Source: Lester and Boukydis (1992).

The results of diagnostic studies conducted on human and rodent infants reported in the following section further support the conclusion that the neural pathways at the core of vocal behaviour, and especially in infants, appear to be highly conserved across mammalian species (Jürgens, 2009; Lester & Boukydis, 1992; Newman, 2007). In the case of the innate and involuntary distress cry response of the human neonate, Lester and Boukydis (1992) assert dominance of the limbic pathway and lack of cortical control. However, a change from involuntary to voluntary control of the cry response occurs in human infants at 2-3 months of age, which corresponds to the developmental milestone associated with transition from physiological to social regulation (Papousek & Papousek, 1984). This "*biobehavioural shift*", thought to involve CNS reorganisation, also involves the repositioning of the larynx in the vocal tract (Lester & Boukydis, 1992). The ontogenic acoustic change occurring as young transition from neonate to juvenile stage, related to dependence and need for caregiver response, also appears common in nonhuman infants (Ehret, 1980; Owings & Morton, 1998).

2.3.5 Mammalian signal types and the neonate distress call

The acoustic signals used by mammalian, and indeed non mammalian, species to convey information are diverse; perform a wide range of functions; and differ greatly in temporal, acoustic and spectral characteristics. In bioacoustics the term "*honest signalling*" is used to describe signals which impart accurate information about environmental conditions or physiological characteristics and need of the caller, as opposed to deceptive signals designed to mislead predators or rivals (Fitch & Hauser, 2003). Physical constraints of production on formant frequencies, for example, can indicate body size, age and reproductive fitness in deer stags (Reby & McComb, 2003); and age, body size and sex in goat kids (Briefer & McElligott, 2011). Some assumptions regarding formant frequency can be misleading where species engage in larynx lowering and vocal tract elongation for certain vocalisation types (Fitch, 2000; Sanvito et al., 2007). Moreover, the difficulties of determining vocal parameters of free-ranging animal species, over widely varying distances and vegetative environments which impact on signal analysis interpretation remains a considerable constraint. In terms of data collected for acoustic analysis, fundamental frequency (*F*0) has frequently been found to be the most consistent and reliable parameter in measurements collected over distance (Maciej, Fischer, & Hammerschmidt, 2011). Similarly *F*0 is the most useful and reliable parameter utilised in clinical assessment of humans (Vogel, 2010).

While the underlying anatomy of the vocal chords and physiology of sound production are similar in all mammals (Fitch & Hauser, 2003), there is great intra- and interspecies variation possible in the type of sound made because of the morphology of the vocal tract and control of signal output by laryngeal, nasal and oral (articulator) movement. Signals can vary from simple to complex sounds with varied concentration of harmonic, melody and frequency components which fall within the range of hearing of the particular species. Harmonic parameters of a signal may change, for example, both with species-related traits and by reshaping of an individual's mouth cavity and positioning of the lips and tongue (Silverman, 2006).

The production of ultrasonic vocalisations (USVs) of small mammals such as rodents and bats enable communication which is not detected by birds or other predators unable to hear signals above 10-20 kHz. Such sounds also do not transmit over distance and are

readily localised - features which assist retrieval of rodent pups and discourage nest departure (Branchi et al., 2001). Alternatively some vocalisations, such as those reported in human and primate studies, can also be described as graded signals (Gustafson, Wood, & Green, 2000; Newman & Goedeking, 1992) which can vary in acoustic parameters such as duration, amplitude or frequency in response to motivational or emotional state. Owings and Morton (1998) described this type of signal production, found in avian and mammalian vocalisation, as adhering to a *motivation-structural* code where the structure and type of signal made at any time by an individual reflects difference in motivation state.

In adherence to the motivation-structural code principal, signals based on function such as predator avoidance, maternal bonding, reproductive behaviour, communication of affective state (aggression, submission for example) are often similar and recognisable across many mammalian and bird species (Owren & Rendall, 2003). There is also evidence that these calls are recognised between species, particularly alarm calls (Seyfarth & Cheney, 1990; Slobodchikoff, 2010) and as recently shown, neonate distress signals (Custance & Mayer, 2012; Lingle & Riede, 2014). Broadly, signals can be categorised into distinct groups including alarm calls (Hollén & Radford, 2009), resource-recruitment signals (Bradbury & Vehrencamp, 1998), aggression/warning signals, mating calls, contact calls and maternal-neonate calls (Lingle et al., 2012; Owings & Morton, 1998). Bioacoustic research documenting the range of call types made by some species is available in considerable detail (Atkeson, Marchinton, & Miller, 1988; Boughman & Moss, 2003; Ehret, 1980; Kiley, 1972), but the vocalisation types of the neonate will be the focus of the remainder of this review.

Neonate vocal signals

The care soliciting vocalisations of mammalian and avian infants have evolved to meet a very specific aim - to attract and compel the parent to provide care and ensure survival of the young. The neonate separation call is considered to be the most primitive of all mammalian vocalisations (MacLean 1985) and studies of separation calls report a similar basic acoustic structure across a range of mammalian species (Lingle et al., 2012; Newman, 2007). Evidence of the same neural circuitry across mammalian and bird species, and alignment of critical periods of vocal behaviour, has been used to support the

conclusion that evolution of the infant cry pathway has remained relatively unchanged or has converged toward a similar configuration which is effective in ensuring reproductive success within a range of environments and social situations (Newman, 2007).

The medical definition of *neonate* in humans implies a newborn aged up to 28 days, as opposed to an infant, a word which interestingly originates from the Latin word *infans* meaning "unable to speak". The age definition of a neonate would thus be expected to differ across species if the transition from neonate to juvenile stage correlates with changes in social communication and reactivity (Ehret, 1980; Papousek & Papousek, 1984). For example the vocal behaviour of young precocious, mobile species like the sheep would be expected to change more rapidly than altricial, nesting species like the rodent. However, age definition of a nonhuman neonate is also commonly up to 28 days (Lingle et al., 2012). Review of mammalian vocal development demonstrates that many species show a transition from neonate distress vocalisation to juvenile and then adult type calls at specific and often common time points (Ehret, 1980). All neonates across the species of Chiroptera, Carnivera, Rodentia and Primate produce at least one type of vocalisation (the isolation distress signal), although in species where this is the only known signal, the full range of signals produced by those neonates may not have been documented (Ehret, 1980).

The distress vocalisation

The neonate distress signal or "cry" is commonly used to describe the sound of a neonate in distress, either from pain or more frequently in nonhumans, from separation or isolation. It is regarded as an honest signal in both the nonhuman (Lingle et al., 2012) and human literature (Furlow, 1997; Soltis, 2004) because it reflects the need and the status of the signaller at the time of emission. The term "contact call" or "attraction call" has also been used in some studies (Lingle et al., 2012) but which can also refer to calls where vocal contact is being maintained but distress is not evident - for example, Briefer and McElligott (2011). The distress vocalisation signal is typically tonal, consisting of clear harmonics and acoustic features associated with intensity, with the aim to be heard by the caregiver and elicit immediate response. Studies have shown that the distress cry of a human neonate associated with pain can be differentiated from a hunger cry on the basis of acoustic structure related to perceptions of urgency, arousal and aversion, with the

initial segments of the pain cry being most potent in evoking a response (Zeskind, Sale, Maio, Huntington, & Weiseman, 1985).

In an interspecies comparison of neonate distress vocalisation structure, Lingle et al. (2012) reported that frequency modulation of most distress signals in the majority of species was relatively simple and exhibited a chevron pattern (rising then falling). Isolation and capture calls of mammalian neonates appear to be fundamentally equivalent, differing only in urgency based on acoustic parameters associated with increased distress such as increase in maximum *F*0, amplitude and upward shift in energy distribution (Lingle et al., 2012). Similar acoustic features have also been found in chickens (Marx, Leppelt, & Ellendorff, 2001) and crocodilians (Vergne, Aubin, Taylor, & Mathevon, 2011). Demonstration that the neural pathways of separation vocalisations are independent to those associated with behavioural expressions of anxiety in adult dogs provides further evidence that the separation distress vocalisation is more innate than the emotion of anxiety (Scott, cited in Newman, 2007).

Crying behaviour associated with a signal of such specific acoustic characteristics as described above has proven to be particularly effective in eliciting responses from caregivers within a species (Soltis, 2004) as well as across species (Lingle & Riede, 2014). The similarity of distress cry acoustic structure and the response of caregivers across species with taxonomically and ecologically diverse physiology, provides further evidence for the same neural foundation for infant distress signal production (Lingle & Riede, 2014; Lingle et al., 2012). The distress vocalisation has been the focus of a large body of research as a measure of neurological functioning in the human and rodent neonate for some years (Branchi et al., 2001; Newman, 2007; Zeskind et al., 2011), as well as the basis of study of maternal-young interaction and maternal behaviour (Rosenblatt, Mayer, & Siegel, 1985; Wiesenfeld & Malatesta, 1982). Studies related to distress vocalisation in human and rat neonates are many and are reviewed in Section 2.3.6.

The close-range vocalisation

While not as well documented acoustically or phonetically in the bioacoustic literature, close contact vocalisations, often made with the mouth closed, have been documented in both adult and young of a number of species including sheep (Sèbe, Nowak, Poindron, & Aubin, 2007); goats (Briefer & McElligott, 2011b); gazelles and deer (Atkeson et al., 1988; Faatz, 1976; Lingle et al., 2007; Richardson et al., 1983; Teichroeb et al., 2013; Volodin et al., 2011); seals (Phillips & Stirling, 2000; Van Opzeeland & Van Parijs, 2004); marmosets (Epple, 1968); chicks (Collias & Joos, 1953; McBride & Lickliter, 1994); dogs and guinea pigs (Ehret, 1980); pandas (Stoeger, Baotic, Li, & Charlton, 2012); cattle, horses and pigs (de la Torre et al., 2015; Illmann, Schrader, Špinka, & Šustr, 2002; Kiley, 1972; Tallet et al., 2013) and primates (Hammerschmidt & Fischer, 1998; Newman, 1985).

These vocalisations are typically of low amplitude and frequency (Sèbe et al., 2010; Volodin et al., 2011) and are reported to be for the purpose of reassurance and comfort of the young at close range (Shillito, 1972; Volodin et al., 2011) and to possibly reduce further loud vocalisation of the neonate and risk of supplying positional information to predators (Kiley, 1972). Different types of close-range vocalisations have been documented in deer (mews, low and high whine sounds) which appear to be utilised in different close range contexts such as nursing (Atkeson et al., 1988; Faatz, 1976) and may have a variety of purposes (Richardson et al., 1983). The function of the low amplitude vocalisation of the neonate is not well reported across species, although in sheep, Nowak (1990a, 1996) suggests that vocalisation of the young in the immediate postpartum phase may serve to strengthen maternal-young bond. Lingle et al. (2012) also reported occurrence of low and high amplitude broadband signals among infants of the species in their study, however, based on the general definition of broadband (refer to Table 2.1), information is not sufficient to allow interpretation of the calls as only close contact reassurance vocalisations. The ability of the neonate to communicate contentment or wellbeing has not been well documented in the nonhuman literature but may be of interest in terms of cognitive state and neurobehavioral responses.

2.3.6 Neonate studies related to neurobehavioral state

Research documenting cry analysis in human and rodent neonates is reviewed below on the basis that it provides opportunity to consider application to other mammalian species. The origins of human cry analysis stem from the earliest observations that human newborns which did not cry immediately and vigorously following birth were at risk of poor survival. Such babies were discarded in African cultures (Basden, 1921) rather than waste parental investment (Soltis, 2004). Virginia Apgar in the 1950s was the first to formally recognise the importance of timely crying of the neonate following birth by developing the APGAR scoring system which remains unadjusted and widely used throughout the world today as a measure of infant viability (Apgar, 1953; Casey, McIntire, & Leveno, 2001). The APGAR assessment is based on a total score out of 10, assessed at birth and at 5 minutes following delivery, where the parameters listed in Table 2.2 are assigned a value of 0 to 2. A score of a maximum of 6 would be given based on lack of cry alone, compared to a normal score of 7 or higher indicating good to optimal neonate condition (Casey et al., 2001).

Researchers assessing neonate vigour in nonhuman mammals have also applied modified APGAR scores to lambs (Dutra & Banchero, 2011; Pfister et al., 2006), piglets (Herpin et al., 1996; Randall, 1971), foals (Vaala, 1999) and dog pups (Silva, Lúcio, Veiga, Rodrigues, & Vannucchi, 2009) but without utilising vocalisation parameters. However more recent APGAR assessments of dog pups have included vocalisation components based on the presence and clearness of cry response to paw tip compression at 10 minutes, 24 and 48 hours postpartum (Groppetti et al., 2010; Veronesi, Panzani, Faustini, & Rota, 2009). An APGAR scale including vocalisation emission is also commonly applied to rodent pups in studies modelling perinatal asphyxia (Herrera-Marschitz et al., 2011; Herrera-Marschitz et al., 2014).

Table 2.2: APGAR sores associated with neonate parameters at 1 and 5 minutes postpartum.

Parameter	0	1	2
Heart rate	absent	below 100 beats/min	over 100 beats/min
Respirations	absent	weak cry	strong cry
Muscle tone	limp	some bending	active motion
Reflex irritability	no response	grimace	cry
Colour	blue or pale	body pink, arms and legs blue	completely pink

Source: American Academy of Pediatrics and American College of Obstetricians and Gynecologists (2006).

Human infant cry analysis

The more advanced study of human infant vocalisation for diagnostic purposes was initiated by reports of abnormal cry features in infants with brain disorders (Fisichelli & Karelitz, 1963) and the use of spectrograph technology in 1968 (Ostwald, Phibbs, & Fox, 1968), although the study of the infant cry has been documented as early as 1906 (Michelsson, 1971). One of the early seminal studies comparing acoustic parameters of healthy, asphyxiated and low birth weight neonates was conducted by Michelsson (1971), the findings of which are summarized in Table 2.3. Since then a range of disorders associated with abnormal cry acoustics have been described (see Table 2.4) and reviewed by Corwin, Lester, and Golub (1996), Furlow (1997) and LaGasse et al. (2005). While there are issues related to change of cry characteristics with age (Kheddache & Tadj, 2013; Zeskind, 1985), and evidence of signal grading associated with arousal states (Zeskind, 1983; Zeskind, Marshall, & Goff, 1996), all studies appear to confirm that cry analysis is a valuable measure of neurobiological and neurobehavioral dysfunction of the neonate. Current assessment of neonate cry primarily involves analysis by automated computer systems (Hariharan, Chee, et al., 2012; Hariharan, Sindhu, et al., 2012) such as that employed by the Cry Research Institute in Brookline, MA; and follows a set procedure (outlined by LaGasse et al., 2005 and Golub & Corwin, 1985) with referral available in extensive databases of pathological neonate cry signals. Most studies using these systems employ analysis of a cry defined as an expiratory utterance of at least 0.5 s duration over the first 30 seconds of the test period, as a decline in response following stimuli application is known to impact acoustic parameters (Prechtl, Theorell, Gramsbergen, & Lind, 1969; Runefors, Arnbjörnsson, Elander, & Michelsson, 2000). The number of signals assessed (a single specific utterance or an average across 2 to 3

signals) may depend on investigator judgment for utterance level variables (LaGasse et al., 2005). For further description of acoustic terms used in this section, refer to Table 2.1.

Temporal features such as latency of the signal following a pain stimulus (such as a pin prick or flick of a rubber band to the foot); duration; inter-cry interval; and the number of short (<0.5 s) and full expiratory cry utterances (>5 s) are routinely measured among a range of other parameters including threshold to elicit the cry; the number of cry mode changes; melody type; *F*0 and formant frequencies *F*1-*F*2; amplitude; resonance, hyperphonation and dysphonation measures (LaGasse et al., 2005; Michelsson, 1971), as listed in Tables 2.3-4. The typical features of a normal cry are reported to have a latency period of 1.2-2.5 seconds, *F*0 ranging from 300-600 Hz, and no evidence of biphonation, gliding or sharp changes in pitch (Fisichelli & Karelitz, 1963; Michelsson, 1971). Low birth weight neonates have been reported to not differ significantly from normal birth weight infants, but *F*0 of premature neonates is higher the more premature the infant (Michelsson, 1971). No evidence of gender or race difference has been found in normal neonate signals (Fuller & Horii, 1986; Murry, Amundson, & Hollien, 1977; Zeskind, 1983; Zeskind et al., 2014).

High *F*0 in particular appears to be the prime indicator of neurophysiological dysfunction in the human neonate distress cry (Corwin et al., 1996; Prechtl, 1968; Zeskind & Lester, 1978), and is often associated with pronounced instability of pitch, also reported as glottal instability, in the case of asphyxiated infants (Golub & Corwin, 1982; Michelsson, 1971). Cry latency from application of the stimulus, a higher threshold to initiate a response, dysphonia and other temporal features associated with *F*0 are also common hallmarks of poor neurological integrity and high-complication infants (Wasz-Höckert, Michelsson, & Lind, 1985; Zeskind & Lester, 1978; Zeskind et al., 2011) as shown in Table 2.4. Rothenberg et al. (1995) reported acoustic anomalies associated with prenatal lead (Pb) exposure and gestational age, including increased median *F*0 and reduction in both the number of distress cry utterances and percent nasalisation (a parameter associated with lowering of the velum and escape of air through the nasal passages, Ladefoged, 1993).

Cry pitch (*F*0) has been reported to be more exclusively indicative of infant abnormality than physical and behavioural symptoms such as lack of eye contact (Morley, Thornton, Cole, Fowler, & Hewson, 1991). Successful automated estimation of acoustic parameters

associated with a range of pathologies including asphyxia, has shown that specific acoustic features are associated with each disorder, and that certain acoustic parameters in healthy infants change with gestational age (Kheddache & Tadj, 2013). These authors also report the finding that the dysphonic cry (characterised by high noise concentration, or low Harmonic to Noise Ratio - refer Table 2.1) is a significant predictor of asphyxia.

Table 2.3: Summary of cry features of healthy and abnormal infants. Values shown as median (25-75th percentile).

Variable	Control n=50	Small for date n=30	Premature [a] n=75	Asphyxiated neonates n=205
Latency (s)	1.8 (1.2–2.5)	1.7 (1.4-2.2)	1.6 (1.3-2.0)	>2.5
Duration (s)	2.0 (1.6–3.6)	3.8 (2.2-4.9)	2.4 (1.3-3.5)	<1.3 or >5.0
*F*0 max (Hz)	620 (580-690)	550 (470-630)	1040 (600-1910)	>700 full term >1500 premature
*F*0 min (Hz)	390 (340-450)	330 (280-390)	450 (340-710)	>450 full term >1500 premature
Vibrato % [b]	Nil	7	3	>2
Biphonation % [b]	Nil	Nil	5	>25
Voiced phonations % [b]	66	50	53	<51

[a] Gestation age <34 weeks.
[b] Prevalence of infants tested in group.
Adapted from Michelsson (1971).

Table 2.4: Range of pathological disorders detected by cry analysis.

Disorder	Aberrant cry characteristic(s)
Asphyxiation (peripheral)	High *F*0 (mean max. of 1,000 Hz) dysphonia
Asphyxiation (severe)	High *F*0 (mean max. of 1,460 Hz), dysphonia
Bacterial meningitis	High *F*0 (mean max. of 860 Hz), biphonation
Cardiopulmonary disorders	High *F*0, biphonation
Cri-du-chat syndrome	High *F*0
Cocaine exposure, prenatal	High *F*0, increased latency, reduced utterance rate, dysphonation
Diabetic mother	High *F*0
Herpes simplex viral encephalitis	High *F*0
Hydrocephalus	High *F*0, increased latency
Hypoglycemia	Biphonation, vibrato
Hyperbilirubinemia	High *F*0, short duration and latency, glottal instability, biphonation
Lead exposure	High *F*0, reduced cry rate and nasalisation
Low birth weight	High *F*0, biphonation, flat melody, gliding
Metabolic disorders	High *F*0
Brain damage	High *F*0, increased latency, short duration, increased threshold
Birth trauma or complications	High *F*0, increased latency, short duration
Premature birth	High *F*0, more % abnormality
Sudden Infant Death Syndrome	High *F*0 in some reports

Adapted from Corwin et al. (1996), LaGasse et al. (2005) and Furlow (1997).

Rodent pup ultrasonic vocalisation (USV) analysis

Initiated by the extensive breeding and utilisation of rodents for laboratory trials, the observation that rodent pup vocal behaviour was influenced by pharmacological treatment during maternal separation (Cuomo, De Salvia, Maselli, Santo, & Cagiano, 1987), has led to the use of the rodent pup vocalisation - USVs with typical frequencies ranging between 30 and 90 kHz - as a model for assessment of neurological insult (Branchi et al., 2001; Ehret, 2005). Vocalisation rate is the most widely utilised acoustic parameter (Scattoni & Branchi, 2010), although duration, amplitude and frequency shift have also been associated with neurobehavioural effect (Scattoni, Crawley, & Ricceri, 2009; Zeskind et al., 2014). Decreased calling rates in pups have been associated with prenatal exposure to conditions and substances which also impact upon human infant acoustic structure such as asphyxia (Calamandrei et al., 2004; Dell'Anna et al., 1997; Engidawork et al., 1997), organophosphates (Venerosi, Ricceri, Scattoni, & Calamandrei,

2009), opiates (Carden & Hofer, 1990), alcohol and cocaine (Barron & Gilbertson, 2005; Cox et al., 2012; Cox Lippard et al., 2015; Zeskind et al., 2014) among others (for a review see Zeskind et al., 2011). Other acoustic markers of neurobehavioral deficit in rodent pups including increased latency and threshold, as well as different waveforms, have also been reported (Barron & Gilbertson, 2005; Vathy & Komisaruk, 2002; Venerosi et al., 2009; Wellmann, Lewis, & Barron, 2010). As in the human model, a decline in pup response following stimuli application is known to impact acoustic parameters (Cox Lippard et al., 2015), although test period duration may range from 30 seconds to 60 minutes among rodent studies (Hahn & Lavooy, 2005). The most common test periods (75% of cases) reported in the review by Hahn and Lavooy (2005) were of less than 5 minutes and an optimal test time of 1 minute to avoid rat pup response reduction, was determined in pilot tests by Cox Lippard et al. (2015).

Rate of USV calling in rodents has been demonstrated to follow an ontogenetic profile (Scattoni & Branchi, 2010) and typically pups in trials used a model for the human infant are assessed at specific development stages best correlated to corresponding human neonate ontogeny, for example experimental results of rat pups assessed on postnatal day 7 are equated to neurodevelopment of the human newborn at birth (Clancy, Finlay, Darlington, & Anand, 2007). Studies are often conducted on a single sex (males) to avoid gender interaction (Hahn & Lavooy, 2005); and vocalisation response is stimulated by removal of a single pup from the mother and nest, placed in temperature regulated sound-attenuated chamber and recorded for 4 minutes (Venerosi et al., 2009). Considerable methodological variation between rodent pup vocalisation studies has been shown to be associated with inconsistency of results, especially in association with length of observation period, sex of animals tested, and situations used to elicit the USVs (reviewed by Hahn and Lavooy, 2005).

Embryo studies of mice and rats have provided detailed information about genetic background, gender and early environmental factors on vocalisation behaviour and maternal response (Scattoni & Branchi, 2010; Stern, 1997; Wöhr et al., 2008; Wöhr, Oddi, & D'Amato, 2010). Reports providing further detailed description of range and function of rodent pup isolation vocalisation acoustics include that by Bell (1974), Ehret (2005) and Portfors (2007). In contrast Blumberg and Sokoloff (2001) have questioned the function of rat pup isolation vocalisations by suggesting that rodent distress USVs are

a result of abdominal compression reactions associated with handling although this theory has not been widely accepted (Panksepp, 2003). Interestingly, laryngeal braking (associated with the mouth-closed, short duration, grunt sounds of human infants (McCune, Vihman, Roug-Hellichius, Delery, & Gogate, 1996) has been reported to be associated with lowered oxygenation in both human and rodent neonates (Blumberg & Alberts, 1990).

Based on the similarity of vocal behaviour between rodents and human infants, the potential of distress vocalisation analysis for neurobehavioral assessment in both human and rodent species has been emphasised by a number of authors (Cox Lippard et al., 2015; Hofer, 1996c; Stern, 1997; Venerosi et al., 2009; Zeskind et al., 2014; Zeskind et al., 2011). A set of measures which could assist standardisation of measurement and comparisons between the two species (shown in Table 2.5) has been proposed by Zeskind et al. (2011) with the view that the common neurobiological basis of vocalisation initiation across mammalian species offers potential to further progress human neonate research by application of a translational research approach.

Table 2.5: Common acoustic measurements of human infant cry and rat pup USV signals.

Human infant crying	**Rat pup USVs**
TEMPORAL MEASURES	
Expiration duration	USV duration
Inter-cry-interval	Interval
Repetition rate	Repetition rate
Latency	Latency
SPECTORAL MEASURES	
Peak *F*0	Maximum *F*0
Peak *F*0 amplitude	Max *F*0 amplitude
Dominant frequency amplitude	Peak frequency
Overall maximum amplitude	Peak frequency amplitude
	Overall maximum amplitude
	Minimum *F*0
	*F*0 std deviation
ACOUSTIC STRUCTURE	
Harmonic structure	Waveform structure
	Number of harmonics

Source: Zeskind et al. (2011).

Other species

Acoustic analysis of mammalian infants for the purpose of neurobehavioral deficit assessment, apart from APGAR assessment in dog pups, appears to have been only applied to nonhuman primates and rodents. A number of studies of domestic chicks have utilised distress vocalisation responses in assessment of antipredatory behaviour and anxiety state (Jones, 1977; Marx et al., 2001), and to investigate postnatal development and neurochemical control of neural pathways associated with reactivity to social isolation (McBride & Lickliter, 1994; Panksepp, Meeker, & Bean, 1980). Of most interest is research in 7 day old chicks by Jones (1977) demonstrating gender and strain differences in distress call rates where female chicks of two strains were markedly more vocal, active and had shorter latencies to commence movement in a novel environment. Males spent a larger proportion of time in the freeze position, had longer periods of eye closure and emitted fewer distress vocalisations. Following studies confirmed the conclusion that male chicks appear to be more fearful than females and that sex differences shown at low-to-moderate fear levels may disappear in situation of greater fear (Jones & Faure, 1981; Jones & Williams, 1982). Later work by Vallortigara and Zanforlin (1988) reported that sex differences in chick ambulation latencies were more likely to be associated with a stronger motivation for social reinstatement. These gender differences suggest that indiscriminate mixing of sexes in behavioural and vocalisation tests should be avoided (Jones, 1977).

The potential for other vocal species to be assessed for neurological deficit by acoustic cry analysis also seems possible as information of species vocalisation characteristics are made available. For example, of note in the veterinary literature is the reported abnormal high pitched “barking” vocalisations of newborn foals associated with peripartum asphyxia syndrome and hypoxic-ischemic encephalopathy (Vaala, 1999). Other species with such potential include follower ungulates, and available research regarding what is currently known of *Ovis* species vocalisation behaviour is reported in the next section.

2.4 Acoustic research related to the *Ovis* species

The majority of published sheep vocalisation literature appears to be focused on maternal behaviour, as most studies concentrate on the dam's role in establishment and maintenance of the mother-young bond (Alexander & Shillito, 1977b; Pollard, 1992; Sèbe et al., 2010; Shillito & Alexander, 1975; Shillito Walser, Walters, & Hague, 1981b). As a consequence, there are few studies which document in detail the vocalisation characteristics of the lamb. Most studies investigate animals at an older age rather than the young neonate, and in context of kin recognition or as a temperament indicator. None have investigated the acoustic characteristics of the lamb signal as a potential marker of vigour or health, although measures of bleat rate (Hergenhan, 2011; Hernandez, Matthews, Oliver, Bloomfield, & Harding, 2009; Nowak, 1990b; Pfister et al., 2006) and latency to the first postnatal bleat (Darwish & Ashmawy, 2011) as indicators of vigour have been reported.

A series of playback experiments reported by Shillito Walser and Walters (1987) is one of few studies to document the timing of the separation bleat of lambs aged between 7 and 30 days of age in response to recordings of their own and an alien dam, where the authors reported that 7 day old lambs responded within 2 seconds of hearing a ewe vocalisation. The timing of this response was similar for both own dam and an alien ewe, and at the later ages of 20 and 30 days postpartum (Shillito Walser, Walters, & Hague, 1982; Shillito Walser, 1978). Lambs also appeared to recognise their own dam's bleat at 7 days as evident in the frequency of response. Breed differences in frequency and responsiveness of bleats were reported, with Border Leicester lambs being slower to respond or being "less vocal" than Dalesbred and Jacob bred lambs at 7 and 20 days of age. A small number of ewe bleats were reported to go unanswered, more often by Border Leicester lambs, and more frequently in the second round of the tests.

Sèbe et al. (2007) investigated bleating behaviours of lambs in the early neonatal period (ranging from birth to 12 hours postpartum) and has reported that lamb bleat rate, while with the dam, was highest during the first 3 hours postpartum. Lambs were documented to make the same types of vocalisations as adult ewes which included high and low pitched sounds, associated with open-mouth and closed-mouth signals, respectively). Intermediate bleats, a mixture of low and high pitch bleats were also reported to be

emitted by both ewes and lambs. Sèbe et al. (2010) also found that ewes of 2 day old lambs made low pitched vocalisations when lambs were less than 1m away, but high pitched vocalisations when separated by more than 1m. Spectrograms of high pitched bleats of lambs aged 15 days (±1 day) were documented but not analysed in any detail by Sèbe, Aubin, Boué, and Poindron (2008) and peak vocal periods were noted to be correlated with nursing (lamb age 0-15 days).

A comprehensive acoustic study of lamb vocalisation parameters is reported by Searby and Jouventin (2003) who were primarily interested in assessing the stereotypy of ewe and lamb signals and the difference between individuals. Lambs were aged between 3 days and 2 weeks of age and the type of call is presumed to be a high pitched, mouth open distress vocalisation although this was not specified. The authors reported that mean *F*0 of lamb signals were higher (345±49.6 Hz) than that of the ewe (152±15.4 Hz) (Table 2.6) and that the sheep acoustic signature appeared to be based on a simple frequency coded system. Discrimination capacity was concluded to be most likely to be enabled by the duration, spectral density and mean *F*0 of the call, which was reinforced by lack of recognition of frequency modified signals by both ewes and lambs. However, subsequent research by Sèbe et al. (2011) suggests that recognition and maternal call identification, by the lamb, occurs by a multiparametric process which more effectively transmits information in a herd situation. Lingle et al. (2012) also reported on data gathered from bighorn sheep reporting a tonal distress call mean *F*0 of 329 Hz (ranging from 278-358 Hz) in lambs aged less than 2 days old. Further details of acoustic parameters of the lamb at the various stages of ontogenic development remain to be documented.

In comparison, a detailed documentation of acoustic analysis of kid goat vocalisations has been reported by Briefer and McElligott (2011). Lower frequency, close contact but open-mouthed calls instead of higher-pitch distress vocalisations were analysed with the intent to encode information about age, sex and body size. Age of kids ranged from birth to 124 days and were grouped into 3 age groups by sex. Mean *F*0 for males was 606.6±2.7 Hz compared to 591.4±4.2 Hz for females in the 0-10 day age group, but female *F*0 was higher in older age groups (27-41 days and 104-124 days). Mean *F*0 and formant frequency lowered as kids aged, and calls became longer and less modulated in frequency and amplitude. Age and sex related changes were evident with *F*0 decreasing faster with

age for males than for females, a difference attributed to body size based on source-filter theory and the possible effect of sex hormones on vocal ontogeny.

Table 2.6: Acoustic characteristics of vocal signatures of ewes and lambs aged 3 days to 2 weeks, n=15 calls per individual.

Acoustic variables	Mean ± sd	
	Lamb	Ewe
Duration (s)	0.71±0.22	0.79±0.23
$F0_{mean}$ (Hz)	345±49.6	152±15.4
$RelF_{start}$ [a]	1.89±0.10	0.99±0.03
$RelF_{end}$ [a]	0.93±0.11	1.00±0.03
$RelF_{minimum}$ [a]	0.84±0.08	0.96±0.01
$RelF_{maximum}$ [a]	1.08±0.04	1.04±0.01

[a] Relative frequency of parameter divided by $F0_{mean.}$. Adapted from Searby and Jouventin (2003).

Developmental changes in goat kid vocalisations have also been described by Lenhardt (1977) and Terrazas, Serafin, Hernández, Nowak, and Poindron (2003) who analysed spectrograms of kid distress and contact bleats over the first 4-5 days of life and older. As in sheep, goat mothers have been reported to be unable to distinguish their own kid signals from alien kids in the first 4 days because they would respond to other kids as well as their own (Lenhardt, 1977; Sèbe et al., 2010). However Terrazas et al. (2003) reported changes in kid acoustic parameters from the first day postpartum and, using kid playback recordings, found that while goat mothers responded to the distress calls of all neonates they showed a distinct preference for their own kid at 48 hours of age. Lenhardt (1977) found that kid orientating calls demonstrated a rapid change in *F*0 and an upper frequency limit in the first day of life and that sex differences appeared after 4 weeks. Male orientating and distress vocalisation maximal energy was at least 3 octaves below that of females (upper range of male call 7.5-10 kHz, compared to female 15k Hz) (Figure 2.5). Lenhardt's research also is also one of the few studies which describes a single distress signal as having both oral and nasal components (Kiley, 1972; Volodin et al., 2011).

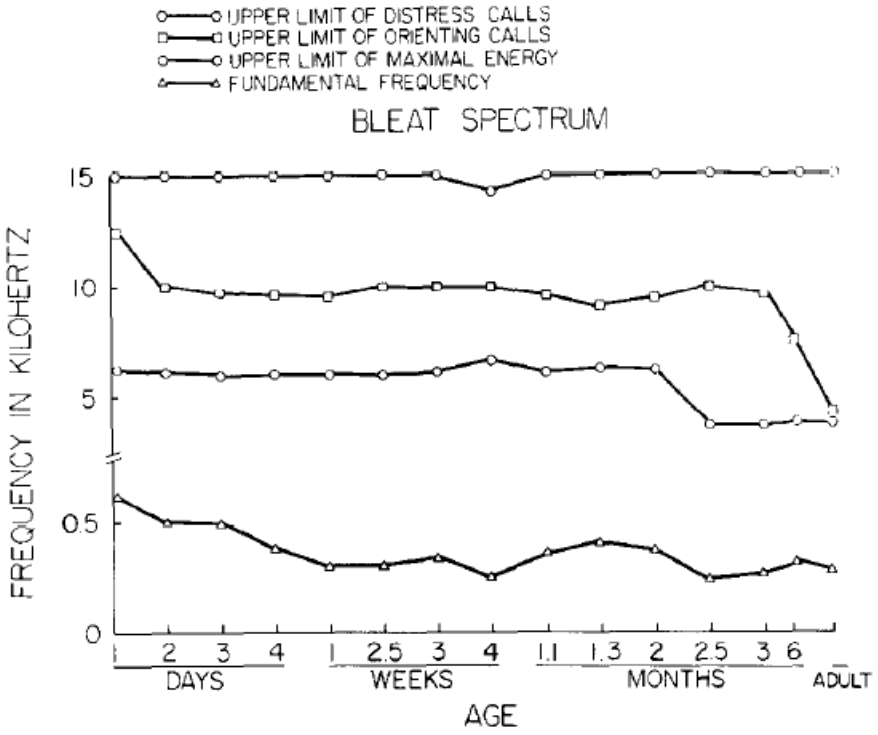


Figure 2.5: Change in goat kid vocal components as a function of age. Source: Lenhardt (1977).

There have also been a number of other studies which have reported variation in rate of bleating in very young lambs. An effect of breed, ewe parity and litter size, but not sex, was reported by Dwyer et al. (1998) in bleat rate of lambs within the first 30 minutes of birth (lambs of primiparous ewes and Suffolk breed had higher bleat rates than lambs of multiparious ewes and Blackface lambs respectively). Darwish and Ashmawy (2011) also reported that lambs of primiparous ewes experiencing prolonged and difficult births vocalised earlier and more often than lambs experiencing short and unassisted births. This was assumed to be because ewes experiencing difficult birth demonstrated less interest and maternal care, and that vocalisations of the difficult birth lambs were an indicator of need. Higher bleat rate in lambs of sires associated with poorer survival also corresponded with slower first standing and sucking latencies in 4-6 hour old lambs in barrier test trials (Hergenhan, 2011) while Nowak (1990b) reported a higher bleat rate in Border Leister merino cross lambs than pure Merino, and implied than higher bleat rates were indicative of better bonding processes.

Another conclusion to be drawn from the above results is that lambs suffering difficult birth and compromised ability either fail to suck properly or elicit a positive response from the ewe and thus would be less content than easy birth lambs, depending on the period of time which had elapsed since birth and temperature regulation demands. This inference appears evident in the longer latencies to suck of difficult birth lambs in the Darwish and Ashmawy (2011) study, and lambs born to Suffolk and primiparous ewes in the studies reported by Dwyer, Calvert, Farish, Donbavand, and Pickup (2005) and Dwyer and Lawrence (1999b).

Deficient vocalisation rates aligned with other behaviours indicative of neurological deficiency have also been reported in neonate lambs exposed to prenatal locoweed (Pfister et al., 2006) and in twin siblings tranquilized with xylazine (Porter, Nowak, Orgeur, Lévy, & Schaal, 1997). Poor vocalisation responses to separation in lambs aged 0-7 days and an associated inability of ewes of different breeds to locate these lambs during discrimination tests were also reported by Shillito Walser (1978). In Shillito's study it was noted that Soay breed lambs were more likely to silent during testing, and that Soay and Dalesbred ewes tended to be better at locating their lamb when hidden or silent. The social behaviour of the Soay, a feral sheep breed noted to be more akin to the goat, a "hider species" (Shackleton & Shank, 1984), may be evident in this trial. A sex difference in bleating rate and activity of lambs aged between 24 hours and 4 weeks has also been reported by Hernandez et al. (2009) where, on average, female lambs had a higher vocalisation rate and were quicker to leave a starting pen than males.

In many studies where bleat rate (the number of bleats averaged over a given period of time) is reported to be low, for example following a separation stimulus in a test arena, temporal measurements may in fact be associated with long latencies to initiate a bleat, depending on the duration of the test. Other studies documenting rates of bleating while the lamb is accompanied by the ewe (for example Dwyer et al., 1998; Sèbe et al., 2008), are not describing a separation distress vocalisation nor latency to initiate a vocalisation following application of a stimulus. Such vocalisations are more likely to indicate either hunger or contentment, which in the context of this review is an important distinction in regard to arousal and neurobehavioural assessment. Furthermore, descriptions regarding the quality of the vocalisations emitted are usually not available in any of the above studies.

Other data of interest relating to lamb vocalisation behaviour, is that available from field lambing studies in Australia where a range of vigour measurements have been assessed in lambs aged less than 24 hours of age (Brien et al., 2014; Brien et al., 2009; Brien et al., 2010; Geenty et al., 2014). Analysis of the time taken for the lamb to initiate a bleat following tagging by the shepherd was reported to be phenotypically correlated with survival (Brien et al., 2014), but influenced by many variables that contribute to large variation in a field environment. Other traits measured simultaneously such as struggle score and time to reunite with the dam showed less correlation with lamb survival at weaning. Estimates of lamb age were determined by coat wetness, evidence of walking and position relative to the birth site (score 1=newborn, not stood; score 2=on birth site, age ~1-4 hours; score 3= following ewe, age >4hours). The procedure of these measures involved capture of the lamb and tagging (piercing of the ear and insertion of an identification tag) before release to assess timed behaviours, which may have influenced levels of arousal and cortisol response in each animal (Beausoleil, Blache, Stafford, Mellor, & Noble, 2012).

2.5 Conclusion and scope of thesis

The preservation of a signal of elemental acoustic structure which functions to elicit a strong maternal response across mammalian species suggests that infant distress vocalisations are innate, dependant on developmental stage and have a commonality of neurophysiological production. If, as has been demonstrated in human and more recently rodent infant distress vocalisations, the acoustic characteristics of this crucial vocal signal can constitute a reliable indicator of infant phenotypic status and vigour, it is hypothesised that this may also apply to neonates of the *Ovis* genus, a species which has already proven to be a suitable physiological model for the human infant in neonate medical research. Given what is currently known of the vocalisation features of neonates of sheep and other ungulates, and the need for robust maternal-young vocal connections in follower prey species, it seems logical to conclude that if the acoustic features of the *Ovis* neonate distress signal related to detection by, and attractiveness to, the dam are compromised, chances of survivability will be reduced. Acoustic data regarding the sheep neonate is noted to be surprisingly sparse considering the large body of research already associated with this species.

Therefore this thesis aims to:

- investigate isolation distress vocalisation latency of neonate lambs in the first 12 hours of life as a potential measure of lamb vigour and viability (Chapter 3)
- investigate the association of vocalisation responsiveness, and other acoustic parameters, with indicators of fetal distress (Chapters 4 and 5)
- identify and quantify characteristics and acoustic structure of neonate lamb vocalisations in greater detail than currently available, including differences associated with sex and genotype (Chapter 5)
- assess whether acoustic structures of distress vocalisations of neonate lambs have potential to be used as a marker of neurological deficit (Chapter 5)
- investigate maternal responsiveness to vocalisation characteristics of the neonate lamb which may influence successful mother-young interaction (Chapter 6).

Chapter 3

Assessment of lamb vigour based on initiation of vocalisation

3.1 Introduction

Lamb survival rates determined at weaning give little information about on-farm factors associated with poor lamb mortality. The timing of lamb loss appears to be concentrated in the first few days of life but importantly, events which precipitate death are more likely to be initiated during birth or in the critical first 6 to 12 hours of life (Dutra & Banchero, 2011; Dutra et al., 2007). While a prime cause of perinatal lamb mortality has been reported to be associated with varying severity of central nervous system injury following hypoxic-ischemic damage during intrapartum processes (Dutra & Banchero, 2011; Dutra et al., 2007; Haughey, 1980; Holst et al., 2002), other factors correlated with lamb survival may relate to the vigour of the lamb itself which has been reported to be influenced by genetic between and within-breed variation (Brien et al., 2014; Hergenhan et al., 2014).

Traditionally vigour assessment of neonate lambs has been based on timed measures of critical early behaviours such as latency to stand, reach the udder and suckle (Dwyer, 2003; Matheson et al., 2011; Owens et al., 1985); and test arena measurement of lamb ability to reunite with, or discriminate between, their own and an alien dam (Bickell et al., 2009; Nowak et al., 1987; Pfister et al., 2006). More subjective measures of vigour related to observed lamb struggle levels and activity during field tagging procedures have also been reported (Alexander & Peterson, 1961; Brien et al., 2010; Cloete, Scholtz, Cloete, & van Wyk, 2005; Stevens, Alexander, Mottershead, & Lynch, 1987) although the difficulty in obtaining standardized measurements during field tagging, due to variation in lamb age, remains a major source of inconsistency. Viability at birth has also been measured by assessment of modified APGAR scores (Dutra & Banchero, 2011; Pfister et al., 2006), rectal temperature (Barlow et al., 1987; Brien et al., 2014; Dwyer & Morgan, 2006), biochemical markers (Barlow et al., 1987), and cold resistance (Slee,

Alexander, Bradley, Jackson, & Stevens, 1991). Many of the above measures are difficult and impractical to assess in commercial production systems, and more practical measurement of neonate survival probability is required.

Amongst a range of potential behavioural field measures of lamb survivability described by Brien et al. (2009), measurement of lamb bleat response following tagging was reported to have a moderate genetic correlation (-0.43±0.32) with lamb survival at weaning (Brien et al., 2014). Distress vocalisation response and signal strength have also been linked to human neonate vigour since the development of the APGAR score and earlier (Apgar, 1953; Basden, 1921). Review of current neonate medical research reveals that there is a well documented association of distress vocalisation latency in human and rodent neonates with central nervous system insult and neurobehavioural deficit including that associated with birth asphyxia (Michelsson, 1971; Newman, 2007; Zeskind et al., 2011). Modified APGAR scores applied to neonate lambs and other mammals including piglets have excluded vocalisation (Trujillo-Ortega et al., 2011), primarily because of poor or absent vocalisation responses following to a pain stimulus. However vocalisation measures are included in rodent pup APGAR scoring systems based on signal responses elicited by separation from the dam (Herrera-Marschitz et al., 2014), and have been applied to a modified APGAR evaluation of canine pups (Veronesi et al., 2009).

Given the high risk of perinatal lamb mortality associated with intrapartum asphyxia and related trauma, it seemed possible that a measure of lamb vocalisation response following separation, as in the rodent model, could be correlated with central nervous system function and subsequent neonate viability. The aim of this study was to investigate in more detail the association of neonate lamb vigour with vocalisation latency, and the potential of vocalisation responsiveness following application of an isolation distress stimulus as a novel tool to assess neurological status and lamb viability in the early postpartum period. It was hypothesised that neonate lambs demonstrating healthy vocalisation reactions when separated (as indicated by a more immediate response) would exhibit superior vigour-related behaviours associated with neurological integrity; including latency to first stand and suckle, and capacity to locate their dam in a test arena.

3.2 Materials and methods

3.2.1 Animals

A total of 280 merino ewes (2 years old n=133, 3-5 years old n=147) were oestrus synchronized and joined by artificial insemination (AI) in 5 weekly cohorts to 6 Merino sires in April/May 2012 at the CSIRO FD McMaster Field Station, Armidale, Australia. Sires were selected for divergent lamb vigour based on ranked estimated breeding values (EBVs) of traits associated with lamb survival; in particular lamb survival at weaning (LSW), bleat response, rectal temperature and crown-rump values (see Table 3.1). These values had been made available from sire comparisons in a large scale artificial breeding program conducted in flocks across Australia (Brien et al., 2010). As sires were selected from data available for AI joinings prior to 2011, selection of sires was also dependant on semen availability. Ultrasonic scanning was performed at 50-57 days after joining to determine pregnancy and litter size status, and only single and twin bearing ewes were retained.

Ewes were maintained at body condition score (BCS) 2.5-3 throughout pregnancy and managed as 5 separate cohorts for the last month of gestation from which time they were supplementary fed a lupin/corn mix at 250 gm per ewe three times weekly. Ewes were housed from day 145 of gestation in individual 1.2 m^2 pens and fed an *ad libitum* lucerne-based feed ration, supplemented with lucerne hay and a 3:2 lupin/corn ration of 200 gm/day. Ewes were monitored by 1 to 2 observers 24 hours/day with numbers in each cohort ranging from 45 to 63 ewes. Lambing assistance was only given if the ewe had failed to deliver within 2 hours of the appearance of lamb body parts, or if no progress was observed one hour after the rupture of membranes. At the completion of tests at 12 hours postpartum ewe/lamb units were released into an enclosed area outside and observed for a further 48 hours to ensure successful bonding.

Table 3.1: Relative ranking of selected sires based on EBVs [a] associated with lamb survival traits.

Sire ID	Relative sire vigour ranking	Sire rank (EBV)				
		LSW	Bleat response	Rectal temp	Crown	Total rank [b]
S1	High	**3** (0.0174)	**2** (-1.793)	**2** (-0.0077)	**5** (0.2386)	**5** (17)
S2	High	**2** (0.0227)	**1** (-2.7730)	**4** (-0.2196)	**3** (1.1550)	**2** (10)
S3	High	**1** (0.0237)	**3** (-0.9295)	**1** (0.2934)	**1** (3.5970)	**1** (6)
S4	Low	**6** (-0.0243)	**5** (0.5960)	**6** (-0.6021)	**6** (0.1617)	**6** (23)
S5	Low	**5** (-0.0235)	**4** (0.1876)	**5** (-0.2493)	**4** (0.8834)	**3** (14)
S6	Low	**4** (-0.0159)	**6** (2.3860)	**3** (-0.1708)	**2** (2.6760)	**4** (15)

[a] Estimated Breeding Values based on available information at time of selection (F. Brien pers. com, March 2012).
[b] Sum of relative ranks for each trait EBV

3.2.2 Data collection

Video monitoring was initiated at the first sign of suspected prepartum behaviour, and maintained until 3 hours postpartum. The time when membranes and lamb body parts were first observed was recorded and progression of events noted. Duration of parturition (duration of stage II labour) was determined to be from the first appearance of body parts to complete expulsion of the lamb. Video data was analysed to confirm time of birth and to determine neonatal behaviours including latency to *stand*, *reach udder* and *suck* as described by Dwyer (2003). Impact on normal bonding behaviour was minimised by lack of interference with the ewe or lamb until 3 hours post birth when a vocalisation (bleat) response measure was collected. This measurement, adapted from lamb test protocols described in Brien et al. (2009), was collected by quickly removing the lamb from its pen with minimal contact and restraining each lamb on its side (head facing away from the dam) at a distance of 1.5 m from the dam's pen. The lamb was held for 5 seconds until release, and the time taken for the lamb to first initiate a vocalisation was recorded by stopwatch to the nearest 0.00 second. If lambs did not vocalise within the first 90 seconds of the test, the measurement was recorded as *time elapsed*. If the lamb was vocalising while being restrained (which was rare), the restraining hand was not released until the lamb had completed the vocalisation. Lambs were then measured for weight, rectal temperature, crown-rump length, chest girth circumference, and tagged as per normal field protocol.

At 4, 8 and 12 hours from birth of the first lamb, the ewe and lamb/lambs were taken to an outside arena where the ewe was restrained in a pen 2 m away from the lamb (see

Figures 3.1 and 3.2). Video monitoring was used to record the time taken for the lamb to stand and then travel to the retrained ewe, and a vocalisation response measure as per above was also recorded at this time. Successful return to the ewe was achieved if the lamb reached the 0.5 m contact zone in front of the penned ewe. *Time to stand* was defined as when lamb had stood on all four legs, and *time to return to ewe contact zone* was defined as when the front 2 legs of the lamb were over the line. If subjects did not reach the ewe within 180 seconds, or vocalise within the first 90 seconds of the test, the measurements were recorded as *time elapsed* (TE). A ewe responsiveness score based on level of vocalisation response was also recorded. Twin lambs were tested individually, and each sibling not being tested was kept separate from the dam while its co-twin was being tested. In 80% of litter tests the first born lamb was the first to undergo assessment in the test arena - in the other 20% of litters (n=6) the test order had been reversed because of twin order misidentification.

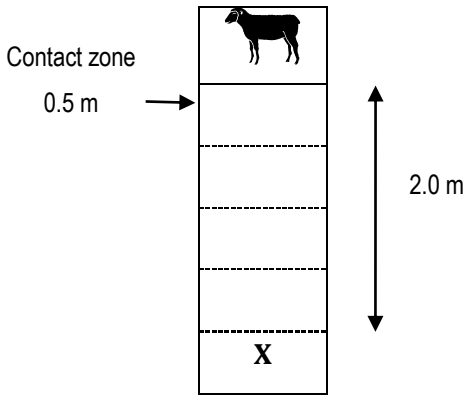


Figure 3.1: Diagram of test arena with distances used for 4, 8 & 12 hour behavioural measurements. X marks position of lamb placement at the commencement of the test.

(a) (b)

Figure 3.2: Vocalisation response and return to ewe testing in progress. (a) Gentle restraint holding lamb for 5 s, (b) Lamb returning to the ewe contact zone.

3.2.3 Statistical analysis

Viable lambs with evidence of fetal growth restriction (n= 5, birth weight <2 kg) were removed from the analysis to avoid effects on sire-related comparisons, and singleton and twin lambs were analysed separately for all comparisons. In this study, lamb data was unbalanced for sire following unexpected post scanning fetal loss (unrelated to sire or parity) which had impacted on cell numbers in the earlier AI cohorts. Lamb body weight and dimensions, rectal temperature and parturition duration were analysed in R (version 3.1.1, R Core Team, Vienna, Austria) using a linear mixed-effects model, *nlme* package (Pinheiro, Bates, DebRoy, & Sarkar, 2011). To investigate effects across all sires, lamb sex and dam age were included as fixed effects, and sire was nested within AI cohort as a random term. Birth weight was included as a covariate for all measures. To analyse differences between sires, sire was included as a fixed effect with AI cohort as the only random term. In twin analysis, lamb identity was nested within ewe identity as a random effect. Model complexity was reduced sequentially by removal of nonsignificant terms and first order interactions were retained in the model if significant at $p<0.05$. Parturition data were transformed to natural logarithms. Data normality assumptions were tested by inspection of Q-Q plots and scatterplots of the residuals, and application of the Shapiro-Wilk's test.

As timed behaviours (longitudinal data) were nonparametric and included censored observations of lapsed test times; and data related to effects were unbalanced, Cox proportional hazard model (Cox PHM) regression, (R *survival* package, Therneau and Grambsch, 2000), was used to develop relative risk models to compare survival curves of behavioural latencies where latency to an event was deemed "failure" and hazard ratios were indicative of comparative likelihood of a shorter latency compared to a reference. Distinct to an odds ratio, the hazard ratio reflects the relative risk or likelihood of an event occurring compared to the reference, at each point in time, for every one-unit increase or decrease in the predictor. Hazard ratios >1.0 indicate, in this study, higher probability of a behavioural event occurring earlier and values <1.0 indicate lower probability of an event occurring earlier. To compare the effect of sex and parity in the model with sire as a random effect, sire was included as a clustered term with birth weight excluded in order to identify collinear associations. The cluster term identifies correlated groups of observations and gives robust sandwich variance estimators (Therneau & Grambsch, 2000).

Terms which were not significant were dropped from the model using backwards step regression. For twin litter analysis, in addition to the above, parity was removed and birth order included. Ewe identity was included as a frailty term with a Gaussian distribution to allow comparisons within each dam litter. Based initial modelling to determine interactions with sire and violation of proportional hazards assumptions, all final models for sire effect were rerun and determined with parity and sex stratified to remove non proportional effects and give adjustment for confounding variables. Statistical significance of regression coefficients was tested using the log-likelihood ratio test and model adequacy by chi-square goodness-of-fit tests. For estimation of the median latency across all variables, sire was fitted into the fitted model as a cluster term. Finally a post hoc test of behavioural associations with labour duration and birth weight and were conducted by applying a Cox PHM model without fitting any other effects. Model proportionality assumptions were assessed by the cox.zph test (based on scaled Schoenfeld residuals) which computes a chi square test for each variable and global test for the model as a whole (Grambsch & Therneau, 1994).

Cox model reference levels for all models were *sex=female*; *parity=2 yr old dam*; and *sire=Sire 1*. Cox PHM results are displayed as hazard ratio$_{effect}$ with 95% confidence

intervals. The minimum recording for vocalisation latency if the lamb had an immediate response upon release was set at 0.1 seconds rather than 0.0. The datasets for time to *stand* and *return to the dam* contained a number of missing values due to loss of video files in the early stages of the experiment, but corresponding vocalisation latency measures recorded by stopwatch were used. In the test arena measures, the return to ewe measure did not include the time taken for the lamb to stand. Means for physical data are displayed as LSmeans±SE determined from fitted models using R package *lsmeans* (Lenth, 2013). All median latency times are shown as Kaplan-Meier estimates with first and third quartiles to describe data distribution. Correlations between vocalisation response and other behavioural measures were also tested using the Pearson correlation coefficient. To compare the relative differences between lambs born to each sire for the likelihood of demonstration of faster or earlier behaviours, the hazard ratio was used as a "relative performance indicator" where performance was based on latency of time to perform a behaviour in the study setting.

To further investigate relationships between behaviours, lambs were divided into "*fast*" and "*slow*" vocalisation response groups based on the first (25^{th} percentile) and third quartile (75^{th} percentile) divisions of the vocalisation latency data for each time point as data were highly skewed. This approach is widely used in medical research to determine reference points (Altman, 1990; Altman & Bland, 1994). The threshold for the "*fast*" bleat latency category was set at ≤2.00 seconds for all comparisons using the median value of the Cox PHM fit accounting for all ages, sex, parity, litter size and sire (n=917 records), and which has previously been described as the typical vocalisation response latency of 7 day old lambs separated from their dam (Shillito Walser et al., 1982). Initial tests run on behavioural correlations between the first two bleat latency quantiles also revealed a lack of significant difference. "*Slow*" vocalisation response categories were determined as latencies higher than the third quartile value at each age time point. Comparisons of standing, suckling and test arena behavioural latencies were then compared between the "fast" and "slow" categories using Cox PHM without fitting for other predictors. Results are shown as Kaplan Meier medians with 95% confidence intervals. For interpretation of Kaplan Meier survival curves describing the data of this study, first quantile values correspond to the point of 0.75 survival distribution and third quantile values correspond to the 0.25 survival distribution. Thus the survival distribution

indicated by the y-axis equates to the proportion of animals which have not initiated a vocal response at the corresponding point in time.

3.3 Results

3.3.1 Differences between singleton lambs

Singleton lambs born to 2 year old ewes assumed to be primiparous (n=72, mean body weight 37.5±0.36 kg) and those born to ewes aged 3-5 years assumed to be multiparous (n=98, mean body weight 42.33±0.28 kg) were included in the analysis. Of the 159 observed deliveries, 15.7% required assistance for malpresentation (n=19) and prolonged parturition (n=6). Five lambs of birth weight <2 kg were excluded from the analysis.

Birth weight, body dimension, parturition duration and rectal temperature

Birth weight of singleton lambs ranged from 2.42-6.56 kg, and rectal temperatures from 36.9-40.9ºC. Mean adjusted birth weights of singletons across all sires are shown in Table 3.2. Males were 176 grams heavier than females (males: 4.61±0.85 kg; females: 4.39±0.91 kg, $p<0.05$) and lambs of older ewes tended to have heavier birth weights (4.58±0.82 vs 4.30±0.94, $p=0.07$). There was a sex x parity interaction for girth ($p<0.05$) with males from older ewes recording a smaller mean girth measures than females. There was also a sex x parity interaction for rectal temperature ($p<0.05$) reflecting a lower mean rectal temperature in male lambs born to primiparous ewes. Rectal temperatures indicating mild hypothermia (37-39ºC, DEFRA, 2004) at 3 hours postpartum were recorded in n=16 lambs (birth weight ranging from 3.1-5.7 kg). One singleton weighing 4.1 kg, born to a first parity ewe, exhibited severe hypothermia (<37ºC, DEFRA, 2004). Mean duration of parturition was twice as long in primiparous ewes compared to multiparous ewes (31.2 minutes compared to 15.9 minutes, $p<0.0001$) and was influenced by birth weight ($p<0.0001$). A sex x parity interaction indicated that male lambs of primiparous ewes had a longer mean length of stage II labour than females ($p<0.05$, Table 3.2).

Table 3.2: Physical and physiological measures of singleton lambs by sex and age of dam (LSmean±SE).

Variable	Dam age: 2 yr old			Dam age: 3-5 yr old				
	Male n=49	Female n=31	Total n=72	Male n=48	Female n=50	Total n=98	Effect	*p* value
Birth weight (kg)	4.46±0.10	4.30±0.11	4.38±0.94	4.60±0.10	4.50±0.98	4.58±0.82	Parity Sex	=0.07 <0.05
Girth (cm)	38.89±0.26	38.56±0.28	38.73±0.22	38.80±0.24	39.34±0.24	39.07±0.20	Birthwt ParityxSex	<0.0001 <0.05
Length (cm)	46.67±0.49	46.55±0.54	46.61±0.45	46.61±0.49	46.49±0.47	46.55±0.40	Birthwt	<0.0001
Rectal temp (˚C)	39.47±0.10	39.83±0.11	39.65±0.07	39.73±0.09	39.55±0.09	39.64±0.07	ParityxSex	<0.01
	n=37	n=27	n=62	n=38	n=42	n=80	n=142	
Parturition duration (mins) [a]	1.58±0.07 (38.17)	1.41±0.08 (25.55)	1.49±0.05 (31.22)	1.14±0.06 (14.11)	1.25±0.07 (17.96)	1.20±0.05 (15.89)	Birthwt Parity ParityxSex	<0.0001 <0.0001 <0.05

[a] Data are presented as log LSmean±SE, back transformed mean in parentheses.

Early neonate behaviours

All lambs included in the analysis had stood and suckled within the first 3.5 hours following birth. Over half of the lambs (58.7%, n=88) stood within 30 minutes and 87.3% (n=131) stood within one hour postpartum. Sixty five percent (65.1%) had suckled within the first hour. Lambs of primiparous ewes were more likely to be slower in reaching the udder by a factor of 1.41 compared to lambs of older ewes (hazard ratio$_{damage>2yrs}$=1.41, 95% CI [1.02-1.95], p<0.05) (Table 3.3). Males were 29.0% more likely to be slower to achieve successful suckle after reaching the udder (hazard ratio$_{sexmale}$=0.61, 95% CI [1.07-2.43], p<0.001); and at greater risk of being slower if born to primiparous ewes (sex x parity interaction, p<0.05).

Table 3.3: Early behaviour latencies of singleton lambs by sex and age of dam shown as median (25-75th percentile).

Behaviour (min)	Dam age 2 yrs		Dam age 3-5 yrs		Cox PHM	
	Male n=38	Female n=27	Male n=40	Female n=44	Predictor	*p* value
Stand	27.0 (15.0-42.0)	18.0 (11.0-38.0)	25.5 (16.5-51.0)	24.0 (15.5-46.0)	NS	NS
Stand to reach udder [a]	7.0 (2.0-15.0)	11.0 (2.0-23.0)	4.0 (1.0-11.5)	4.5 (2.0-9.0)	Parity	<0.05
Reach udder to successful suckle [b]	10.0 (3.0-22.0)	5.0 (3.0-13.0)	8.0 (1.0-17.5)	4.5 (2.0-11.0)	Sex ParityxSex	<0.001 <0.05
Total time (from birth to suckle) [c]	52.5 (43.0-77.0)	42.0 (31.0-59.0)	46.0 (34.0-74.0)	46.5 (27.0-78.0)	Birthwt	<0.01

[a] p=0.04 for parity effect (hazard ratio$_{damage>2yrs}$=1.41, 95% CI[1.02-1.95]).
[b] p<0.001for sex effect (hazard ratio$_{sexmale}$=0.61, 95% CI[1.07-2.43]).
p=0.02 sex x parity interaction (hazard ratio$_{sexmale:dam>2yr}$=1.61, 95% CI[1.07-2.43]).
[c] p=0.08 for sex x parity interaction (hazard ratio$_{sexmale:dam>2yr}$=1.65, 95% CI[0.94-2.91]).
NS = Non significant.

Vocalisation latency

Table 3.4 summarises the proportion of lambs responding with a vocalisation within 2 and 5 second timeframes following test commencement at the various stages postpartum. The proportion of animals with shorter bleat latency increased significantly (p<0.0001) over the 12 hours. There was a marked increase in lambs responding quickly at the test

time at 4 hours compared to an hour earlier. In lambs of older ewes, average median latency to bleat was 3.47 seconds at 3 hours of age, and <2 seconds when tested at 4 hours postpartum and older. In lambs of primiparous ewes, average median bleat latency was 4.66 seconds at age 3 hours, 2.17 seconds at 4 hours of age and <2 seconds when older (>8 hours postpartum).

There was a clear sex effect with male lambs being more likely to have a longer latency to vocalise at each age of testing. At 3 hours postpartum, males were 47.0% more likely to be slower to respond than females (hazard ratio$_{sexmale}$= 0.53, 95% CI [0.32-0.89], p<0.05); and at 12 hours of age, 49.0% more likely to be slower (hazard ratio$_{sexmale}$ =0.51, 95% CI [0.36-0.69], p<0.71) (Table 3.5). There was also a sex x parity interaction at 3, 8 and 12 hours postpartum indicating that male lambs of older ewes were more likely to have faster vocalisation responses than male lambs of younger ewes (at 3 hours: hazard ratio$_{male:dam>2yrs}$=1.94, 95% CI [1.22-3.05], p=0.005; at 8 hours: hazard ratio $_{male:dam>2yrs}$=1.65, 95% CI [1.12-2.45], p<0.02; at 12 hours: hazard ratio$_{male:dam>2yrs}$=2.27, 95% CI [1.09-4.76], p<0.05) but that this likelihood decreased over time (reflected in declining hazard ratios). Labour duration (hazard ratio$_{labourlength}$=0.99, 95% CI [0.99-1.00], p<0.05) was a significant predictor of slower vocalisation latency at 3 hours of birth, only when adjusted for birth weight.

Median vocalisation latency across all singleton lambs, fitting sex, parity, sire, birth weight and age was estimated to be 2.03 s, 95% CI [1.88-2.25]. All terms were required in the model but only sex (p<0.05), age (p<0.001), sire (p<0.001) and a sex x parity interaction (p<0.01) were significant.

Table 3.4: Proportion of singleton lambs vocally responsive within 2 and 5 second timeframes following test commencement.

Age postpartum	Responding in <2 s		Responding in <5 s		Non responsive lambs	
	n	% of total	n	% of total	n	% of total
3 hrs	52	30.77	97	57.40	12	7.10
4 hrs	92	54.76	139	82.74	1	0.59
8 hrs	96	57.49	149	89.22	2	2.98
12 hrs	98	57.65	144	84.71	5	2.94

Table 3.5: Vocalisation response latencies (s) of single lambs by sex and age of dam shown as median (25-75th percentile).

Age Post partum	Dam age 2 yrs		Dam age 3-5 yrs		Cox PHM	
	Male n=41	Female n=31	Male n=47	Female n=49	Predictor	*p* value
3 hrs [a]	7.70 (2.60-17.89)	3.19 (1.16-5.50)	3.47 (1.49-11.69)	3.49 (1.31-9.42) [g]	Sex ParityxSex	<0.02 <0.005
4 hrs [b]	2.63 (1.31-5.25)	1.69 (0.81-3.66)	1.87 (0.94-3.19)	1.90 (0.98-2.97)	Sex	=0.06
8 hrs [c]	1.75 (1.06-3.30)	1.56 (0.79-2.60) [e]	1.50 (0.81-3.38)	1.67 (0.78-3.67)	Sex Parity ParityxSex	<0.0001 <0.01 <0.05
12 hrs [d]	2.00 (1.00-5.84)	1.22 (0.78-2.53)	1.51 (0.85-3.07) [f]	1.91 (0.79-3.03) [g]	Sex Parity ParityxSex	=0.0001 <0.05 <0.01

[a] p=0.02 for sex effect (hazard ratio$_{sexmale}$=0.53, 95% CI[0.32-0.89]).
p=0.005 for parity x sex interaction (hazard ratio$_{male:dam>2yrs}$=1.94, 95% CI[1.22-3.05]).
[b] p=0.06 for sex effect (hazard ratio$_{sexmale}$=0.79, 95% CI[0.62-1.01]).
[c] p<0.0001 for sex effect (hazard ratio$_{sexmale}$=0.65, 95% CI[0.52-0.80]).
p=0.002 for parity effect (hazard ratio$_{damage>2yrs}$=0.74, 95% CI[0.61-0.90]).
p=0.011 for parity x sex interaction (hazard ratio$_{male:dam>2yrs}$=1.65, 95% CI[1.12-2.45]).
[d] p=0.0001 for sex effect (hazard ratio$_{sexmale}$=0.51, 95% CI[0.36-0.71]).
p=0.03 for parity effect (hazard ratio ratio$_{damage>2yrs}$=0.61, 95% CI[0.39-0.95]).
p=0.03 for parity x sex interaction (hazard ratio$_{male:dam>2yrs}$=2.27, 95% CI[1.09-4.76]).
[e] n=30, [f] n=48, [g] n=50.

Test arena behaviour

More than 50% of lambs had stood within 5 seconds (52.9%, n=74) when released in the test arena at 4 hours postpartum; and only one lamb did not stand within the 181 second test period. A larger proportion of lambs at 4 hours postpartum did not reach the ewe contact zone within the allocated time of 181 seconds (18.1%, n=25); but by 8 hours only n=15 lambs did not achieve successful reunion with their dam. At 12 hrs postpartum n=9 lambs did not reach the contact zone, and a number of these appeared to be unconcerned by separation (pers. observation, C. Morton).

Median latencies to stand and return to the dam in the test arena decreased over time (Table 3.6) and at all periods of testing the slowest female lamb returned to the ewe in faster times than the slowest male. Male lambs were 21.0% more likely to be slower to stand than females (hazard ratio$_{male}$=0.79, 95% CI [0.64-0.96], p<0.05) at 4 hours postpartum, and at 8 hours postpartum lambs of mature ewes were more likely to be

slower to stand than those of young ewes (hazard ratio $ratio_{damage>2yrs}$=0.84, 95% CI [0.72-0.97], p<0.05).

Table 3.6: Test arena latencies (s) of single lambs by sex and age of dam shown as median (25-75th percentile), n.

Behaviour and lamb age	Dam age 2 yrs		Dam age 3-5 yrs		Cox PHM	
	Male	Female	Male	Female	Predictor	p value
Stand (s)						
4 hrs [a]	5.0 (4-12), 32	6.0 (3-9), 26	5.0 (3-14), 39	5.0 (3-12), 41	Sex	<0.05
8 hrs [b]	4.0 (2-5), 30	3.0 (2-5), 24	3.0 (2-8), 38	4.0 (2-6), 45	Parity	<0.05
12 hrs	3.0 (2-3), 29	2.0 (2-4), 23	3.0 (2-5), 39	2.0 (2-3), 47		NS
Return to dam (s)						
4 hrs [c]	50.0 (18.5-na), 32	19.0 (11-115), 26	24.0 (11-91), 39	31.0 (12-74), 41	Parity	<0.05
8 hrs	19.0 (7-39), 30	12.0 (5-64), 24	16.0 (5-48), 38	13.0 (6-50), 45		NS
12 hrs	11.0 (5-30), 29	7.0 (4-26), 23	13.0 (4-49), 39	9.0 (4-25), 47		NS

[a] p = 0.03 for sex effect (hazard $ratio_{sexmale}$=0.79, 95% CI[0.64-0.96]).
[b] p = 0.02 for dam effect (hazard ratio $ratio_{damage>2yrs}$=0.84, 95% CI[0.72-0.97]).
[c] p = 0.012 for parity effect (hazard $ratio_{damage>2yrs}$=1.25, 95% CI[1.05-1.49]).
NS = Non significant.

3.3.2 Differences within a litter

A total of 66 twin lambs (33 complete sets) born to multiparous ewes only were included in the twin analysis because there were insufficient sample numbers born to first parity ewes. There were 4 lambs which required assisted birth (second born n=3). Data for physical measures by sex and birth order are shown in Table 3.7.

Table 3.7: Within-litter physical measures of twin lambs by sex and birth order (LSmeans±SE).

Variable	Sex		Birth order		Effect	p value
	Male n=41	Female n=25	First born n=33	Second born n=33		
Birth weight (kg)	3.60±0.13	3.43±0.15	3.60±0.13	3.43±0.14	Birthorder	<0.05
Girth (cm)	35.56±0.27	36.54±0.32	36.20±0.27	35.90±0.28	Birthwt Sex	<0.0001 <0.01
Length (cm)	43.32±0.43	43.69±0.51	43.59±0.43	43.42±0.44	Birthwt	<0.0001
Rectal temp (˚C)	39.35±0.21	39.19±0.22	39.39±0.22	39.16±0.22		NS

NS = Non significant.

Birth weight, body dimensions and rectal temperature

Mean birth weight of twin lambs was lighter than single lambs by an average of 0.79 kg for males (twin: 3.77±0.12 kg; singleton: 4.56±0.85 kg) and 0.75 kg for females (twin: 3.64±0.14 kg; singleton: 4.39±0.91 kg). Rectal temperature was also significantly lower in twin lambs (p<0.01), ranging from 33.5 to 40.8°C. Four lambs were severely hypothermic (<37°C, birth weight 2.9 to 4.1 kg) at 3 hours postpartum and n=10 were mildly hypothermic (<39°C). First born lambs were significantly heavier (p<0.05) than the second born, and male lambs were non-significantly heavier than females within a litter, although mean girth circumference of females was significantly larger (p<0.01).

Early neonatal and separation behaviours

Early behavioural, vocalisation and test arena median latencies by birth order and sex within a litter are shown in Table 3.8 with the corresponding model hazard ratios for this data shown in Table 3.9. There was considerable variation in vocalisation response time within a litter, with 30 seconds being the largest difference measured between siblings. All second born lambs responded vocally within 10 seconds. The only non responding animal of all twin animals was a first born lamb. Second born lambs were more likely to initiate a bleat earlier by a factor of at least 2, at most time points, when compared to the first born in each litter (refer to hazard ratios 3.9). Second born lambs tended to be more likely to return to the ewe at 4, and 12 hours postpartum in less time but this was only significant at p<0.08 level. Test arena results were quite variable and at 8 hours there appeared to be little significant difference.

Males co-twins generally had a marginal probability of being slower (hazard ratios <1) to initiate a vocalisation and this was more evident at 12 hours postpartum (hazard $ratio_{male}$=0.55, 95% CI [0.33-0.80], p<0.07). But a significant birth order x sex interaction at 4 hours postpartum indicated that male co-twins were only more likely to initiate a vocalisation faster if they were second born (hazard $ratio_{male:birthorder}$=3.86, 95% CI [1.08-13.81], p<0.002). Apart from vocalisation, males also often displayed longer median latencies for early and test arena behaviours although significance levels were p>0.10 or marginal and impacted by birth order interaction, i.e. test arena behaviour at 4 hours postpartum (hazard $ratio_{male:birthorder}$=1.72, 95% CI [0.36-3.36], p=0.08). Birth weight of co-twins impacted upon test arena return latency and bleat initiation at 12 hours,

indicating that the heavier twin was more likely to be quicker in returning to the dam and initiating a vocalisation than lighter weight siblings ($p<0.01$).

Table 3.8: Within-litter twin lamb latencies for early and test arena behaviours shown as median (25-75th percentile).

Behaviour	Birth order 1			Birth order 2		
	Male n=19	Female n=14	Total n=33	Male n=22	Female n=11	Total n=33
Stand (min)	20.00 (11.0-29.5)	22.50 (8.5-49.5)	20.00	17.00 (13.0-25.0)	12.00 (7.0-20.0)	15.00
Suck (min)	43.50 (33.5-90.5)	43.50 (28.0-62.5)	43.00	51.00 (36.0-83.0)	36.00 (16.0-45.0)	38.00
Vocalisation latency 3 hrs (s) [a]	2.06 (1.4-3.6)	4.05 (2.0-7.6)	2.62	1.26 (0.9-2.7)	1.64 (1.0-2.4)	1.37
4 hrs (s) [ab]	3.31 (2.0-7.5)	1.98 (1.1-2.8)	2.69	1.00 (0.6-2.0)	1.76 (0.9-5.7)	1.34
8 hrs (s) [a]	1.22 (0.9-2.3)	1.44 (0.8-2.5)	1.22	1.31 (0.8-2.4)	1.28 (0.6-2.1)	1.28
12 hrs (s)	2.53 (1.0-8.2)	2.28 (1.9-3.5)	2.28	1.58 (1.3-3.1)	1.64 (0.7-2.1)	1.58
Return to ewe latency 4 hrs (s)	32.00 (27.0-na)	16.00 (11.0-47.0)	29.00	25.00 (14.0-52.0)	21.00 (14.5-32.0)	21.00
8 hrs (s)	13.50 (11.0-5.5)	12.00 (6.0-18.0)	12.00	12.00 (7.0-29.0)	9.00 (8.0-45.0)	12.00
12 hrs (s)	12.50 (6.0-34.0)	10.00 (7.0-26.0)	12.00	6.00 (4.0-20.0)	9.00 (6.0-14.0)	6.00

[a] Birthorder effect ($p<0.05$); See Table 3.9 below.
[b] Sex x birthorder interaction ($p<0.05$) See Table 3.9 below.
na = Unable to be calculated because of censored data.

Table 3.9: Within litter hazard ratios and 95% confidence intervals for twin lamb behavioural latencies at various ages postpartum. Medians shown in Table 3.8.

Behaviour	Predictor variable	Hazard ratio	95% CI	Significance
Bleat 3 hrs	Birthorder2	2.07	1.17-3.66	<0.03
Bleat 4 hrs	Birthorder2	2.27	1.41-3.37	<0.004
	Birthorder2 x Sexmale	3.86	1.08-13.81	<0.002
Bleat 12 hrs	Birthorder2	2.75	1.46-5.81	<0.02
	Sexmale	0.55	0.33-0.80	<0.07
	Birth weight	1.0009	1.00-1.001	<0.0008
Return to ewe 4 hrs	Birthorder2 x Sexmale	1.72	0.36-3.36	=0.08
Return to ewe 12 hrs	Birthorder2	1.86	0.93-3.75	=0.08

In summary, the median vocalisation response latency across all twin lambs, fitting sex, birth order, sire, birth weight and age into the model was predicted to be 1.72 s, 95% CI [1.47-2.03]. First born lamb latency median was 2.16 s, 95% CI [1.89-2.82] and second born median latency was 1.37 s, 95% CI [1.25-1.68]). Birth order was the only significant term in the full model ($p<0.001$) and in general, males were more likely to be slower in behaviours when they were the first born.

3.3.3 Relationships between vocalisation latency and vigour measures

When singleton lambs were grouped into slow and fast bleat response groups, there was a significant association between vocalisation and test arena reunion latencies (Table 3.10). Lambs who were less responsive vocally were also slower to return to the dam compared to more responsive lambs at all periods of testing ($p<0.05$) (refer to Table 3.10). While median latency to reunite with the dam at 4 hrs postpartum was also slower in lambs with delayed vocalisation initiation an hour earlier, this was not statistically significant except at the 4 hr return test ($p<0.05$). There were also slightly lower median suckle latencies in lambs slow to initiate a bleat but this difference was not statistically significant ($p>0.10$).

In twins, more responsive lambs tended to have faster medians for return to the dam when compared to less responsive animals, but with small numbers data should be viewed with caution. Significant differences in Kaplan Meier distributions were only evident for some measures (see Table 3.11).

Table 3.10: Relationship between vocalisation latency threshold groups and vigour-related behaviours in singleton lambs shown as median [95% confidence intervals], n. Bold types indicate significant (p<0.05) and marginally significant (p<0.01) differences.

Age postpartum/latency group		Stand (min)	Suckle (min)	Return to dam (s)		
				Age 4 hrs	Age 8 hrs	Age 12 hrs
3 hrs	Fast (≤ 2 s)	24.5 [20-36], 42	44.0 [36-63], 42	**20.0 [15-38], 43 ***	12.0 [7-28], 43	9.0 [7-19], 43
	Slow (>11 s)	24.0 [20-38], 43	49.0 [37-66], 43	**37.0 [37-66], 34**	22.0 [13-37], 45	14.5 [9-31], 34
4 hrs	Fast (≤ 2 s)	25.0 [22-36], 76	49.0 [41-61], 77	**19.0 [14-35], 78 ******	**10.0 [8-14], 76 ****	**7.0 [6-11], 76 ****
	Slow (> 3 s)	25.0 [18-35], 48	47.0 [42-60], 48	**71.0 [43-na], 41**	**23.0 [13-66], 40**	**15.0 [9-54], 40**
8 hrs	Fast (≤ 2 s)	24.0 [21-33], 80	45.0 [38-53], 80	**22.0 [17-33], 81 ***	**11.0 [8-17], 81 ****	**7.0 [5-10], 78 ***
	Slow (> 3 s)	27.0 [18-42], 40	51.0 [44-68], 41	**67.0 [48-na], 33**	**42.0 [24-75], 33**	**19.0 [11-49], 34**
12 hrs	Fast (≤ 2 s)	22.0 [18-29], 79	42.0 [37-52], 80	**18.0 [12-26], 75 ********	**10.5 [8-16], 76 *****	**7.0 [5-10], 79 *****
	Slow (> 3 s)	25.0 [20-33], 42	48.0 [45-60], 42	**74.5 [43-na], 38**	**39.5 [24-86], 36**	**24.5 [16-49], 36**

na = Unable to be calculated because of censored data.
* p<0.05
** p<0.01
*** p<0.001
**** p<0.0001.

Table 3.11: Relationship between vocalisation latency and test arena behaviours in twin lambs shown as median [95% confidence intervals], n. Bold types indicate significant (p<0.05) and marginally significant (p<0.01) differences.

Bleat latency groups		Return to dam (sec)		
		Age 4 hrs	Age 8 hrs	Age 12 hrs
4 hrs	Fast (≤ 2 s)	**18.0 [14-41], 22** [t]	**11.0 [7-34], 21** [t]	6.0 [5-21], 22
	Slow (> 3.6 s)	32.5 [21-na], 12	**12.0 [11-na], 13**	19.5 [9-na], 12
8 hrs	Fast (≤ 2 s)	**20.0 [14-36], 24** *	12.0 [8-29], 26	8.0 [6-21], 24
	Slow (> 2.3 s)	**35.5 [25-na], 12**	16.0 [8-na], 12	9.0 [4-na], 12
12 hrs	Fast (≤ 2 s)	25.0 [14-49], 21	**11.0 [8-29], 21***	10.5 [6-35], 30
	Slow (> 3.5 s)	32.0 [12-na], 8	**30.0 [12-na], 10**	17.5 [12-na], 10

na = Unable to be calculated because of censored data.
*p<0.05; [t] p=0.08.

There were also significant associations between vocalisation and test arena latencies as indicated by correlations between these measures at various times postpartum (Table 3.12). Behaviours measured at 4 and 8 hours postpartum showed the closest associations between vocalisation and behavioural measures. Vocalisation at 4 hours postpartum was positively correlated with return latency at 4 hours and 8 hours postpartum (r=0.37, p<0.0001). Vocalisation latency at 8 hours was more closely associated with return to dam measures at all test times (4 hours: r=0.30, p<0.001; 8 hours: r=0.26, p<0.001; 8 hours: r=0.35, p<0.0001). Vocalisation associations reflecting a weaker relationship with return latencies were evident at 12 hours postpartum (p<0.05). Early suckling latency was not well correlated to vocalisation except for demonstrating a weak association at 8 hours postpartum (r=0.18, p<0.05).

Table 3.12: Pearson correlation coefficients and significance (p<0.05) for relationship of vocalisation latency with behavioural latencies in singleton lambs.

Bleat latency	Return to dam (hours postpartum)			Early behaviour
/age (s)	4 hours	8 hours	12 hours	Suckle
Log Latency to bleat 3 hrs	0.24 **	NS	NS	NS
Latency to bleat 4 hrs	0.37 ****	0.37 ****	0.26 **	NS
Latency to bleat 8 hrs	0.30 ***	0.26 ***	0.35 ****	0.18 *
Latency to bleat 12hrs	0.19 *	0.18 *	0.24 *	NS

*p<0.05; ** p<0.01; *** p<0.001; **** p<0.0001.
NS = Non significant.

3.3.4 Sire influence

Sire effect - singletons

There was a significant sire-associated difference between singleton lambs for birth weight ($p<0.05$) and girth circumference ($p<0.01$) (Table 3.13). Lambs of S2 and S3 had significantly smaller girth measures but were also in the higher birth weight group, along with S5. Thus these animals had a significantly lower mean girth:body weight ratio ($p<0.01$) than lambs of the other 3 sires. Lambs sired by S2 had the smallest mean girth circumference and lambs sired by S4 had the largest mean girth circumference. Lambs sired by S1 had the largest girth:body weight ratio. There were no significant differences in rectal temperature or parturition duration associated with sire.

All lambs of S2, S4 and S5 had responded vocally in response to separation by 12 hours postpartum (by 5, 20 and 9 s respectively), and all lambs of S2 had initiated a bleat response within 23 seconds at the earliest age of testing. Three lambs of S1 did not respond during any of the tests, except at 4 hrs postpartum where latency for these 3 individuals was higher (>22 s) than the overall singleton median or 50th percentile value (2.03 s, 95% CI [1.88-2.25]). One hundred percent of S2 and S3 sired lambs had achieved reunion with their dam within the allocated test time at 12 hours of age, however a small proportion of lambs sired by the other 4 sires did not achieve reunion within this timeframe (180 s).

Median latency values for early and test arena behaviours of singletons born to each sire, with model hazard ratios and 95% confidence intervals are presented in Appendix 1. There were differences in the rate of change and median response times for vocalisation response and ability to re-join the dam of lambs sired by the different sires as illustrated in the figures in Appendix 2. Lambs of S2 and S5 demonstrate significantly steeper survival curves and higher hazard ratios than those of other sires, indicating that S2 lambs were more likely to have faster reactions times in most measures. There was a significant effect of sire in all vocalisation and test arena return measures, except vocalisation response measures at 4 hours postpartum. A summary of the hazard ratios for singleton early behaviour, bleat and test return latency measures to compare sires are shown in Figure 3.3 indicating likelihood of faster response times and significant sire difference for most measures. S2 was ranked best or second best among all sires and across all

measures when compared to the reference sire S1. There were no significant differences in stand or suckling latencies related to sire.

Sire effect - twins

Sire distribution was highly variable in twin litters, and only n=3 sets of twins sired by S2 and S5 were available for analysis. There was a significant difference in twin lamb birth weight associated with sire ($p<0.05$) with lambs of S5 and S6 having heavier birth weights. As in singleton lambs, twin lamb mean girth circumference was smallest in lambs sired by S2 and largest in lambs sired by S4, although this difference did not reach statistical significance ($p>0.01$). There also appeared to be a sex x birth weight interaction for girth measurement (Table 3.14), but small numbers many have influenced this result. In most behavioural measures there was no effect of sire, and where an effect was significant sire ranking was inconsistent (data not shown). The most marked effect of sire was shown in the measure for latency to suck where lambs of S2 ($hazardratio_{sire2}=2.44$, 95% CI [1.59-3.77], $p<0.001$) were more likely to suckle earlier (median latency of first born vs second born lambs, 38.0 and 34.00 s respectively). Any sire differences reported here should be viewed with caution because of the small cell numbers.

Table 3.13: Physical measures of singleton lambs by sire (LSmeans±SE).

	Sire						
	S1	S2	S3	S4	S5	S6	*p* value
	n=28	*n=30*	*n=20*	*n=29*	*n=36*	*n=27*	*n=170*
Birth weight (kg)	4.18±0.14	**4.58±0.16** [a]	**4.79±0.16** [a]	4.29±0.14	**4.60±0.12** [a]	4.43±0.14	Sire: *p*<0.01
Girth (cm)	39.45±0.29	**38.24±0.29** [a]	**38.55±0.34** [a]	39.57±0.29	38.85±0.27	38.88±0.30	Sire: *p*<0.01
Length (cm)	46.65±0.67	47.09±0.65	46.37±0.79	46.85±0.66	45.97±0.60	46.63±0.68	Sire: NS
Girth: weight (cm/kg)	9.49±0.23	**8.61±0.23** [a]	**8.41±0.27** [a]	9.23±0.27	**8.66±0.21** [a]	8.89±0.23	Sire: *p*<0.01
Rectal temp (˚C)	39.45±0.10	39.59±0.10	39.66±0.12	39.73±0.10	39.63±0.09	39.67±0.10	Sire: NS
	n=18	*n=28*	*n=17*	*n=24*	*n=34*	*n=23*	*n=144*
Parturition duration (mins) [b]	1.15±0.10 (14.12)	1.29±0.08 (19.60)	1.53±0.10 (33.84)	1.41±0.08 (25.81)	1.41±0.07 (25.58)	1.28±0.08 (18.88)	Sire: *p*=0.08

[a] Different superscripts within rows indicate means which differ significantly (*p*<0.05).
[b] Data are shown as log LSmean, back transformed means in parentheses.

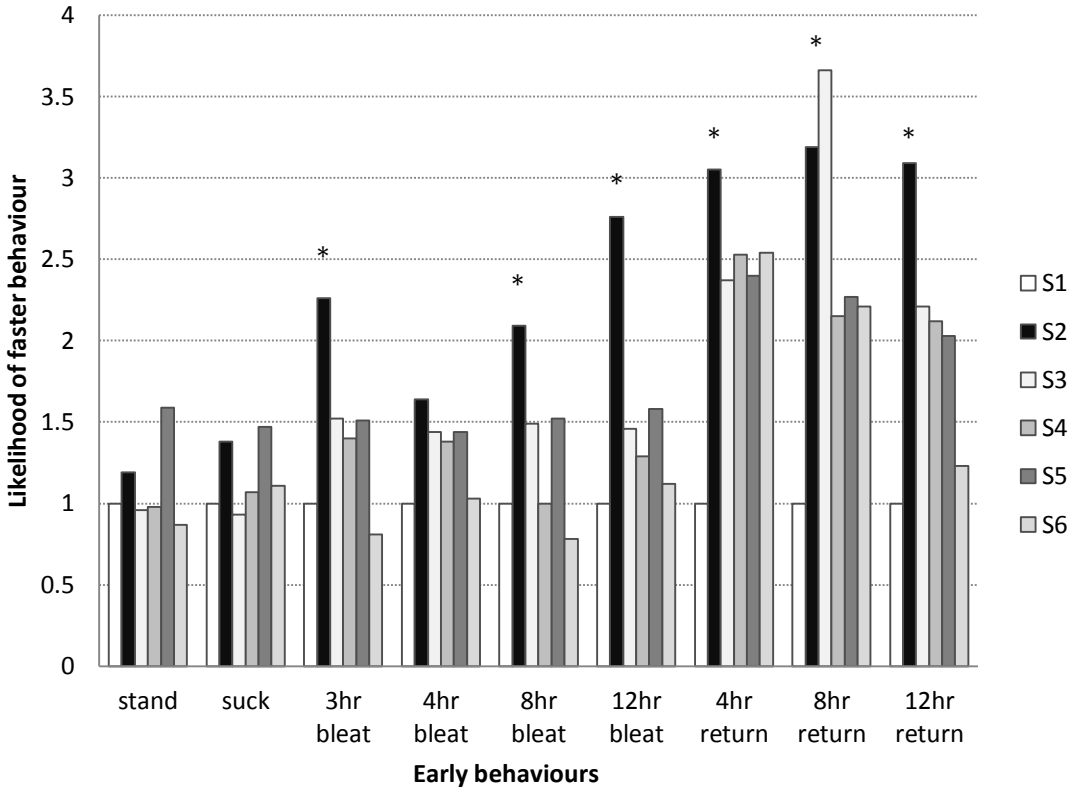


Figure 3.3: Relative performance of sires based on latency of singleton behaviours. Asterisk indicates significant difference between sires (p<0.05), compared to reference S1. Full data shown in Appendices.

Table 3.14: Within-litter physical measures of twin lambs associated with sire (LSmeans±SE).

	Sire						
	S1	S2	S3	S4	S5	S6	*p* value
Variable	*n*=14	*n=6*	*n=12*	*n=20*	*n=6*	*n=8*	*n=66*
Birth weight (kg)	3.32±0.22	3.90±0.34	3.25±0.24	3.17±0.18	**4.25±0.34** [a]	**4.31±0.29** [a]	Sire<0.05
Girth (cm)	35.87±0.39	33.47±0.85	35.66±0.44	36.86±0.36	36.63±0.72	35.55±0.73	Sire x birthwt <0.01
Length (cm)	41.99±0.69	45.35±1.08	44.61±0.74	43.13±0.59	45.24±1.01	43.03±0.96	Sire=0.09
Rectal temp (˚C)	39.73±0.37	39.17±0.58	39.42±0.41	38.98±0.32	37.88±0.59	40.06±0.52	NS

[a] Different superscript within rows indicate means which differ significantly (p<0.05).

3.3.5 Lambs with a consistently poor vocalisation response

Lambs which demonstrated consistently retarded behaviours until 12 hours postpartum are listed in Table 3.15. All but one of the 5 lambs responded with a vocalisation only when tested in the outdoor test arena environment at 4 hours postpartum - the first time lambs had been exposed to this environment. Latency of each vocalisation response was slower than the 75th percentile value (less than 4 s) for each time point, and all other behavioural latencies were similarly slower than each relevant behavioural median (reported in Table 3.6). Two additional lambs (IDs 4 and 5 with incomplete observations) were originally excluded from the analysed data set because they were born to twin-bearing primiparous ewes. A number of the delayed response animals had experienced difficult parturition and had required assisted delivery (all sired by sire S4 which was associated with the largest mean girth measure in both singleton and twin lambs). No lambs exhibited behavioural latencies within the fastest 25^{th} percentile of measures for that behaviour.

Table 3.15: Measurements of lambs with consistently delayed vocalisation responses up to 12 hours postpartum.

ID [a]	Sex	Dam age (yrs)	Birth weight (kg)	Rectal temp. (˚C)	Labour duration and delivery	Bleat latency (s) at each age postpartum				Early behaviour category [b]		Return to ewe category at each age postpartum [b]		
						3 hrs	4 hrs	8 hrs	12 hrs	Stand	Suckle	4 hrs	8 hrs	12 hrs
1-S4	M	2	4.13	36.9	68 mins/ assisted	TE	30.66	TE	TE	slow	slow	TE	na	na
2-S1	F	>2	5.16	39.0	na	TE	83.30	45.58	TE	medium	medium	TE	TE	slow
3-S6	M	2	3.05	39.1	11 mins/ normal	TE	21.45	TE	TE	slow	slow	TE	slow	TE
4-S4 [c]	M	>2	2.51	38.5	>10mins/ assisted (weak lamb)	TE	TE	TE	TE	na	na	na	na	na
5-S4 [c]	F	2	4.13	39.6	120 mins/ assisted & malpresented	TE	8.61	na	na	slow	slow	na	na	na

[a] ID number also identified by sire number.
[b] Slow = latency >75th percentile; medium = latency >25th and <75th percentile, refer Table 3.6 for values.
[c] Excluded from statistically analysed data.
TE = Time elapsed (>90 seconds for vocalisation latency; >180 seconds for return to ewe latency).
na= Datum not available.

3.4. Discussion

Factors affecting lamb arousal and test response

The method of separating the lamb a distance of 2 meters from the dam to elicit a distress vocalisation, and initiating a timed response by removal of restraint, appeared to be successful in identifying differences in vocal responsiveness between groups of animals in this study. For the sheep, a predated species, the state of separation is extremely stressful in both young and adults (Ligout, Foulquié, Sèbe, Bouix, & Boissy, 2011; Porter, Nowak, & Orgeur, 1995; Price & Thos, 1980; Rault, Boissy, & Boivin, 2011) and a capacity for arousal will contribute to survival success (Dwyer & Lawrence, 2005b; Lingle et al., 2012). The condition of separation in a precocial animal such as the neonate lamb appears to be recognised by acute visual and auditory senses (Nowak, 1990b, 1991; Shillito & Alexander, 1975; Shillito, 1975; Vince, Lynch, Mottershead, Green, & Elwin, 1985), although lamb olfactory senses responding to ewe inguinal wax odour may also play a role (Vince, Lynch, Mottershead, Green, & Elwin, 1987; Vince & Ward 1984). While breed or genotype differences have been reported in discrimination ability of 12 hour old lambs (Nowak et al., 1987) and vocalisation behaviour of older lambs (Dwyer et al., 1998; Shillito Walser, 1978; Shillito Walser, Walters, & Ellison, 1984), the degree to which very young lambs aged between 3-4 hours old are sensitive to perception of isolation, movement and auditory cues has not been well documented.

The procedure reported on here was designed to limit any effect of handler movement once the test was commenced as extraneous visual or auditory stimuli may have had an impact on test results (Nowak, 1991; Vince et al., 1985). Handling of animals during testing was also limited as much as possible in the period following birth and during test procedures, as tactile stimulation facilitated by the mother's grooming is known to effect lamb and rodent pup behaviour (Van Oers, de Kloet, Whelan, & Levine, 1998; Vince et al., 1985). There were a number of personnel conducting the tests, particularly in operation of timing devises; and handler bias may have associated with some animal behavioural responses. To allow for this variability it may have been more accurate to include tester identity as a fixed effect in the modelling of results, however there would have been a loss of statistical power with such an inclusion, if found to have an impact.

The results of this study also suggest that an impact associated with environment change occurring at 4 hours postpartum, when lambs were first exposed to different visual and sound stimuli in the outdoor conditions of the test arena, may have occurred. It appeared that lambs with slower responses at other test periods were more reactive when introduced to a foreign environment as indicated by survival curve comparisons. Changed environments can lead to inaccurate test results in gregarious animals and domestic species (Forkman, Boissy, Meunier-Salaün, Canali, & Jones, 2007) but in this study, while there was less variability between groups, the relative rankings within groups could still be identified at this point in time. In mammalian neonates separation promotes a two phase reaction initiated by intense stress and higher reactivity (Hofer, 1996c; Kanitz et al., 2009) and corticosterone response to mild or more severe stressors in rodents are reported to be highest in the initial days after birth (Hofer, 1996c). The first 4 hours following birth are also likely to be a period of rapid developmental change for such lambs, corresponding to the maximum time period that Merino ewes typically remain on the birth site (Nowak, 1996). Similarly it is possible that separation stress in a novel environment could elicit a higher arousal state and more rapid behavioural responses in lamb neonates. These propositions infer that delayed response lambs may require a higher threshold to elicit reactivity and that visual or auditory stimuli in the sheep neonate may have a significant influence on behaviour at this early stage.

The strength of maternal vocal cues has also been reported to be involved in readiness of lambs to respond and reunite (Pollard, 1992). However this does not explain why in this study, male or first born co-twins would exhibit significantly slower responses, nor why lambs born to particular sires should be alike. Levels of ewe responsiveness were also recorded in this study during testing procedures and there did not appear to be any obvious variation in dam vocalisation stimuli for the lamb – all ewes responded to the separation of their lamb with agitation and repeated bleating, and the greatest diversity observed was in the behaviour of the lamb. An undisturbed and intimate bonding process facilitated by the small pens of the animal house (Alexander & Peterson, 1961) as well as hormonal influences on the ewe (Dwyer et al., 1998) appeared to promote universally strong maternal responses following removal of the lamb.

Significance of within-litter difference

Difference in lamb arousal or reactivity associated with temperament (Bickell et al., 2009; Plush, Hebart, Brien, & Hynd, 2011) and specifically sire-related genotype, also seems unlikely when consistent differences were found to exist between co-twins of a litter in this study. A faster vocal responsiveness and ability to reunite with the dam was evident in the second born co-twin, and this difference was more obvious in male animals. The first born co-twin in this trial was also more likely to have a heavier birth weight than the second born, a finding which has been inconsistently reported in human twin studies (Boomsma, Orlebeke, & Van Baal, 1992). Few studies have identified or investigated differences in birth order of lambs, or partitioned the effect of birth order appropriately by use of paired statistical tests. However significantly longer parturition duration and higher incidence of birth difficulty (Arnold & Morgan, 1975; Grommers et al., 1985 ; Owens et al., 1985); and higher cortisol levels (Kerslake, 2010; Mellor, Matheson, & Small, 1977) have been reported in the first born co-twin of Merino and other breeds of sheep.

While there is some uncertainty that the birth order effect on vocalisation latency of this trial was entirely without bias due to methodological protocol, the reported difference in vocalisation response was also evident in the cases where testing order had been reversed. This study also corroborates results of other studies suggesting that unhoused twins compared to singletons could be more compromised (Barlow et al., 1987; Dutra & Banchero, 2011; Hinch, Kelly, Davis, Owens, & Crosbie, 1985) because a larger number of animals had rectal temperatures in the hypothermic range, although overall twin median behavioural indices were higher. Sire-related confirmation differences may also have been reflected in twins but did not appear to impact on behaviours, although any sire-related generalisations are difficult to make because of low sample size. While efforts were made to allow for adequate statistical power to detect differences within the effects of sex, parity, litter size and sire for this experiment; post conception loss and poor conception rate of first parity ewes prevented adequate investigation of sire differences within twin litters, and the effect of parity in twin bearing animals, which may have provided greater variation in lamb behaviours.

Vocalisation latency as an indicator of vigour

Comparisons of fast and slow animals based on vocalisation latency indicated that vocally responsive animals were more inclined or motivated to reunite with their dam than lambs with slow or delayed vocalisation reactions. Delayed reactions or an inability to recognise the danger of separation from the dam implies that these lambs may be more likely to suffer abandonment as the ewe moves off the birth site, depending on prevailing environmental conditions, than those who attract the dam with an immediate and urgent sounding signal (Lindsay, Nowak, Putu, & McNeill, 1990). Merino ewes in particular have been associated with a tendency to abandon weak or less vigourous lambs (Alexander & Peterson, 1961; Stevens et al., 1982), and although the role of the lamb in this process has also been considered (Nowak et al., 1987) there is little available data to demonstrate this.

The results of this study suggest that lambs with a shorter latency to initiate a separation distress vocalisation are responding to separation more appropriately both in the timing of the signal and their capacity to locate and return to their mother. Vocalisation responsiveness and desire to reunite with the dam appeared to increase over time in lambs with delayed vocal initiation, although there remained individuals who continued to show significantly delayed reactions, independent of sire, parity or sex up to 12 hours post-partum. This is the time at which lambs have been reported to discriminate between their own and an alien ewe (Nowak et al., 1987; Val-Laillet & Nowak, 2006), and some animals could be expected to display habituated behaviours related to the repeated testing procedure (Nowak 1994). However, in general initially less responsive animals demonstrated a capacity to vocalise and return to their dam with improved rapidity over time; which implies that that either rapid maturation processes (Terrazas et al., 2003) or recovery from the birth event may be occurring over 12 hours postpartum, and that a significant proportion (two thirds) of animals were already capable of this level of responsiveness or cognition at 4 hours postpartum. This finding suggests that such differences may be reflective of variation in vigour or cognitive ability and could provide useful information in making an assessment regarding lamb viability.

Lowered rectal temperature in the immediate postpartum period has also been reported to have an association with poor neonate lamb viability and behavioural deficiency (Dwyer

et al., 2005; Eales & Small, 1980; Slee & Springbett, 1986) even up to 72 hours of age (Dwyer & Morgan, 2006) or in field studies (Brien et al., 2010; Dwyer, Lesage, & Richmond, 2014). Mean lamb rectal temperature measured at 3 hours postpartum in this study was found to be slightly lower in male lambs born to primiparous ewes compared to those born to mature ewes, although our results showed a narrower range in rectal temperature than that shown in housed singleton Suffolk lambs (Dwyer et al., 2005) and field studies of merino lambs (Slee & Springbett, 1986). While comparisons between studies are difficult because of variables such as ewe nutrition, body condition and environmental conditions; there were a number of animals in the current trial displaying temperatures indicative of severe hypothermia and these lambs also demonstrated significantly retarded, or absent, vocal and behavioural responses. Lowered ability to maintain rectal temperature following neonatal asphyxia up to one day of age has been also been reported in piglets (Herpin et al., 1996) and up to an hour in pups (Silva et al., 2009). A number of explanations for lowered rectal temperature impacting on neonate lamb viability have been given by researchers including smaller birth weight (Barlow et al., 1987), and low summit metabolism following prepartum hypoxia (Eales & Small, 1980). Low rectal temperature of neonate lambs could also be indicative of anapyrexia, the autonomic lowering of body temperature which has been well established in mammals as a regulated protective response to reduce oxygen demand following hypoxia (Branco et al., 2006; Steiner et al., 2002).

Risk factors associated with vocalisation and behavioural deficit in this study

While there was some variability in results of this study using modelled hazard ratios, there were consistent trends in both singleton and twin data regarding vocal responsiveness and capacity to reunite with the dam following separation. Most test results indicated that vocalisation responsiveness and reuniting behaviours were more likely to be slower in male lambs and in particular male lambs born to primiparous ewes, where duration of parturition was longer and impacted by birth weight. Early standing and suckling latencies were not clearly indicative of significant differences although male lambs appeared more likely to be slower to achieve successful udder attachment in both older and inexperienced ewes. The lambs born to older ewes in this study showed similar median stand latencies to those reported by Dutra and Banchero (2011) but slower latencies to achieve successful suckle, which may have reflected slower behaviours of

Merino lambs compared to the breeds used in Dutra's study. It is also possible that in this trial there were fewer lambs with severely retarded early behaviours. Delayed early milestone behaviours of lambs have also been reported to be associated with male sex, first parity and prolonged parturition in other studies (Dwyer, 2003; Dwyer & Bünger, 2011; Dwyer et al., 1996). Moreover, the ability of the lamb to stay with the ewe has been reported to have a poor relationship with lamb activity in the early postpartum period (Nowak, 1990b).

Birth weight alone did not appear to be associated with delayed early vigour measures or vocalisation responses although a number of lambs in the study weighed over the reported thresholds associated with increased risk of dystocia (Alexander, 1984, 2008) and mortality (Hatcher, Atkins, & Safari, 2009; Hinch, Crosbie, Kelly, Owens, & Davis, 1985b). Lambs of the sires which rated better regarding faster latencies for many measures also had comparatively higher average birth weights. While birth weights over 5 kg are clearly associated with longer parturition duration in a number of studies (Dwyer & Lawrence, 1999b; Dwyer et al., 1996) there may be some difficulty in interpretation of birth weight trends in association with mortality risk because of maternal and other factors (Grommers et al., 1985; Hinch et al., 1985b). Neonate conformation factors such as skeletal shape, neck muscularity and maturity may also be associated with dystocia risk and fetal distress in sheep (Dutra & Banchero, 2011; Dutra et al., 2007; Speijers et al., 2010) as is reported in cattle (Barrier, Haskell, Macrae, & Dwyer, 2012). These factors can influence the potential to reduce brain oxygenation by prolonged fetal hypotension (Lou, 1988) at the critical phase of fetal thoracic engagement in the cervical region (Fraser & Terhune, 1977b; Grommers et al., 1985). The results of this study suggest that low girth:body weight ratio or an associated sire-linked conformation trait may have contributed to better behavioural indices in progeny, which corroborates previous findings where higher mortality rate has been associated with heavier lambs of shorter length (Dutra et al., 2007).

The above evidence does support the possibility that vocalisation responsiveness of lamb neonates in the first 12 hours of life may show similarity to the human model where delayed cry latency of over 2.5 seconds following application of a pain stimulus, and a higher threshold to initiate a response, are evidence of poor neurological integrity and

neurological deficit (Fisichelli & Karelitz, 1963; Lester & Boukydis, 1992; Michelsson, 1971; Zeskind et al., 1996; Zeskind et al., 2011). The data reported here does not directly indicate that fetal distress or hypoxic effects were associated with lamb behaviour, but a supposition could be made because the risk factors common to vocalisation delay are also linked to longer parturition. Direct comparisons with rodent models are difficult to make as vocalisation rate rather than latency is more commonly reported (Hahn & Lavooy, 2005) although latency to emit vocalisations within the first 5 mins have been reported in rodent pups following a 20 minute perinatal asphyxia period (Calamandrei et al., 2004).

Sex related differences in babies aged less than a month are not commonly reported in human neonate cry analysis although Davis and Emory (1995) found indications of greater cortisol responses of 40 hour old male human neonates related to stress reactivity. While the causal agents associated with the results of that study could not be confirmed, Davis proposed a number of possible theories including greater neurophysiological and adrenocorticoid maturity in females, greater impact of labour variables in males or other genetic and hormonal influences. These factors may be related to the sex differences observed in this study although if greater cortisol reactivity to separation stress was occurring in male neonate lambs it would be logical to conclude that this should increase vocalisation reactivity rather than delay it. There are no comparable studies providing age-matched data of young animals to this study but the reason for sex differences and consistently slower behaviours in males is a question which should be further examined.

Conclusion

Lamb vocal responsiveness in this study did appear to indicate neonatal behavioural status associated with capacity or motivation to reunite with the dam. Such behaviour, if also reflected in the field, should enhance following behaviour and chances of survival for the lamb especially while the ewe is moving off the birth site. The between and within-litter differences in animals associated with sex, parity and birth order suggest that delayed vocalisation may be an indicator of neurobehavioural deficit as in the human and rodent model rather than direct genetic influence, and that body dimension effects related to body weight and girth:body weight ratios may contribute to ease of labour and degree of fetal distress experienced during birth. There were some lambs with poor behavioural and vocalisation measures with evidence of hypothermia in this study but there were not

enough individuals demonstrating this association to draw firm conclusions. Vocalisation latency following a separation stimulus as performed in this study may prove to be a simple, practical test to indicate lamb vigour or viability within the first 12 hours of birth, however developmental milestones and age postpartum are important factors to consider if undertaking vocal assessment of neonate lambs in the field. Additional research is required to confirm the effect of birth order difference in twin and triplet litters, investigate biochemical indicators associated with distress vocalisation delay and assess the effect of stressors such those imposed by tagging procedures or other environmental stimuli on vocalisation responsiveness.

Chapter 4

Blood chemistry of neonate lambs associated with vocalisation latency

4.1 Introduction

Central nervous system injury as a result of dystocia and birth trauma has been reported to be one of the main causes of perinatal mortality in neonate lambs (Dutra & Banchero, 2011; Dutra et al., 2007; Haughey, 1973; Haughey, 1973b; Haughey, 1980). Prolonged parturition, retarded early behaviours, rectal temperature and blood chemistry indicative of acid-base disturbance and hypoxemia associated with fetal distress have been used as clinical markers to indicate intrapartum asphyxia and neurological damage in the neonate lamb (Barlow et al., 1987; Dutra & Banchero, 2011; Dwyer et al., 1996). Latency of the distress cry response, and other acoustic parameters, has also been used as a sensitive indicator of CNS damage resulting from birth asphyxia and a range of other pathologies in the human neonate (Corwin et al., 1996; Furlow, 1997; Golub & Corwin, 1982; LaGasse et al., 2005; Lester & Boukydis, 1992; Zeskind et al., 2011). Latency of response and number of vocalisations following application of an isolation distress stimulus have also been shown to reflect neurobehavioral deficit in the rodent pup (Brudzynski, 2009; Zeskind et al., 2011). These measures are now commonly applied in the research setting to investigate prenatal influences on CNS development in the human infant (Cox Lippard et al., 2015; Ehret, 2005; Zeskind et al., 2014).

In an initial study investigating differences in distress vocalisation of neonate Merino lambs (reported in Chapter 3), there appeared to be a difference in latency associated with birth order of twin litters, with the first born lamb being more likely to be slower to initiate a vocalisation in response to an isolation distress stimuli. Experimental methodology may have been confounded with the birth order results of that study and more rigourous testing of within-litter birth order effects in vocalisation initiation is required. Few studies have identified distinct differences between co-twins of the same litter, however first born lambs have been reported to be at a greater risk of birth difficulty (Grommers et al., 1985)

and longer parturition duration (Owens et al., 1985). This indicates that delays in distress vocalisation initiation in the first born lamb may reflect greater trauma associated with being the first to traverse the birth canal during labour. The process of birth imposes enormous stress on the fetus throughout the second stage of labour (Aldrich et al., 1995) and current evidence supported by other research (Mellor, 1988) suggests that this may be greater for the first born.

Furthermore, differences in bleating behaviour based on rate of vocalisation have been reported to be associated with breed in neonate and older lambs (Dwyer & Lawrence, 1998b; Nowak, 1990b; Shillito Walser, 1978). These reported differences may also reflect delayed vocalisation initiation or bleat response latencies, which in some cases may be associated with prenatal or intrapartum effects as opposed to genetic or breed specific traits. The following study seeks to investigate differences in vocalisation latency and blood biochemical markers associated with litter size, birth order, sire breed and application of an additional pain-related stressor in neonate lambs (as was applied in the bleat score procedure of Brien et al, 2009). It was hypothesised that latency to initiate a distress vocalisation will be correlated with blood chemistry results indicative of greater fetal distress in lambs; and that first born co-twins within a litter, and terminal sire cross lambs born to merino ewes will be at greater risk of vocalisation delay or poor vocal responsiveness when subjected to isolation stress. Other markers of fetal distress such as length of labour, body size and rectal temperature may also demonstrate an association with delayed vocal responses in neonate lambs. The application of an additional stressor was expected to have some effect on some animals - either to delay vocalisation response in rapidly responding lambs or to promote faster vocalisation initiation in delayed response animals with a higher threshold of response.

4.2 Materials and method

4.2.1 Animals and maintenance

The experiment was conducted at the University of New England rural property Kirby, Armidale, Australia in two lambing periods: 14-24th September 2013 and 5-20th October 2013. In the earlier lambing group, multiparous Merino ewes were joined by AI to Merino sires in April (ewe n=50; sire n=24) and pregnancy scanned at 85 days gestation to select twin bearing ewes. For the later lambing group, multiparous Merino ewes had been joined by AI to Merino sires in May to White Suffolk sires (ewe n=50; Merino sire n=10; White Suffolk sire n=14) and pregnancy scanned at 70 days gestation to select single-bearing ewes only. This experiment was part of a larger AI program where sires were randomly allocated to each ewe. Both groups of animals were maintained on pasture at BCS 2.0-2.5 for the last month of gestation when they were supplementary fed a lupin/corn mix at 250 gm per ewe three times weekly in preparation for housing. Ewes were individually housed in an indoor facility from day 145 of gestation in 2 m^2 pens and fed a 50:50 lucerne and oaten chaff mix supplemented with a 3:2 lupin/corn ration 200 gm/day and *ad libitum* lucerne hay. Ewes were monitored 24 hrs/day and allowed to proceed unaided throughout labour unless assistance was required as described in Chapter 3, pp 45. Twelve hours after lambing at the completion of tests ewe/lamb units were released into an enclosed area outside and observed for a further 48 hours to ensure successful bonding. Lamb survival data was determined at ~one month postpartum when lambs were castrated and tail-docked (twins aged 44 -54 days; singletons aged between 20-30 days postpartum).

4.2.2 Animal measurements

The first sign of lambing behaviour, timing of presentation of membranes and appearance lamb body parts were recorded where possible on all ewes, and time of birth defined as in Chapter 3, pp 45. A jugular blood sample was collected within the first 5 minutes of complete expulsion of the lamb at birth and rectal temperature for each lamb measured at this time. Collection of heart rate and other APGAR score-related viability data were attempted but extremely cold temperatures made the lambs shiver so excessively that electrocardiogram (ECG) and electronic monitoring of neonate vital signs was abandoned.

At 3.5 hours post birth two bleat latency measure as described in Chapter 3, pp 46, were recorded on all lambs. Each lamb was taken with minimal handling to a 83 x 83 cm hessian enclosed pen at the end of the animal house (see Figure 4.1) and restrained with gentle pressure on its side, head facing toward the recorder, for 5 seconds until release with the verbal cue "*Go*" while the tester remained hidden behind the hessian. The distance between the lamb testing pen and the pen holding the dam ranged between 5 to 17 m, with all animals being within hearing distance of each other. In Experiment 1, the test order of each twin had been randomly allocated prior to sampling to assess birth order effect on vocalisation latency. Audio recording of the test using a Roland R-05 2 microphone recorder was commenced prior to lamb testing and ceased at completion of the 90 seconds test period. Vocalisation latency to the first signal emitted by the lamb from hand release was measured by spectrographic analysis using Praat linguistic software (version 5.3.65, Boersma and Weenink, 2014) and "proofed" against manual stopwatch records. The commencement of the test was marked by the verbal cue "*Go*" simultaneous to hand release. Lambs were then immediately measured for weight, rectal temperature, crown to rump length, girth circumference; and finally tagged (placement of an identification tag in right ear using tagging pliers) while at the site of vocalisation recording. Lambs were then placed immediately back in the recording pen after tagging and another vocalisation latency measure was recorded as above.

Figure 4.1: Lamb testing pen with recorder placed 50-70 cm above lamb head.

4.2.3 Blood sampling

A jugular blood sample (1-1.5 ml) was collected from each lamb into preheparinised syringes immediately after birth. Jugular blood sampling in this trial was preferred to umbilical sampling both for ease of sampling and accuracy to indicate acid-base status at the time of birth (Dutra & Banchero, 2011; Westgate, Garibaldi, & Greene, 1994). Blood samples remained on ice until a packed cell volume (PCV) was measured via haematocrit and the sample centrifuged (15 min, 1000 g) as soon as possible. Plasma was separated and stored at -20°C. Plasma was assayed for glucose and lactate using the SIEMENS Dimension clinical chemistry system. The glucose assay used an adaptation of the hexokinase-glucose-6-phosphatedehydrogenase method (inter assay coefficient of variation <1.5%) and the lactic acid assay employed a modified oxidation of lactate to pyruvate method (inter assay coefficient of variation <3.3%).

Singleton lambs

Due to availability of equipment, additional measures were collected on singleton lambs. Jugular blood samples were collected within 2 minutes of birth and blood samples were first analysed immediately with an iSTAT handheld blood analyser (Abbot Point of Care, Australia) to measure pH, partial pressure of oxygen (pO_2), oxygen saturation ($satO_2$%), partial pressure of carbon dioxide (pCO_2), total carbon dioxide (tCO_2), bicarbonate (HCO_3^-), extracellular fluid base excess (BE_{ecf}), and blood lactate using an EG4+ cartridge (Abbot Point of Care System, Australia) as per method described in Dutra and Banchero (2011). Environmental temperatures of less than 16°C overnight affected the operation of the iSTAT analyser and resulted in missing datum for some samples. The rest of the blood sample was measured, stored and analysed as above.

Twin lambs

Jugular blood samples were collected within the first 5 minutes of birth, and kept on ice until they were centrifuged and measured as above.

4.2.4 Statistical analysis

Twin and singleton lamb data were analysed separately for all measurements because of the potential difference in fetal distress/physiological mechanisms. To analyse differences between breed, singleton lamb data were analysed using general linear regression with sex and breed as fixed effects, and birth weight included as a covariate for body dimension parameters (girth and length) and parturition duration. Best model fit was determined by backwards step model selection and first order interactions were retained in the model if significant at $p<0.05$. To achieve normal distribution of singleton lamb data, lactate was subjected to square root transformation, and parturition duration to natural logarithm transformation. Means for physical data are displayed as LSmeans±SE determined from fitted models using R package *lsmeans* (Lenth, 2013). Analysis of twin lamb body weight and dimensions, rectal temperature and blood metabolites data were undertaken using a linear mixed-effects model, *nlme* package R (version 3.1.1, R Core Team, Vienna, Austria) to investigate the effect of birth order. Lamb sex and birth order were included as a fixed effects, with ewe identity fitted as a random term and birth weight as a covariate. To achieve normal distribution of twin data, plasma glucose was subjected to logarithm transformation. Parturition progress data were missing for a large number of twin lambs so this data set was not included in twin lamb analysis.

Cox proportional hazard model regression (R *survival* package, Therneau, 2013) was used to develop relative risk models for delayed vocalisation latency. Covariates included sex, birth order (twin data) and breed (singleton data). Birth weight was included as a covariate and weight categories of below 2.0 kg and above 5.0 kg body weight were used to assess the relationship of birth weight with vocalisation latency and blood chemistry data in single and twin lambs. Non proportionality of data (n=3 twin lambs for weight category <2 kg; n=15 single lambs for weight category >5 kg) was checked by use of model proportionality tests (cox.zph). Test order was initially included in all vocalisation latency models and then excluded as it was not significant. Variance within a litter associated with birth order, and time of test (pre or post tagging) in singletons, was analysed by inclusion of ewe identity as a frailty term with Gaussian distribution as a robust covariance estimator (Grambsch & Therneau, 1994). Terms which were not significant were dropped from the model using backwards stepwise regression, and log likelihood comparisons (as per Chapter 3). The minimum recording for vocalisation

latency if the lamb had an immediate response upon release was set at 0.1 seconds rather than zero. All median vocalisation latency times are shown as Kaplan-Meier estimates with 25^{th}-75^{th} percentiles to describe data distribution. To check the correlation of vocalisation latency with blood parameters and birth weight, each blood parameter was fitted into the model separately with first order interactions between sex, breed and birth weight. Linear correlations between blood measures and other measures were tested using the Pearson correlation coefficient. To compare differences in blood and physiological parameters between fast (<2 s) and slow (>5 s) vocalisation categories a Wilcoxon ranked sum test was used as data were non parametric.

4.3 Results

A total of 42 singleton and 74 twin lambs surviving birth in the early postpartum period were included in the analysis. In the singleton lamb data set, there were n=20 purebred Merino lambs (MxM) (male n=11, female n=9) and n=22 Merino/White Suffolk cross lambs (WSFxM) (male n=12, female n=10). The two heaviest singleton WSFxM lambs weighing 5.8 and 6.7 kg were the only lambs assisted at birth. In the twin lamb (all MxM) data set, n=37 lambs were male and n=36 were female.

4.3.1 Lamb physical measures and parturition duration

Birth weight in the 42 singleton lambs ranged from 3.0-6.6 kg, with 15 lambs weighing over 5.0 kg. All lambs weighing over 5.5 kg (n=6) were sired by WSF rams. Lambs born to WSF sires weighed significantly more than pure Merino lambs (p<0.05), and males of both breeds tended to be heavier than females (p=0.06) (Table 4.1). Without adjustment for birthweight, the girth circumference of the average Merino-sired lamb was 1.26 cm less than that of the average WSF-sired lamb (MxM lambs 38.35±0.44 cm; WSFxM lambs 39.61±0.42 cm, p<0.05 - data not shown in Table 4.1). Rectal temperatures of singletons ranged from 37.8-40.6°C and mean rectal temperature was higher in WSFxM lambs (p<0.05). Accurate timing of stage II labour duration was recorded in a total of n=35 singleton bearing ewes. Parturition duration was longer in heavier lambs (p<0.0001) and there was a birth weight x breed interaction (p<0.05) associated with longer delivery time in heavier WSFxM lambs.

Birth weight across all twin lambs was a mean 3.41 kg, ranging from 1.2-5.0 kg. Three twin lambs (first born n=2, second born n=1) weighed less than 2.0 kg, with rectal temperature and a blood sample at birth not collected on one first born animal. First born lambs were significantly heavier than the second born by an average of 0.34 kg (p<0.01) and male co-twins were slightly heavier than females by 0.09 kg (Table 4.2) but this difference was not significant (p>0.10). Girth and crown rump measures were dependent on birth weight and there was a birth order x birth weight interaction (p<0.05) for girth, and a sex x birth order interaction for length (p<0.05). Twin lamb rectal temperatures ranged from 36.1-40.7°C, were positively correlated with body weight (p<0.0001), and on average significantly higher in second born lambs after adjustment for birth weight and sex (p<0.01). A sex x birth order interaction (p<0.50) indicated that mean rectal temperature of male lambs was lower than females in first born lambs (male 38.06±0.08°C; female 38.21±0.19°C), but higher than females in second born lambs (male 38.84±0.19°C; female 38.22±0.19°C). Four lambs, including the low birth weight lambs, recorded rectal temperatures below 37.0°C.

4.3.2 Blood metabolite correlations

Plasma metabolite data were collected from 41 singleton lambs and on-site blood gas data were available from 34 singletons. In single lambs, plasma lactate levels ranged from 2.30-13.30 mmol/L, were non significantly higher in male lambs (male: 7.04mmol/L; female: 6.28mmol/L, p>0.10) and were not correlated with rectal temperatures (Table 4.3). Plasma glucose levels in single lambs ranged from 16.18-70.82 mg/dL and were correlated with plasma lactate (r=0.69, p<0.0001) but not linearly with birth weight. Table 4.4 shows the range and mean values of blood gas, blood lactate and acid base variables as well as plasma glucose and plasma lactate levels measured in single lambs. Blood pH was negatively correlated with blood lactate (r= -0.49, p<0.01) and pCO_2 (r= -0.79, p<0.0001) and positively correlated with BE_{ecf} (r=0.38, p<0.05). There was also a significant negative correlation between blood and plasma lactate (r= -0.35, p<0.05).

Table 4.1: Singleton lamb physical measures (LSmean). Bold type indicates significant differences (p<0.07).

Variable	Birthweight (kg) n=42		Girth [a] (cm) n=42		Crownrump [a] (cm) n=42		Rectal temperature (°C) n=41		Stage II labour duration (min) [a b] n=35	
	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value
Breed		**<0.05**		NS		NS		**<0.05**		NS
MxM	**4.48**		38.97		48.86		**39.19**		2.31 (10.11)	
WSFxM	**4.98**		38.96		48.81		**39.64**		2.79 (16.31)	
Lamb sex		**=0.06**		NS		NS		NS		NS
Male	**4.91**		38.96		49.53		39.36		2.56 (12.89)	
Female	**4.54**		38.97		48.15		39.47		2.55 (12.79)	
Covariates										
Birthwt	-	-		**<0.0001**		**<0.0001**	-	-		**<0.001**
Interaction										
Birthwt x breed	-	-		NS		NS	-	-		**<0.05**
Birthwt x sex	-	-		NS		**<0.05**	-	-		NS

[a] Birthweight included as a model covariate. [b] Data are log transformed LS means (backtransformed in parentheses). NS = not significant.

Table 4.2: Twin lamb physical measures [a] (LSmeans). Bold type indicates significant differences (p<0.05).

Variable	Birth weight (kg) n=74		Girth (cm) n=74		Crownrump (cm) n=74		Rectal temperature (°C) n=60	
	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value
Birthorder		**<0.01**		NS		NS		**<0.01**
Firstborn	**3.59[a]**		34.90		44.34		**38.10**	
Secondborn	**3.25[a]**		35.03		45.26		**38.56**	
Lamb sex		NS		NS		NS		NS
Male	3.46		34.99		44.80		38.47	
Female	3.37		34.95		44.81		38.19	
Covariates								
Birthwt	-	-		**<0.0001**		**<0.0001**		**<0.0001**
Interactions								
Birthwt x birthorder	-	-		**<0.05**		NS		NS
Sex x birthorder	-	-		NS		NS		**0.05**

[a] Low birth weight animals <2kg included in data. NS = Not significant.

Table 4.3: Singleton lamb blood assays collected within the first 2 minutes of birth. Data presented to facilitate comparisons with Dutra and Banchero (2011).

Jugular assay	N [a]	Mean	SD	Range	5th percentile	Median	95th percentile
pH	35	7.29	0.06	7.19 to 7.43	7.20	7.28	7.39
BE_{ecf} (mmol/L)	35	-0.06	2.80	-7.00 to 6.00	-5.40	0.00	5.20
pO_2 (mmHg)	34	20.15	4.89	8.00 to 31.00	10.25	20.00	28.00
$satO_2$ (%)	34	26.82	10.93	6.00 to 52.00	7.50	26.00	51.25
pCO_2 (mmHg)	35	55.86	8.66	34.40 to 75.40	38.80	56.90	70.12
tCO_2 (mmol/L)	35	28.17	2.84	22.00 to 33.00	22.00	28.00	33.00
HCO_3 mmol/L	35	26.53	2.58	20.90 to 31.00	20.90	26.80	30.92
Blood lactate mmol/L	33	4.65	1.82	1.22 to 9.94	1.88	4.16	8.68
Plasma glucose mg/dL	40	40.18	13.79	16.18 to 70.82	16.31	41.48	67.34
Plasma lactate mmol/L	40	6.91	2.30	2.30 to 13.30	3.15	6.50	11.27
Packed cell volume (%)	39	42	6.03	24 to 54	32.4	43	49.2

[a] Missing values are associated with low environmental temperatures and clotted blood samples.

Plasma metabolite data were collected from 66 twin lambs (Table 4.5) including 2 of the low birth weight animals. Plasma lactate levels ranged from 2.6-15.5 mmol/L, and were significantly higher in male lambs ($p<0.05$). Mean plasma lactate was non-significantly higher in first born lambs (8.49±0.54 vs 7.59±0.54 mmol/L, $p>0.10$) and plasma lactate was negatively correlated with rectal temperature (r= -0.24, $p=0.06$). Plasma glucose levels ranged from 11.85-93.20 mg/dL and were significantly higher in first born (40.03±1.08 vs 32.83±1.08 mg/dL, $p=0.01$) and male lambs (40.11±1.09 vs 32.79±1.09 mg/dL, $p<0.05$). Twin lamb birth weight had no effect on plasma lactate or glucose levels ($p>0.10$). Lactate and glucose levels were highly correlated (r=0.67, $p<0.0001$). PCV ranged from 25.5-55.5%, although only one sample from a lamb weighing <2 kg was available (PCV=31.0%). Haematocrit values were correlated with lactate concentration (r=0.28, $p<0.05$) but did not differ due to sex, breed or birth weight.

Table 4.4: Singleton lamb plasma and blood metabolite concentrations. Data presented as LS means (lactate values backtransformed). Bold type indicates significant correlations (p<0.05).

Variable	Plasma (n= 42)				Blood [a] (n=35)									
	Lactate [b] (mmol/L)		Glucose (mg/dL)		pH		pO_2 (mmHg)		$satO_2$ (%)		pCO2 (mmHg)		HCO3 (mmHg)		Lactate (mmol/L)	
	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value	LSM	*p* value
Breed		NS		**<0.05**		NS		NS		NS		NS		NS		NS
MxM	6.61		**42.40**		7.29		21.54		27.86		55.37		26.33		4.62	
WSFxM	6.71		**34.42**		7.28		18.92		22.06		56.66		26.86		4.64	
Lamb sex		NS		NS		NS		NS		NS		NS		NS		NS
Male	7.04		40.57		7.29		19.52		24.26		55.06		26.21		4.83	
Female	6.28		36.01		7.28		20.84		25.49		56.97		26.98		4.43	
Covariate																
Birthweight		NS		**<0.05**		NS		**<0.05**		**<0.05**		NS		NS		0.08
Wgt group [c]		NS		NS		NS		**<0.01**		**<0.01**		NS		NS		0.09
<5.00kg n=27	6.51		36.96		7.28		**21.52**		**28.69**		55.87		26.34		4.11	
>5.00kg n=15	6.85		42.13		7.29 [d]		**17.38** [d]		**21.32** [d]		56.26 [d]		27.05 [d]		5.05 [d]	

[a] Whole blood measurement using iSTAT portable blood analyser, collected within 2 minutes of lamb expulsion.
[b] Data are presented as log LSmeans, back transformed.
[c] Weight category factor in models instead of birth weight.
[d] Sample number n=13 for blood gas and metabolite data in weight category >5.0 kg.

Table 4.5: Twin lamb plasma metabolite concentrations (LSmeans±SE). Low birth weight lamb <2kg included in data.

	Birthorder 1		Birthorder 2			
	Male n=17	Female n=16	Male n=14	Female n=18	Effect	*p* value
Plasma lactate (mmol/L)	9.06±0.57	7.17±0.57	8.71±0.58	6.82±0.56	Sex	=0.0001
Plasma glucose (mg/dL) [a]	3.80±0.08 (44.92±1.08)	3.57±0.08 (35.47±1.08)	3.61±0.08 (37.10±1.08)	3.38±0.08 (29.30±1.08)	Birthwt Sex Birthorder	<0.01 <0.001 <0.001
Packed cell volume (%)	42.32±1.17	41.04±1.15	41.93±1.22	40.65±1.09		NS

[a] Data are presented as log LSmean±SE, back transformed mean in parentheses.

4.3.3 Lamb vocalisation latency

Vocalisation latencies of singleton lambs, by sex and breed, are shown in Table 4.6. One singleton lamb exhibited excessively nervous behavioural reactions to handlers while in the presence of the dam and "freezing" behaviours during testing, so this individual was eliminated from the vocalisation analysis (sex male; breed MxM; birth weight 4.5 kg). Male lambs appeared 44% more likely to be slower to respond than females (hazard ratio$_{sexmale}$=0.56, 95% CI [0.28-1.55], p=0.06) but all sex and breed differences in vocalisation latency were accounted for by birth weight (hazard ratio$_{birthwt}$=0.99, 95% CI [0.99-0.99], p<0.05) or more specifically birth weight category. Lambs over 5 kg were 43% more likely to have a longer vocalisation latency (hazard ratio$_{birthwt>5kg}$=0.57, 95% CI [0.30-1.07], p=0.05) than lambs weighing between 3 and 5 kg.

Twin lamb latencies to vocalise following separation, by birth order and sex are shown in Table 4.7. Three twin lambs weighing under 2 kg had delayed vocalisation responses (no response, 51.56 and 8.28 s) but were kept in the vocalisation analysis unless where stated. Vocalisation latency at 3 hours following birth showed no effect of testing order in twin lambs. Only 5 second born co-twins had a vocalisation latency longer than the first born by more than 2 seconds, and these included n=3 animals with birth weight <2 kg. Lambs with low birth weight <2 kg were 78% more likely to have a slower vocalisation response (hazard ratio$_{birthwt<2kg}$ =0.22, 95% CI [0.04-1.09], p<0.05). There was also a sex x birth order interaction with male lambs being more likely to be slower than females if they were the first born lamb (hazard ratio$_{sexmale:birthorder2}$=4.25, 95% CI [0.96-18.75], p<0.001).

To compare animals without those demonstrating fetal growth restriction (n=3 animals with birth weight <2 kg, reduced head circumference, high plasma lactate and glucose) the model was rerun with these animals excluded (Table 4.7). With removal of these 3 second born animals, birth order was a significant predictor of vocalisation latency - second born twins more likely to initiate a vocal signal faster than their first born co-twin by a factor of 1.4 (hazard ratio$_{birthorder2}$=1.39, 95% CI [0.52-3.73], p<0.05).

Table 4.6: Vocalisation latency of singleton lambs shown as median (25-75th percentile).

	MxM		WSFxM			
Bleat latency (s)	Male n=11	Female n=8	Male n=12	Female n=9	Effect	*p* value
Singletons	1.44 (1.21-7.12)	1.40 (1.30-2.57)	9.43 (2.65-27.23)	1.40 (0.52-1.55)	Birthwt Sex	<0.05 =0.06

Table 4.7: Vocalisation latency of twin lambs, with and without low birth weight animals, shown as median (25-75th percentile).

	Firstborn		Secondborn			
Bleat latency (s)	Male n=17	Female n=16	Male n=15	Female n=19	Effect	*p* value
All twin lambs	7.00 (3.02-19.04)	2.54 (1.36-5.61)	2.37 (1.50-6.56)	2.80 (1.90-5.09)	Birthorder	NS
Low birthwt lambs excluded [a]	7.00 (3.02-19.04)	2.55 (1.36-5.61)	2.21 (1.34-4.03)	2.47 (1.84-3.71)	Birthorder BirthorderxSex	<0.05 <0.001

[a] Data calculated with removal of n= 3 lambs (<2 kg birth weight and plasma lactate >10mm/L).

4.3.4 Relationship of fetal distress indicators with vocalisation latency

In singleton lambs there was a clearer relationship between vocalisation latency and blood parameters. Plasma glucose, labour duration, and birth weight were significant predictors of vocalisation latency in singletons (Table 4.8 and Figures 4.2 and 4.3). Cox modelling predicted that for every unit increase in glucose, lambs would be at a 4% greater risk of a longer vocalisation latency (hazardratio$_{glucose}$=0.96, 95% CI [0.94-0.99], p<0.01) (Figure 4.2), independent of birth weight. Lambs experiencing a longer parturition were more likely to exhibit delayed vocalisation with the model predicting that for every extra 10 minute of labour, lambs would be at a 10% greater risk of a longer vocalisation latency (hazard ratio$_{labourduration}$=0.99, 95% CI [0.98-1.00], p<0.05). Animals weighing >5 kg were

50% more likely to be slower to initiate a vocalisation than those weighing <5 kg (hazard ratio$_{weight>5kg}$=0.50, 95% CI [0.25-0.99], p=0.01).

Oxygen perfusion (pO_2 and $satO_2$%) was also a metabolic predictor of vocalisation delay risk in association with birth weight interactions. There was a greater risk of longer latency associated with poorer oxygenation in higher birth weight lambs (evident as a significant oxygenation x birth weight interaction for both pO_2 and $satO_2$%, Table 4.8). Other interactions involving rectal temperature; pH; pCO_2 and HCO_3 in the higher birth weight group are also indicated in Table 4.8. However as sample numbers were low, these data should be viewed with caution as collinear associations between rectal temperature, birth weight and delayed latency may also be associated with interaction significance. Nonetheless all lambs with blood pH <7.22 (n=5) also exhibited delayed vocalisation responses of >4.5 s, and all weighed >4.5 kg. There were no significant interactions with sex or breed in any of the blood parameters which were not accounted for by birth weight or birth weight category.

Table 4.8: Predictors of vocalisation latency hazard in singleton lambs (hazard ratios and 95% confidence intervals).

Predictor - singletons	Coefficient	Hazard ratio	95% CI	*p* value
Birthwt (kg)	-0.0005	0.99	[0.99-1.00]	=0.04
Birthwt >5 kg	-0.69	0.50	[0.25-0.99]	=0.01
Plasma glucose [mgl/dL]	-0.03	0.96	[0.95 - 0.99]	=0.009
Labour duration [min]	-0.01	0.99	[0.98–1.00]	=0.005
pO_2 [mmHg]	0.24	1.27	[1.07 - 1.52]	=0.007
pO2 x birthwt	-4.67	1.00	[0.99-1.00]	0.03
satO2%	-0.00005	1.19	[1.04-1.35]	0.02
satO2% x birthwt	-0.00003	1.00	[0.99-1.00]	0.01
pCO2 x wt>5 kg	-0.013	0.98	[0.97-1.00]	0.05
HCO3 x wt>5 kg	-0.03	0.97	[0.94-1.00]	0.04
pH x birthwt	-1.35	0.99	[0.99-1.00]	0.004
Rectal temp. x birthwt>5 kg	-0.02	0.98	[0.96-0.99]	0.04

Correlations between vocalisation latency and some fetal distress parameters including oxygenation, plasma glucose and parturition duration were more distinct when measurement of vocalisation latency was measured following tagging (Table 4.9 and Figure 4.2). But overall, lambs were not significantly faster to initiate a vocalisation following tagging (hazard ratio$_{pretaglatency}$=0.85, 95% CI [0.52-1.38], p>0.10). Seventy three percent (n=11) of lambs with delayed responses (>5 s) prior to tagging also demonstrated responses >5 s following tagging. Median vocalisation latency before tagging was 1.87 s vs 1.93 s post tagging, 95% CI [1.58-2.94], [1.62-3.43] respectively.

Table 4.9: Comparison of vocalisation latency correlations in singleton lambs, before and after tagging (Pearson correlation coefficients). Bold type indicates significant correlations (p<0.05).

Variable	Pre tagging latency			Post tagging latency		
	r	df	p<	r	df	p<
Plasma glucose (mg/dL)	**0.47**	**35**	**0.004**	**0.51**	**35**	**0.002**
Plasma lactate (mmol/L)	0.21	37	0.20	0.23	37	0.13
pO2 (mmHg)	-0.24	30	0.13	**-0.46**	**30**	**0.009**
satO2 (%)	-0.27	30	0.14	**-0.37**	**30**	**0.04**
Labour duration (min)	**0.47**	**32**	**0.006**	**0.61**	**32**	**0.0002**

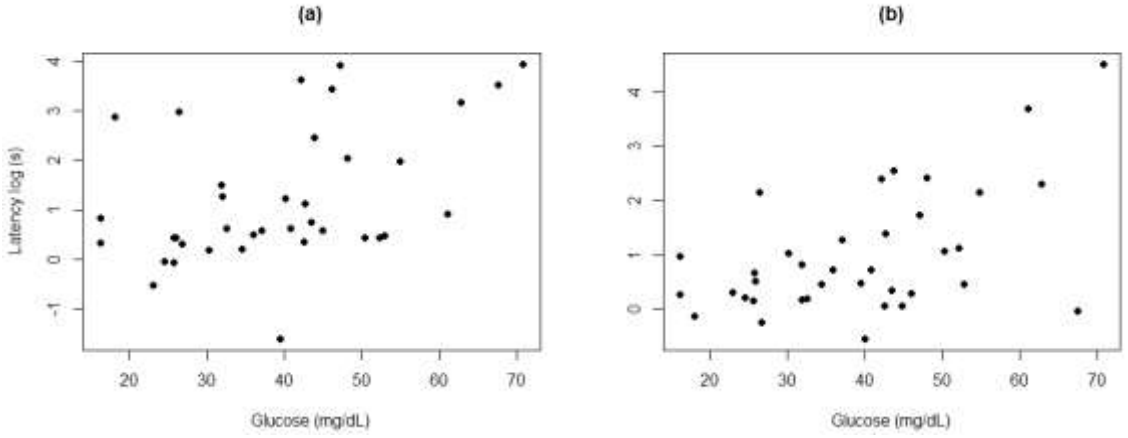


Figure 4.2: Relationship between plasma glucose and vocalisation latency in singleton lambs (a) before and (b) after tagging.

In twin lambs direct associations between vocalisation latency and physiological indicators of fetal distress were not significant. However there was a significant association between vocalisation latency, birth order and log plasma glucose (hazard ratio$_{log(glucose):birthorder2}$=1.23, 95% CI [1.06-1.47], p<0.001) (Table 4.10); and similarly plasma lactate (hazard ratio$_{lactate:birthorder2}$ =1.12, 95% CI [1.03-1.21], p<0.0001). These associations reflect the difference between vocalisation latency and plasma metabolite levels in the second born co-twin compared to the first born; there is a greater likelihood (hazard ratios >1) of the second co-twin in a litter having shorter vocalisation latency as well as lower plasma glucose (p<0.001) and plasma lactate levels (p<0.0001) when compared to the reference (firstborn lamb). This was also true for rectal temperature (hazard ratio$_{rectaltemp:birthorder2}$, =1.01, 95% CI [0.99-1.02], p<0.01). Birth weight was not significant in these interactions following removal of the <2kg birth weight animals.

Table 4.10: Predictors of vocalisation latency hazard in twin lambs [a] (hazard ratios and 95% confidence intervals).

Predictor –twin lambs	Coefficient	Hazard ratio	95% CI	*p* value
logGlucose (mg/dL)	-0.72	0.53	0.22-1.23	<0.05
logGlucose x birthorder2	0.24	1.23	1.06-1.51	=0.0003
logGlucose x sexmale	-0.06	0.94	0.78-1.13	=0.09
Lactate (mmol/L)	-0.10	0.90	0.79-1.03	=0.09
Lactate x birthorder2	0.11	1.12	1.03-1.21	<0.0001
Rectal temperature (°C)	0.03	1.03	0.68-1.57	NS
Rectal temperature x birthorder	0.02	1.01	0.99-1.02	=0.0045

[a] Data calculated with removal of lambs < 2 kg birth weight. NS = not significant.

4.3.5 Comparison of fast and slow bleating lambs

When singleton lambs were grouped into slow and fast vocalisation response groups (fast <2 s; slow >5 s), there were significant differences between the two categories regarding plasma glucose (Wilcoxon test: n=30, W=49, p<0.02) and birth weight (Wilcoxon test: n=33, W=38, p<0.001); and marginal differences in $satO_2$% (Wilcoxon test: n=27, W=116, p=0.06) and pO_2 (Wilcoxon test: n=27, W=113, p<0.09). Plasma lactate, pCO_2 or any other blood variables and parturition duration were not significantly different

between the two groups. Figure 4.3 shows the relationship between birth weight and the above measures in all single lambs, with values identified as those corresponding to fast and slow first vocalisation latency groups. The remainder of the lambs demonstrating vocalisation latencies >2 and <5 seconds are also shown.

4.3.6 Lambs with a consistently poor bleat response

Measurements of singleton and twin lambs which demonstrated consistently retarded vocalisation measures until 12 hours postpartum are presented in Table 4.11. The two twin lambs (Table 4.11, IDs 1-2) presenting delayed vocalisation responses had rectal temperatures reflecting hypothermia (<37°C), very low birth weight and elevated plasma lactate and glucose levels. They also displayed clinical signs of severe fetal growth retardation including disproportionate head circumference and low haematocrit (PCV 31%, available for one lamb only). One single lamb (Table 4.11, ID 3) demonstrated evidence of severe hypoxemia (pO_2=8mg/dL) as well as clinical signs of dystocia and a stressful birth (assisted birth, head oedema, presence of meconium discharge, elevated plasma glucose, and low haematocrit). All singleton lambs with consistently retarded vocalisation measures weighed >5.0 kg.

4.3.7 Lamb survival to 1 month postpartum

Data were available for the number of lambs which had survived to 1 month of age. Twin lamb mortality rate was 17.14% (n=12) and single lamb mortality rate was 2.33% (n=1). The heaviest twin lamb (birth weight 4.45 kg) which did not survive had initially been rejected by the dam following birth in preference for the co-twin, and recorded a vocalisation latency of 6.56 seconds. All lambs weighing <2 kg did not survive to one month of age, and 72.73% (n=8) of non-surviving lambs recorded a vocalisation latency >2 seconds. Ninety one percent (90.9%) of twin lambs, n=10) which did not survive had lactate levels above the study median (6.5 mmol/L), and 54.5% (n=6) had glucose levels above the study median (41.48 mg/dL).

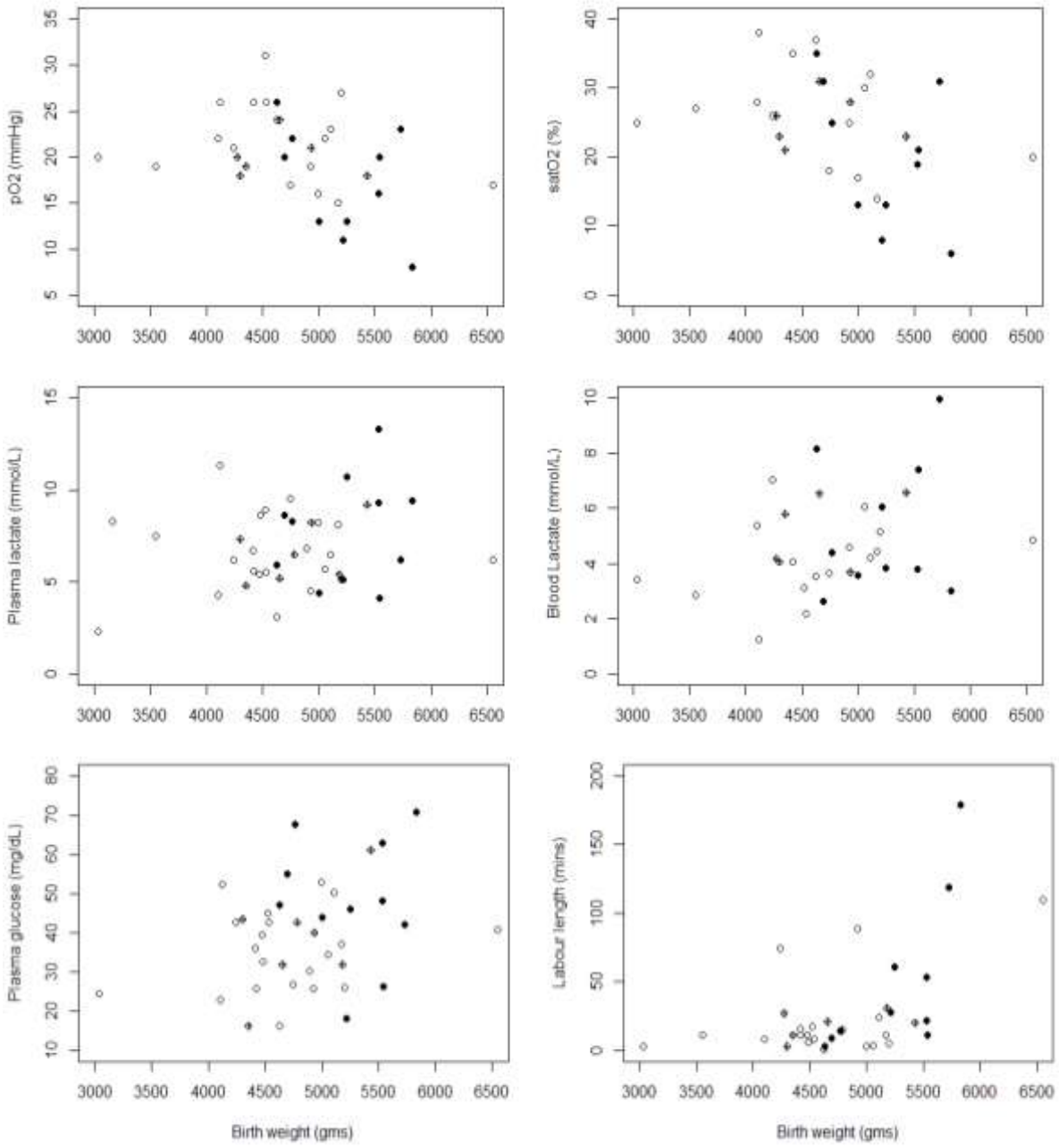


Figure 4.3: Relationship of birth weight with pO2; satO2%; plasma and blood lactate; plasma glucose and parturition duration in singleton lambs. Vocalisation latency: Rapid= ○, Delayed= ● and Medium (between 2 and 5 s)= ⌖.

Table 4.11: Physiological and vocalisation measures of at risk lambs based on consistently poor vocalisation response (>5 s before and after tagging). Bold type indicates values above or below thresholds.

ID	Breed	Litter size	Sex	Birth weight	Temp.	Blood variables						Vocalisation latency [a]	
						Plasma glucose	Plasma lactate	pO2 (hypoxemia< 10)	pCO2	pH	PCV	Pre tagging	Post tagging
				(kg)	(°C)	(mg/dL)	(mmol/L)	(mmHg)	(mmHg)		**(%)**	(s)	(s)
Median [b]				*4.75*	*39.40*	*41.48*	*6.50*	*20.00*	*57.15*	*7.28*	*43*	*1.89* [a]	*1.94* [a]
ID1	MxM	Twin	Male	1.18	**36.1**	**93.2**	**15.5**	na	na	na	na	No response [c]	9.20
ID2	MxM	Twin	Female	1.91	**36.2**	50.89	**12.5**	na	na	na	31	51.56	33.62
ID3 [d]	WSFxM	Single	Male	5.83	39.4	**70.82**	9.4	**8.0**	53.7	7.32	35	51.60	No response [c]
ID4	WSFxM	Single	Male	5.53	39.7	62.84	13.3	na	na	na	24	23.66	9.97
ID5	WSFxM	Single	Male	5.43	**38.0**	61.07	9.2	18	46.6	7.33	36	2.49	40.38
ID6	WSFxM	Single	Male	5.72	40.2	42.12	6.22	23	55.7	7.27	na	37.97	10.94
ID7	MxM	Single	Male	5.00	39.2	43.82	4.4	13	41.2	7.31	44	11.81	12.78

[a] Kaplan–Meier median.
[b] Median value for all single lambs (n=41, refer Table 4.3).
[c] No vocalisation elicited.
[d] Assisted birth.
na= Data not available.

4.4 Discussion

A relationship between delayed vocalisation latency in the early postpartum period and degree of intrapartum trauma or hypoxia, indicated by metabolic markers of fetal distress in singleton and twin born lambs, has been demonstrated for the first time in this study. Singleton lambs with higher levels of plasma glucose and lower blood oxygen saturation demonstrated longer latencies to initiate a distress vocalisation, and these characteristics were more likely to be found in lambs of birth weight >5.0 kg, and those experiencing longer parturition. Lambs with clinical signs of severe fetal growth restriction also exhibited delayed vocalisation responses. In twin lambs weighing >2 kg, the first born co-twin was more likely to have higher plasma lactate and glucose assay than the second born, as was also demonstrated in male twins, and this was associated with a greater risk of delayed vocalisation latency.

Blood gas & acid base data

While there are limited records available for neonate lamb blood gas and acid base data, direct comparisons of these variables measured in singleton lambs of this study can be made with the results of (Dutra & Banchero, 2011) who used similar blood sampling methodology to assess early postpartum viability in Texel and Polworth breed cross lambs at birth. The lambs in the study reported here were found to have concordant median values for most blood gas and acid base parameters with Dutra's study. Based on thresholds utilised by Dutra and Banchero (2011); Gardner, Fletcher, Bloomfield, Fowden, and Giussani (2002) and Helwig, Parer, Kilpatrick, and Laros (1996), there was one lamb in this study demonstrating evidence of severe hypoxemia (pO_2 <10 mmHg) and another with respiratory acidosis (pCO_2 >70 mmHg) which also had a low pO_2 (11mm Hg). The lamb with severe hypoxemia also had the highest plasma glucose level (70.82 mg/dL), weighed over 5.8 kg, and had clinical signs of dystocia (head oedema following a prolonged and assisted birth). While there were no lambs which could be described as exhibiting clinical acidosis based on arterial blood pH thresholds (pH<7.1, Helwig et al., 1996), a number were below the umbilical venous blood pH threshold (pH <7.27), reported by D'Souza, Black, Cadman, and Richards (1983) for acidosis in human neonates, and retinal venous pH associated with poor vitality score in piglets (Trujillo-Ortega et al., 2011). Difficulty in standard definition of acidosis (Westgate et al., 1994) and values relating to either cord or jugular blood (D'Souza et al., 1983; Thorp, Sampson,

Parisi, & Creasy, 1989) make it difficult to quantify exact thresholds for the lambs reported on here - but certainly correlations between blood pH and pCO_2 and BE_{ecf} in this study closely reflect those that were found by Dutra and Banchero (2011).

While a high incidence of dystocia was not expected because of the prior lambing history of the study ewes, these comparisons indicate that a small proportion of lambs in this study experienced severely compromised oxygenation during birth, while others appear to have suffered a mild to moderate degree of hypoxemia, which was not evident by any other visual signs. A limitation of the study was the low statistical power associated with missing blood gas data, and a larger sample number may have demonstrated greater variation between animals. This was evident in the significance of interactions at higher birth weight involving a number of variables indicating fetal distress, and while these results indicate expected relationships, they also reflect the status of a relatively small number of animals. Although pO_2 was not viewed as a reliable predictor because of possible contamination by Herpin et al. (1996), the blood gas analysis method of this study demonstrate meaningful correlations. In summary, while the iSTAT handheld analyser failed to function at low temperatures, the available results appear to provide valid comparisons with Dutra's study.

Plasma glucose

Elevated plasma glucose and catecholamine levels indicative of stressful parturition have been linked to fetal asphyxia in sheep (Dutra & Banchero, 2011; Gardner et al., 2002; Jones & Ritchie, 1978), pigs (Herpin et al., 1996; Trujillo-Ortega et al., 2011), calves (Bellows & Lammoglia, 2000), humans (Swanstrom & Bratteby, 1981) and rodents (Jansen, Hayden, & Ogata, 1984; Vannucci & Vannucci, 1997). A number of lambs in the current trial had values above the mean fetal plasma glucose levels of non asphyxiated lambs (30±8 mg/dL) reported by Grajwer, Sperling, Sack, and Fisher (1977). All singleton lambs with plasma glucose levels above 54.00 mg/dL had delayed vocalisation responses, with the exception of one animal who responded with a deficient signal (refer to Chapter 5). Four twin lambs reported glucose levels >70 mg/dL as well as corresponding high lactate levels (>10 mmol/L) which clearly reflected a level of fetal distress. Mean plasma glucose values above 70.3mg/dL have been reported in non

surviving lambs and those with acute intrapartum hypoxia via jugular blood sampling by Barlow et al. (1987).

These findings, and the correlation between glucose and corresponding lactate levels in both twin and singleton lambs in this and other neonate studies (Jones & Ritchie, 1978; Swanstrom & Bratteby, 1981; Trujillo-Ortega et al., 2011), indicate that conditions leading to varying degrees of fetal distress, oxygen depletion and metabolic compensation were implicated in these animals, and that the first born co-twin may experience a relatively more difficult birth than the second born. Additionally, elevated glucose levels associated with lower oxygenation in singleton lambs weighing >5 kg indicates a greater degree of fetal distress during parturition for these animals. Similarly, indicators of hypoxia, including elevated plasma glucose and poorer vitality scores, have been reported in heavier piglets by Trujillo-Ortega et al. (2011) who proposed that greater trauma while traversing the birth canal had been experienced by those animals. During periods of reduced fetal oxygen availability, blood flow in the fetal lamb is redirected to preserve heart muscle and circulatory function (Dawes, Mott, & Shelley, 1959; Ikeda et al., 1998b; Ley et al., 2004), a response associated with the superior ability of fetal and newborn animals to survive anoxia (Mott, 1961). The resultant reduction in hepatic blood flow and a decline in liver oxygen uptake initiate a proportional glucose surge from the liver (Bristow, Rudolph, Itskovitz, & Barnes, 1983) in order to maintain the high cerebral glucose uptake of the fetal brain in late gestation (Jansen et al., 1984; Jones Jr, Burd, Makowski, Meschia, & Battaglia, 1975; Shelley, 1961; Vannucci & Vannucci, 1997). Such redistribution has been found to result in lowered circulating plasma glucose during the recovery phase (Herpin et al., 1996; Jansen et al., 1984) which could promote an ongoing cascade of effects elevating mortality risk if the neonate lamb does not access further carbohydrate supply.

Glucose levels in the fetus can also be associated with circulating maternal blood glucose levels (Plank, Boskovic, Sowers, & Angeles, 2008) but this possibility does not explain the disparity evident in twin lambs, nor the large differences between singleton lambs correlated with plasma lactate level and independent of birth weight. As reported in a study of asphyxia in rodent pups (Jansen et al., 1984), pre-existing glycogen or glucose status in the current study would appear not to be associated with elevated plasma glucose at birth, so it is concluded that these results are reflective of glucose regulation by the

liver in response to varying levels of fetal distress. Merino lambs were found to have higher mean circulating plasma glucose levels than the terminal cross breed in this trial, following adjustment for birth weight and sex, which would not be expected if glucose levels were only linked to substrate reserves or maternal status. Why Merino lambs may be more susceptible to hypoxia or fetal stress indicated by plasma glucose when compared to Merino cross lambs is of interest, and could be linked to conformational differences as proposed by Dutra and Banchero (2011) or metabolic capability determined by fetal growth regulation (Gluckman, 1997; Harding, Liu, Evans, & Gluckman, 1994). Elevated glucose in the first born fetus has also been reported to be associated with a higher rate of increase in prenatal corticosteriod concentrations in the days preceding birth of lambs although the mechanisms associated with this disparity are not clear (Mellor et al., 1977; Schwartz & Rose, 1998).

Plasma lactate

In the current study a greater proportion of twin lambs had lactate levels above 10 mmol/L compared to singletons, suggesting an increased risk of hypoxia for twin lambs (Dutra & Banchero, 2011; Everett-Hincks et al., 2007; Gardner et al., 2002). Fetal growth retardation was also implicated as a risk for hypoxia based on the lactate levels of lambs weighing <2 kg, corroborating the findings of previous studies (Barlow et al., 1987; Mellor, 1988; Stafford, Kenyon, Morris, & West, 2007; van Os, Liem, Hopman, Klaessens, & van de Bor, 2005). Developmental immaturity would make such individuals particularly susceptible to hypoxia due to their limited ability to increase blood flow to brain white matter via vasodilation and cerebral blood flow autoregulation (Berger & Garnier, 1999; Szymonowicz et al., 1990).

It is also probable that all lambs in this study weighing >2 kg with lactate levels >10mmol/L had experienced varying periods of oxygen depletion and mild to severe hypoxemia even though blood pH levels were not significantly low (Seidl et al., 2000). Low haematocrit in these animals could also indicate acute oxygenation deficit (Barlow et al., 1987; Mellor, 1988), as was found in the low birth weight animals of this trial. Plasma lactate has also been reported to be a better predictor of hypoxic impact in the human neonate than blood pH (Kruger, Hallberg, Blennow, Kublickas, & Westgren, 1999; Nordström, 2001; Plank et al., 2008). Plasma lactate levels in the lambs of this trial

may also have continued to elevate further to peak later in the postnatal period as reported in other studies (Comline & Silver, 1972; Ikeda et al., 1998b; Jansen et al., 1984; Seidl et al., 2000), but this data is not reported on here. Marginal differences indicated by blood lactate associated with birth weight >5 kg may also have been more reflective of immediate fetal distress impact than plasma lactate levels. A gender effect associated with plasma lactate, implying a greater risk of mortality in the interpartum period for males in this study, is consistent with lamb and piglet survival studies proposing prolonged parturition and poor vitality at birth as causal factors associated with newborn male mortality (Dutra & Banchero, 2011; Trujillo-Ortega et al., 2011).

Vocalisation latency correlations

Delayed vocalisation latency was associated in this trial with low birth weight subjects displaying clinical signs and blood biochemistry associated with fetal growth restriction. Due to the low number of animals in this category these results should interpreted with caution, although delayed vocalisation response in very low birth weight lambs had also been noted in the first study (C. Morton, unpublished data). Similarly, delayed latency and other acoustic cry anomalies have also been reported in premature human neonates weighing <2.5 kg in association with retarded nervous system development (Golub & Corwin, 1982; Lester et al., 1991; Michelsson, 1971). Delayed vocalisation latency in the current study was also evident in lambs classified as severely hypoxemic or suffering respiratory acidosis by metabolic thresholds; and in association with fetal distress risk factors (high plasma glucose, high body weight, low blood pH and longer parturition). This evidence suggests that, as in human (Fisichelli & Karelitz, 1963; Michelsson, 1971; Zeskind et al., 1996) and rat neonates (Calamandrei et al., 2004; Engidawork et al., 1997; Venerosi, Cutuli, Chiarotti, & Calamandrei, 2006), neurological integrity may have been compromised by resulting hypoxic impacts on the lamb CNS. A vocalisation latency of less than or approximate to 2 seconds may be a possible upper threshold for optimal normality or viability. This value, determined as the median latency in Chapter 3 and similar to distress cry latency demonstrated in the healthy human neonate (Michelsson, 1971; Zeskind & Lester, 1978), also appears to be associated with significantly fewer risk factors or indicators of fetal distress. This response time is also reported as typical reactive behaviour in older lambs (Shillito Walser & Walters, 1987) whom would be expected to

have recovered from any birth related deficits in vocalisation behaviour, as is indicated by the results of Chapter 3.

Of interest was the more closely correlated relationship between indicators of fetal distress and vocalisation latency subsequent to the tagging procedure, an additional stress stimuli which may have elicited higher arousal among subjects and highlighted more deficient animals. Longer delays in reactivity and higher thresholds to elicit vocalisation are believed to reflect a poorer capacity to integrate sensory stimuli compared to typical neonates, and may even be associated with less developmentally mature infants (Zeskind et al., 1996). In this study a poorer capacity for arousal appeared to be associated with extent of fetal distress. Even if fetal distress was not an evident factor, the wider implication could be that such lambs would most likely be associated with poorer survival chance under adverse conditions.

The lack of breed difference in vocalisation latency in this trial may reflect that distress vocalisation in the early neonate is dominated by neurobehavioural integrity rather than by genetic variation. In contrast to other ovine studies which have reported vocalisation rate differences associated with breed and vigour (Dwyer et al., 1998; Nowak, 1990a; Shillito Walser, 1978), this study found that birth weight had a greater impact on vocalisation delay initiated by isolation stress than breed or lamb sex. However a greater risk of vocalisation delay in male lambs, even if in association with higher mean birth weight, is consistent with trends in blood parameters and the findings reported in Chapter 3. In the current trial, a large proportion of lambs weighing over 5 kg were male which may have influenced any gender disparity. Certainly lambs weighing over 5 kg are associated with greater risk of dystocia (Alexander, 1984; Cloete, 1993; Hatcher et al., 2009) and both the vocalisation latency and blood results of this study support this observation. Gender and sex effects in human or rodent neonate cry studies are also not often reported until later postnatal ages associated with ontogenic change (Cox Lippard et al., 2015; Lester & Boukydis, 1992; Zeskind et al., 2014).

Vocalisation response differences between co-twins in this trial confirmed that there was no effect of test order associated with the greater probability of a faster response in the second born twin. The results reported here thus repeat those found in the previous experiment (Chapter 3) where the first born co-twin was more likely to have a slower

vocalisation response than the second born. In addition the first born co-twin was more likely to have a lower mean rectal temperature than the second born, despite being heavier on average, indicating a possible subtle thermoregulatory effect associated with parturition stress and hypoxia (Branco et al., 2006; Steiner et al., 2002) which has also been implicated in neonate pigs with poor vitality scores (Trujillo-Ortega et al., 2011). Interestingly both experiments of this dissertation have found that the first born co-twin was more likely to be heavier, a trend which is not consistently reported in studies of human twins (Usta et al., 2001). While noting that methodological deficiencies appear common in both human (Smith, Fleming, & White, 2007) and ovine research in the poor use of paired statistical tests to compare co-twins; in humans the risk of mortality and neurological impact as a result of hypoxia and fetal distress during twin birth (where most infants are caesarean-delivered) appear to be consistently greater for the second born (Armson et al., 2006; Young et al., 1985).

Conclusion

The findings reported here indicate that both high birth weight and very low birth weight in the neonate lamb may be associated with increased risk of fetal distress and delayed vocalisation response initiated by an isolation stimulus. This conclusion confirms previously established causal factors related to neonate lamb mortality, many of which are also analogous to the human neonate, and the results are particularly compelling in light of the differences between co-twins. The most closely correlated indicator of delayed vocalisation in singleton lambs in this trial was elevated plasma glucose. The association of delayed vocalisation latency with blood pathology indicating minor to more severe fetal hypoxemia, longer parturition and high birth weight, support the hypothesis that a delayed vocalisation response appears likely to reflect a neurological impact of fetal distress. While the incidence of asphyxia and hypoxemia based on established thresholds was low in this study, the distinct linear relationship of plasma glucose with lactate and correlated delayed vocalisation suggests a graduated effect, which has also been reported in neonate asphyxia models in both rodents and sheep. The significance of this effect may be minimal under environmental conditions which favour neonate survival. However, delayed communication, cognitive functioning and neurobehavioural deficiency in the lamb neonate at critical periods and up to at least 3-4 hours postpartum could contribute to greater risk of mortality under adverse conditions in the field, which may have been

reflected to some degree in the twin lamb mortality rates of this trial. The likelihood of a slower vocalisation reaction and higher mortality rates in male lambs may be associated with subtle effects occurring during parturition linked to birth weight, which was also indicated as a fetal distress risk factor by biochemical markers in this trial. Further research validating these findings with a larger number of samples, including confirmed cases of hypoxia, would be useful.

Chapter 5

Acoustic analysis of the neonate lamb distress vocalisation

5.1 Introduction

The reporting of mammalian vocalisation features has been greatly advanced with the development of remote recording and computerised analytical technology over the past few decades (Blumstein et al., 2011). Based on bird song analysis, these technologies have primarily been applied to investigate within-species communication (Briefer & McElligott, 2011b; de la Torre et al., 2015; Ehret, 1980; Kiley, 1972), caller identity or phenotypic qualities (Briefer & McElligott, 2011; Owren & Rendall, 2003; Reby & McComb, 2003) and animal affective state (Manteuffel et al., 2004; Schön et al., 2004). Such information can provide valuable information on population dynamics, reproductive behaviour, maternal-young interaction and animal welfare. In the sheep, mother-young recognition studies have reported varying modes of signal coding (Searby & Jouventin, 2003; Sèbe et al., 2011); and acoustic cues common to adult and young related to degree of mouth-opening, pitch and separation context (Dwyer et al., 1998; Sèbe et al., 2007).

Despite the expiratory and vocal tract limitations related to the position of the larynx compared to humans (Fitch, 2000; Fitch, 2006), neural control and production of vocalisation in the sheep and other mammals share common physiology and mechanisms (Jürgens, 2009; Riede et al., 1997). While acoustic parameters of sheep neonates have been documented to a limited degree (Searby & Jouventin, 2003; Sèbe et al., 2008; Shillito Walser et al., 1982; Shillito Walser, Walters, & Hague, 1983), factors impacting upon characteristics and developmental changes in the vocalisation signals of *Ovis* species juveniles have not been reported in detail, although some data is available regarding goat kid vocal ontogeny (Briefer & McElligott, 2011; Lenhardt, 1977; Terrazas et al., 2003).

In addition, little research applying acoustic analysis as a tool to detect pathology, as in the human model (Titze, 1994b), has been undertaken in nonhuman species. Application of principals utilised in spectral study of the human voice has revealed that acoustic analysis of the human neonate distress cry is a sensitive indicator of neurobehavioural deficit in the infant (LaGasse et al., 2005; Lester & Boukydis, 1992; Rothenberg et al., 1995; Zeskind et al., 2011). Apart from delayed initiation of responses, higher thresholds required to initiate a response, and other acoustic parameters; high fundamental frequency (*F*0) heard as an irritating high pitch cry appears to be the most commonly reported indicator of neurological insult in human neonates including that associated with birth trauma, asphyxia and a range of other deficits (Corwin et al., 1996; Fisichelli & Karelitz, 1963; Furlow, 1997; Golub & Corwin, 1982; Lester et al., 1991; Lester & Dreher, 1989; Michelsson, 1971; Rothenberg et al., 1995; Vohr et al., 1989; Wasz-Höckert, Koivisto, Vuorenkoski, Partanen, & Lind, 1971; Zeskind & Lester, 1978; Zeskind et al., 2014).

The neonate distress cry has been found to demonstrate a similar acoustic structure across a range of mammalian species (Lingle, Wyman et al., 2012), and translation of the human neonate model to acoustic analysis of rodent pup distress vocalisations induced by separation from the dam has demonstrated that comparable acoustic characteristics (Brudzynski, Kehoe, & Callahan, 1999; Hofer, 1996a; Zeskind et al., 2011) are associated with rodent infant neurobehavioural deficit and prenatal impact (Barron & Gilbertson, 2005; Calamandrei et al., 2004; Venerosi et al., 2006; Wellmann et al., 2010; Zeskind et al., 2014). As the neonate distress cry appears to be a highly conserved infant response, believed to share a common ancestry and neural circuitry across mammalian and avian species (Jürgens, 2009; MacLean, 1985; Newman, 2007), this model has potential to be applied to the study of the distress vocalisation responses of neonates of other species. While it has been reported that foals diagnosed with hypoxic-ischemic encephalopathy make abnormal characteristic vocalisations (Vaala, 1999; Wong, Wilkins, & Bain, 2011), as far as we know no research has applied acoustic analysis of distress vocalisation as a potential indicator of health and neurological deficit in sheep, or other production livestock or non rodent species.

The previous chapters have indicated that delayed vocalisation initiation does appear to be linked to low vigour and biochemical markers indicative of fetal distress and hypoxia in the early neonate lamb. The purpose of the current investigation was to study the

variation in acoustic parameters of lamb neonate distress vocalisations using principles of human voice and neonate cry analysis in an attempt to compare and identify acoustic features associated with normal and delayed vocalisation initiation, and corresponding biochemical markers indicative of fetal distress and asphyxia. It was hypothesised that neonate lambs with delayed distress vocalisation responses would show significant differences in fundamental frequency (*F*0) or other temporal and acoustic parameters compared to lambs with immediate distress vocalisation responses.

5.2 Materials and methods

5.2.1 Animals and experimental groups

The design of this experiment was based on the acoustic cry comparison of asphyxiated, low birth weight and normal human neonates by Michelsson (1971). Criteria used to determine the groups in Michelsson's study included APGAR score rating, so it seemed valid to test vocalisation latency of lamb neonates as an indicator of normality in a similar way (refer to Chapter 2, pp 27-28). The subjects for this study were selected from the experiment described in Chapter 4; and to compare acoustic features associated with vocalisation latency, two experimental groups were determined based on availability of non contaminated audio recordings samples from MxM and WSFxM lambs. The samples were then subdivided into two groups on the vocalisation response criteria described below:

1. *RAPID group*: animals with rapid vocalisation responses (latency <3 s)
2. *DELAYED group*: animals with delayed vocalisation responses (latency >5 s).

Five animals from the original singleton dataset (n=43) were excluded on the basis of vocalisation latency (latency >3 and <5 s); a further 10 singleton animals were excluded because audio files were contaminated or incomplete. Although groups for comparisons in previous experiments (Chapters 3-4) have compared animals below the vocalisation response median (<2 s) determined in Chapter 3, for this study a threshold of 3 seconds was set to capture as many samples as possible (additional animals latency >2 s and <3 s, n=4). This value was based on Chapter 4 study findings of singleton lambs following the tagging stimulus (median latency 1.93 s, 95% CI [1.58-2.94 s]). To ensure vocalisation

categories were mutually exclusive, we retained the lower threshold for the DELAYED category as >5.00 seconds. To source adequate subject x sex samples, recordings of twin lamb vocalisations were also sourced from the experiment described in Chapter 4 on the basis of the above selection criteria. In total, audio files from n=28 singleton and n=17 twin born lambs (first born n=8; second born n=9) were available for analysis. While equal group numbers were available, data were unable to be completely balanced for sex or breed (see Table 5.1).

Table 5.1: Number of animals used for acoustic comparisons.

Group	Criteria	Breed = MxM		Breed = WSFxM	
		Male	Female	Male	Female
RAPID (n=21)	Vocalisation latency <3 s	6	7	4	4
DELAYED (n=21) [a]	Vocalisation latency >5 s	6	6	8	1
Low birth weight (n=3)	Vocalisation latency >5 s, birth weight <2 kg	2	1	0	0

[a] Data concerning acoustic variables was incomplete for n=5 lambs (MxM n=3, 2 male, 1 female; WSFxM n=2, 2 male).

There were no obvious signs of physical abnormality, abnormal behaviour or distress at birth in any of the lambs from which recordings had been selected, although one animal (DELAYED response group) had been assisted at birth and had meconium stained birth fluids. To exclude the effects of obvious prenatal developmental complications, n=3 twin born lambs with low birth weight <2kg (all demonstrating delayed vocalisation latency) were retained in a separate group for comparison of acoustic variables as per Michelsson (1971). These lambs had also shown no obvious signs of gross abnormality, complicated delivery or distress apart from a physical appearance typical of lambs suffering fetal growth restriction (as described in Chapter 4).

5.2.2 Audio recordings

Recordings were collected at 3.5 hours postpartum upon application of an isolation distress stimulus as described in Chapters 3 and 4. A second recording was collected within 5 minutes when the lamb was replaced on the ground following tagging.

Recordings were made on a Roland R-05 dual microphone recorder at a sample rate of 44.1 kHz and saved as MP3 files at a bit rate of 128 kbps to preserve relevant acoustic properties associated with human voice analysis (Cervera, 2001; Gonzalez, Cervera, & Llau, 2003). The recorder was placed 70 cm above the floor and the distance from the lamb muzzle to the microphones ranged from 50-60cm depending on orientation of lamb which was usually toward the microphone for the first vocalisation. Recording ceased at 90 s following commencement of the test (hand release and verbal cue). If lambs were observed to face away from the microphone a note was made verbally on the recording.

5.2.3 Acoustic analysis

Determination of vocalisation latencies from verbal cue given as the test was commenced, as well as bleat rate and spectrographic analysis were performed using the linguistic software Praat (version 5.3.65, 2014, Boersma and Weenink). Vocalisations were visualised as broadband spectrograms in Praat which allowed better definition of temporal measures and glottal functioning as shown in Figure 5.1 (FFT method, window length=0.005 s, time step=1000, frequency steps=250, Gaussian window shape, dynamic range=70 dB). Frequency and amplitude parameters were measured on the mid section of the sustained open-mouthed (*tonal*) portion of the vocalisation (sound "*a-a-a-a-a-a*"), as in line with human voice analysis of the sustained vowel (Titze, 1995), using the Praat commands 'Pulses command: Voice report' and 'Intensity command: Mean and Maximum' to obtain intensity values (cross correlation method, pitch floor=100 Hz, pitch ceiling =500 Hz). As vocalisation duration was not able to be controlled in lambs as in human voice analysis (Titze, 1995), where possible the segment length of the section analysed was standardised at 0.3 s where *F*0 and amplitude intensity were more stable (Figure 5.1). It was important to use the section of the signal when the lamb's mouth was fully open so that parameters associated with vocal cord function were not affected by vocal tract resonance (Fitch, 2006). To obtain measures for nasally-emitted signals, frequency and amplitude measurements were also taken from the stable 0.3 s mid section of the signal spectrograph.

The total duration of the signal from commencement of the first nasally-emitted sound ("*mm*", mouth closed) to reclosing of the mouth was measured, and the duration of the

nasally-emitted "*mm*" section at the beginning of the vocalisation, were measured by assessment of the spectrogram changes (Figure 5.1) in association with auditory assessment by the researcher. The proportion of the total signal during which a nasally-emitted sound "*mm*" was made was calculated as MCLOSE (%) = *(duration of mouth closure segment) ÷ (duration of complete signal) x 100*. In some cases a lamb would commence a vocalisation with a "*mmamam…* " sound where the mouth or lips would partially open (although this was not common). This segment would also be classified as nasally-emitted and contribute to the MCLOSE variable. Acoustic parameters measured on each signal are listed and summarised in Table 5.2. Formants, which relate to the quality of vowel production, were not relevant to this study which focuses on pitch, glottal pulsing and vocal fold function. Median value of *F*0 from the Praat voice report was selected as a parameter for comparison rather than mean *F*0 because it may more closely represent the commonly perceived pitch (Grawunder & Bose, 2008), although statistically there was found to be no significant difference between the two measures in this study (mean difference=0.16 Hz, paired Student's t-test $p>0.5$).

In addition, temporal measures associated with signal initiation and rate (Table 5.2) were also measured from the spectrographic data. Bleat rate was determined from the number of vocalisations made by a lamb, both open and closed-mouthed, from the time of the first vocalisation for a period of 30 seconds (as in human infants per Rothenberg et al., 1995) which allowed a more robust determination of the initial intense bleat rate of rapidly responding lambs before decline in vocalisation rate, which has also been reported in rodent trials (Cox Lippard et al., 2015). If a lamb did not bleat within the first 30 seconds, then the total number of vocalisations emitted over the entire recording period (90 s) were calculated so that this data could be captured. Bleat rate was expressed as bleats per second as these periods of measurement were unequal. Latency to the first open-mouthed, tonal vocalisation (LATDS) as opposed to latency to the first vocalisation (LATB1, measured and described previously in Chapters 3-4) was also measured. Emission of any tonal or nasally-emitted signal was the accepted latency time point for LATB1, whereas LATDS (a variable able to be determined from the audio recordings in this experiment) was a measure of the latency to emit an appropriate or "true" tonal distress signal.

Tonal and nasally-emitted signals were analysed from each lamb where available to describe vocalisation parameters in these signals. The first non contaminated tonal signal

emitted by the lamb upon the commencement of the test (release of the hand), and the first non contaminated nasal signal emitted were selected for analysis. To compare acoustic parameters of control and delayed group vocalisations as per Michelsson (1971), only tonal signals were analysed to allow accurate determination of perturbation measures (Titze, 1995; Titze, 1994b). If the mid section of the first tonal signal had been partially contaminated, but signal parameters were similar in the following signal (as had transpired in n=6 animals), spectrographic analysis was conducted on the comparable, non contaminated sample. Calculation of variable values from a mean of n=3 signals (as per infant cry analysis conducted by Zeskind and Lester, 1978) was not possible as a large number of delayed response animals emitted n=<3 tonal signals during the recording periods. Signals where the lamb faced away from the microphone (as verbally noted on audio recording or identified by auditory analysis) were not sampled so that standardised amplitude parameters could be compared across animals.

The ability of the lamb to respond with an immediate and appropriate response (an open-mouthed tonal distress signal) following isolation from the dam was investigated by analysis of tonal distress signal latency (LATDS), and the order in which the signal occurred. Tonal distress signals initiated immediately (ie bleat number 1) were categorized as "*Immediate*" and tonal signals not initiated immediately (bleat n>1 and following earlier nasally emitted, mouth-closed response/s) were categorised as "*Subsequent*". If a lamb did not make a distress signal during the pre tagging recording, a signal was sampled from the post tagging recording where possible and noted (refer Chapter 4, pp 80).

5.2.4 Physiological and biochemical measures

Data for venous whole blood and blood plasma measures were available for each lamb as described in Chapter 4, along with lamb birth weight (measured at 3.5 hours). Also available were corresponding data on length of stage II labour and rectal temperature (refer Chapter 3 pp 45; Chapter 4 pp 79-81). A number of observations for animals in this experiment were missing, including data for labour duration (twins: n=14, singletons: n= 6) and venous whole blood data (twins: n=14, singletons: n=7).

Table 5.2: Description and definition of acoustic variables. Refer also to Table 2.1.

Variable	Units	Description
Frequency measures:		
*F*0Max	Hz	Maximum fundamental frequency - highest *F*0 (pitch) measured in the signal.
*F*0Med	Hz	Median fundamental frequency- most commonly represented *F*0 value measured in the tonal segment of the signal.
Amplitude measures:		
INTmax	dB	Maximum intensity, relating to degree of energy in a sound wave and directly proportional to squared amplitude. Intensity in Praat is defined as the root mean square (RMS) pressure relative to 0.00002 Pascal (converted to dB).
INTmean	dB	Mean intensity – mean intensity measured in the tonal segment of the signal. Calculated as above.
Signal modulation/instability:		
Jitter	%	Periodic deviation in the signal *F*0 (pitch perturbation). In Praat, Jitter (local) is the average absolute difference between consecutive periods, divided by the average period (refer Table 2.1).
Shimmer	%	Cycle to cycle variation in the signal amplitude (amplitude perturbation). In Praat, Shimmer (local) is the absolute difference between the amplitudes of consecutive periods, divided by average amplitude (refer to Table 2.1).
Harmonic to noise ratio (HNR)	dB	Degree of acoustic periodicity and measure of hoarseness (poor functioning of vocal folds). Represents proportion of energy in harmonics relative to noise (refer to Table 2.1).
Temporal measures:		
DUR	s	Duration or length of signal from commencement to end.
DURmc	s	Duration of the nasally-emitted, mouth-closed segment at the beginning of the signal.
MCLOSE	%	Proportion of nasally-emitted segment at the commencement of the signal (DURmc÷DUR x100).
LATB1	s	Latency from start of test to first vocalisation response (any type of signal).
LATDS	s	Latency from start of test to first distress vocalisation response (open-mouthed tonal signal only).
Bleat rate	(bleats/s)	Number of signals emitted in the first 30 s of the test (any signal type). If no vocalisation was emitted, rate was calculated for that animal over the total 90 seconds of the test (pertinent to delayed response lambs only).

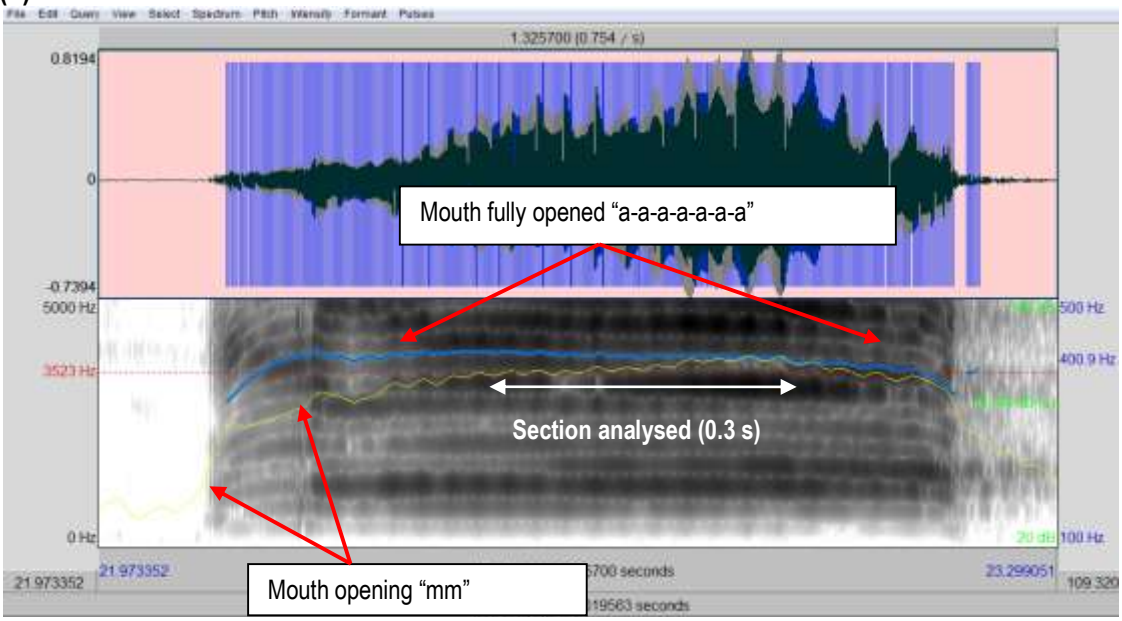
5.2.5 Statistical analysis

All statistical analyses were conducted using R statistical package (version 3.1.1, R Core Team, Vienna, Austria). Comparison between nasally-emitted and tonal signals were made using a Wilcoxon signed rank test for each acoustic variable as the data were nonparametric. Data are presented as median, 25-75th percentiles and range. For between-group analysis, a binomial test was used to analyse appropriateness of vocal response related to sequence of signal type, and the Fisher exact test was used to assess proportions

of lambs in each group related to sex and breed. A series of linear models were performed to investigate the effect of group, birth weight, breed and sex on each acoustic variable. Initial stepwise modelling (with group, sex and breed as factors and birth weight as a covariate), repeated for each variable, established that an effect of sex was not evident in any of the models and breed was not significant once adjusted for birth weight. Interactions between group and sex were unable to determined accurately as cell numbers in the delayed group were $n<3$ (Table 5.1).

As birth weight appeared to be a significant covariate associated with between-group difference, and was collinear with both breed and sex, a final linear mixed effect model *lmer* (Bates, Maechler, & Bolker, 2013) was performed on each variable of interest, with group as the main effect and sex and breed fitted as random effects to report significant levels and LSmeans, unadjusted for birth weight. The same data were also compared using the Wilcoxon ranked sum test, achieving similar results. Cox PHM (*survival* package, Therneau and Grambsch, 2000) was used to investigate the relationship between latency variables, and group, sex and breed differences. Data normality assumptions were tested by inspection of Q-Q plots and scatterplots of the residuals. Where necessary data were log transformed, and are presented as back transformed means with corresponding LS means and standard error. Latency data are presented as Kaplan-Meier distribution median values. Data for linear associations between acoustic variables and physiological variables including blood metabolites, body weight, rectal temperature and labour length (natural log transformed) are shown as Pearson correlation coefficients. Simple correlations between acoustic variables and signal initiation latencies are described by Kendall correlation coefficients to account for non normality of the two disparate populations (Kowalski, 1972).

(a)



(b)

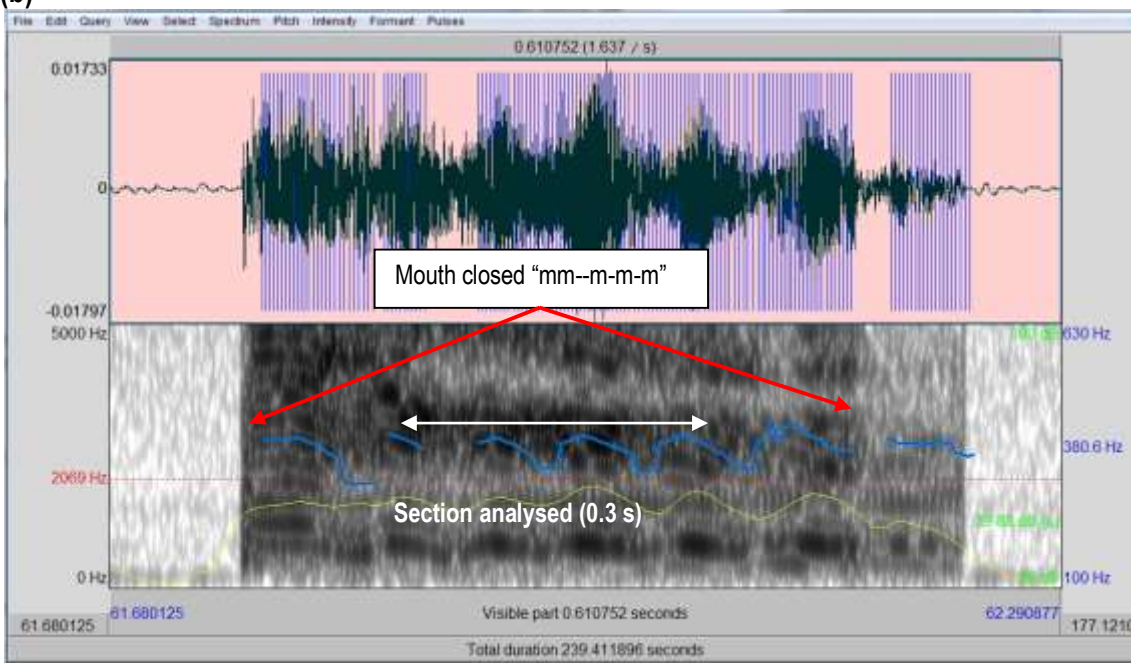


Figure 5.1: Praat spectrogram of (a) tonal and (b) nasally-emitted lamb bleat showing vocal tract opening and vocal fold vibration. Pitch tracking line in blue, intensity in yellow. Formants (not analysed) are the darker shade bands of energy.

5.3 Results

Data for acoustic variables (complete and incomplete) were available from a total 45 lambs. A number of lambs did not emit a nasally-emitted signal (n=25) or a tonal signal (n= 4) during the recording period either before or after tagging.

5.3.1 Comparison of tonal and nasal signals

Lamb vocalisations were classified into 2 main types of signal associated with closure and opening of the mouth, which consequently generated different signal acoustic characteristics. For comparison between nasally-emitted and tonal vocalisations, a total of 22 nasal (WSFxM n=7, MxM n=15; male n= 14, female n=8) and 39 tonal (WSFxM n=15, MxM n=24; male n=23, female n=16) signals were compared. All vocalisations commenced with a nasally-emitted segment where sound was initiated while the mouth was closed (characterised by the "*mm*" sound). Table 5.3 summarises values for various parameters to allow comparison of the two signal types.

Nasally-emitted vocalisations

These signals normally associated with close contact communication were classified as vocalisations produced where the lamb did not open its mouth, and sound was emitted through the nasal passages only. These signals were generally of lower *F*0 (*F*0Med: 342.5 Hz, $p<0.05$; *F*0Max: 351.6 Hz $p<0.09$), although high *F*0 values (>400 Hz) were also produced by nasally-emitted signals (Table 5.3). Amplitude measures in nasally-emitted signals were significantly lower than in tonal signals (INTmax: 70.88 dB, $p<0.0001$) with a maximum intensity level of 84.7 dB being measured in a nasal signal. Signal duration was also significantly shorter (DUR: 0.587 s, $p<0.01$) in nasally-emitted signals. Signal pulsing (refer to pulsed phonation in Table 2.1, pp 18-19) with voice breaks was also observed (also described in Kiley, 1972 and Volodin et al., 2011) so that the sound made could be heard phonetically as "*m---m-m-m*".

Tonal vocalisations

These signals, often associated with isolation distress and distance communication, were classified as those produced when the lamb opened its mouth during the vocalisation, and sound was emitted through the oral aperture. All tonal signals were initiated with the mouth closed (the "*m*" segment of the vocalisation); therefore the commencement segment of the signal (ranging from 0.027 to 0.800 s, and comprising 3.9-76.0% of the signal) had nasal resonance, characterised by rising amplitude and *F*0 prior to full oral opening (see Figure 5.1a). The tonal segment of the signal could usually be easily identified by the flatter pitch and intensity curve of the signal spectrogram. In less intense vocalisations, longer initial nasally-emitted segments were observed, sometimes exhibiting a pulsed signal. In Figure 5.1a, the *F*0 and intensity curves can be observed to fade as the mouth closes at the end of the signal. Tonal signals were significantly higher in amplitude measures or intensity ($p<0.0001$), longer in duration ($p=0.01$) and more likely to be of higher median *F*0 ($p<0.05$) (Table 5.3). The highest *F*0Max measurement in this trial (487.8 Hz) was associated with a tonal vocalisation.

Table 5.3: Descriptive and acoustic parameters [a] of lamb vocalisation types. Data shown as median (25-75th percentile).

Descriptives	Nasal (n=22)				Tonal (n=39)	
Oral status	Mouth closed				Mouth initially closed → to open	
Sound heard	*"m-m-m-m"*				*"mmaaaaaaaaaa"*	
Glottal activity	Signal pulsing, voice breaks				Continuous signal, may commence with signal pulsing	
Variables	median	range	W [b]	P	median	range
Signal duration (s)	0.587 (0.497-0.796)	0.186-1.025	236	0.01	0.765 (0.640-0.892)	0.377-1.631
DURmc (s)	0.595 (0.457-0.733)	0.186-1.025	700	<0.0001	0.219 (0.136-0.310)	0.027-0.800
Mouth closure (%)	100	100	732	<0.0001	28.9 (15.7-35.4)	3.9-76.0
F0Max (Hz)[c]	351.6 (326.1-372.5)	143.6-473.3	204	0.09	380.6 (347.7-419.6)	313.4-487.8
F0Med (Hz) [c]	342.5 (301.9-357.8)	142.3-467.9	191	0.05	377.3 (337.2-405.3)	302.3-481.3
INTmax (dB) [c]	70.88 (68.65-75.31)	58.43-84.74	62	<0.0001	86.56 (78.15-89.42)	61.49-92.74
INTmean (dB) [c]	67.98 (65.00-71.15)	58.47-81.31	50	<0.0001	82.59 (75.66-87.95)	57.91-92.44

[a] Measures collected following application of an isolation stress stimulus.
[b] Wilcoxon rank sum test statistic.
[c] Measured on sustained vowel of tonal, or stable mid section of nasal vocalisation.

5.3.2 Appropriateness and promptness of response

All lambs included in the experiment emitted a vocal signal in response to separation but not all lambs responded with a distress signal (tonal) when isolated from their dam. Eleven percent (n=5) did not respond with a distress signal during the two 90 s test periods (before and after tagging), and 8.9% (n=4) only emitted a distress signal after they had been subjected to the tagging procedure. All these animals had delayed first vocalisation responses prior to tagging (which were nasally-emitted close-contact vocalisations, see Figure 5.2). Significantly, 100% of RAPID group lambs (n=21) emitted a tonal signal for their first vocal response (*immediate* response) but only 33.3% (n=7/21) of DELAYED group lambs responded with an immediate tonal signal (x^2=18.11, df=1, p<0.0001). All RAPID group lambs initiated distress vocalisations during the first test (pre-tagging) within <3 seconds. Appropriateness of response (*immediate* or *subsequential* distress signal emission) was not associated with sex (odds ratio 1.48, Fisher exact test, p>0.50) or breed (odds ratio 1.42, Fisher exact test p>0.50). Time taken to initiate a tonal distress vocalisation was highly correlated with initiation of the first vocalisation (r=0.68, p<0.0001) across all lambs.

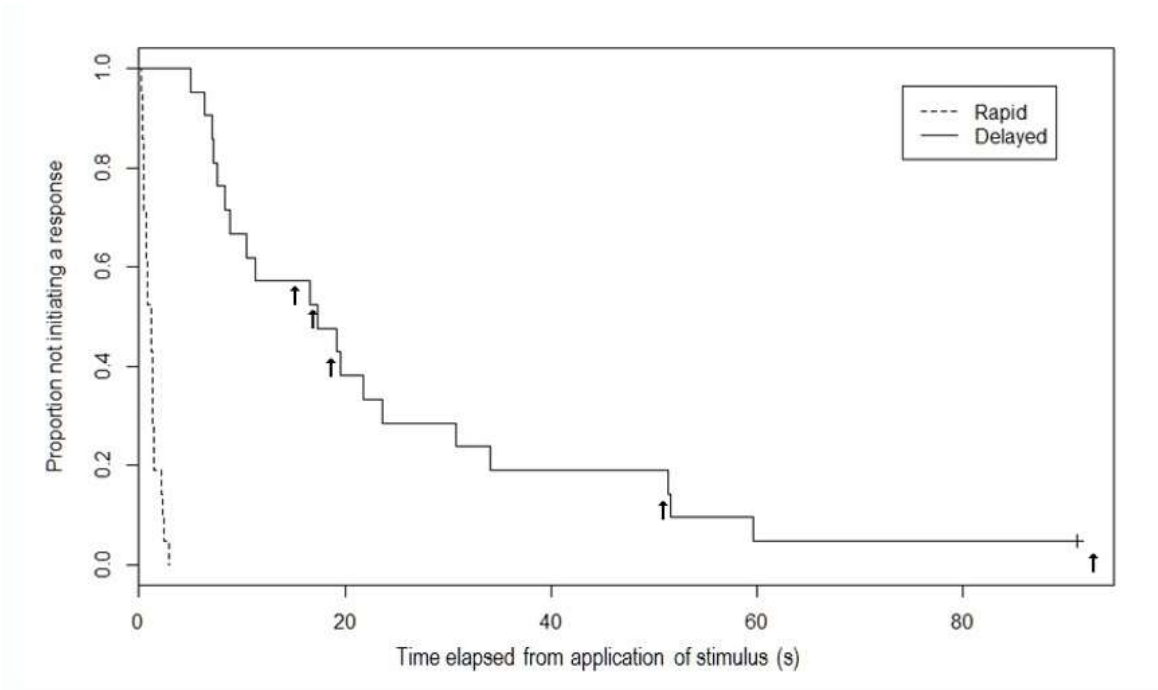


Figure 5.2: Kaplan-Meier survival distribution for first signal response latencies of RAPID and DELAYED latency group lambs prior to tagging. ↑ indicates response latency of lambs which only emitted nasal signals (n=5). Note one lamb with censored datum marked at 90 s did not respond until 129 s (far right).

5.3.3 Difference in acoustic measures associated with vocalisation latency category

Data from n=24 male and n=18 female lambs weighing >2 kg were available for comparison of acoustic parameters associated with bleat latency (described in Table 5.1). Data for n=3 low birth weight Merino lambs weighing <2.0 kg (1.18-1.94 kg, male n=2, female n=1) are also shown separately for comparisons. Five lambs in the DELAYED group, which recorded bleat latencies >16 seconds (including the assisted birth animal) did not make a tonal vocalisation at 3 hours after before or after tagging so signal acoustic data for these animals was not available for comparison (temporal data only for these lambs are included in Table 5.4).

There were significant differences in all acoustic parameters of lambs weighing >2 kg with the exception of maximum *F*0 (*F*0Max) and bleat duration (DUR) when signals of DELAYED group animals were compared to RAPID group animals (Table 5.4). RAPID lambs had signals with higher median *F*0 ($p<0.05$) and signal intensity ($p<0.01$), less modulation or perturbation in the signal (*F*0 modulation: jitter, $p<0.0001$; amplitude modulation: shimmer, $p<0.005$), and shorter segments of mouth closure at the commencement of their signals ($p<0.05$). RAPID lambs also demonstrated a higher rate of vocalisation in the first 30 seconds ($p<0.0001$), and none of these animals failed to make a tonal distress vocalisation following separation from their dam.

When compared to values across all lambs, the three low birth weight animals had comparatively high *F*0 measures (*F*0Max and *F*0Med values higher than the 75th percentile), comparatively high amplitude intensity measures (INTmean and INTmax higher than the 75th percentile), jitter and shimmer measures less than the median, and HNR values between the mean and 75th percentile. However, latency to initiate a signal and emit a tonal vocalisation were delayed (>8 s) in low birth weight animals, and bleat rate was below the median (Table 5.4).

Table 5.4: Acoustic and temporal characteristics of lamb vocalisations following application of an isolation distress stimulus. P values relate to differences between the RAPID and DELAYED groups only. Data shown as LSmean±SE, or median (25-75th percentile) for latency data where noted.

Variable		RAPID n=21	DELAYED n=21	*p* value<	Birth weight <2 kg n=3
Temporal measures:					
Latency first vocalisation (s) [a]	median range	1.18 (0.71-1.50) [0.16-2.92]	17.32 (8.84-34.1) [5.02 - >91.00]	<0.0001	15.06 [8.08-95-25]
Latency tonal vocalisation (s) [abc]	median range	1.18 (0.76-1.50) [0.16-2.92]	44.27 (25.59-na) [6.35 - >91.00]	<0.0001	> 91 [13.8->129.6]
Sequence of tonal vocalisation [bcd]	median range	1st [1st only]	>3rd [1st – 7th]	<0.0001	2nd [1-3rd]
Duration tonal bleat (s) [bc]	mean range	0.818±0.06 [0.377-1.631]	0.740±0.07 [0.499-0.997]	NS	0.856 [0.843-0.868]
Duration mouth closure (s) [bce]	mean range	0.154 (-1.87±0.16) [0.027-0.501]	0.244 (-1.410±0.20) [0.091-0.800]	0.05	0.211 [0.135-0.288]
Percent mouth closure (%) [bce]	mean range	19.93 (2.99±0.16) [3.9-68.6]	33.62 (3.53±0.20) [10.5- 76.0]	0.03	25 [15.5-34.8]
Bleat rate/s	mean range	0.376±0.02 [0.176-0.580]	0.117±0.02 [0.008 -0.307]	<0.0001	0.080 [0.025-0.125]
Frequency and amplitude measures:					
*F*0 max (Hz) [bc]	mean range	388.6±11.4 [315.1- 487.7]	371.3±13.6 [313.4- 437.5]	NS	453.3 [436.6-470.0]
*F*0 med (Hz) [bc]	mean range	382.05±14.8 [308.317-481.3]	353.04±16.6 [302.255-426.7]	0.04	445.2 [431.6-458.9]
INT max (dB) [bc]	mean range	86.17±1.55 [76.40-92.74]	80.11±1.84 [61.49-89.03]	0.005	90.55 [90.38-90.72]
INT mean (dB) [bc]	mean range	84.23±1.65 [73.86-92.44]	76.82±2.01 [57.91 -89.03]	0.003	88.86 [88.70-89.03]
Jitter % [bce]	mean range	0.14 (-1.98±0.17) [0.05-0.41]	0.42 (-0.86±0.21) [0.10-3.93]	<0.00001	0.14% [0.10-0.14]
Shimmer %[bc]	mean range	5.24±1.00 [1.62-13.23]	9.09±1.16 [2.6-15.87]	0.002	3.70% [2.78-4.61]
HNR [bc]	mean range	14.00±1.33 [3.31-22.39]	8.68±1.57 [2.37-16.66]	0.002	14.72 [12.03-17.42]
Voice breaks (% of animals) [bcd]	mean	4.76 (n=1)	25 (n=4)	NS	0

[a] Significance levels determined by Cox PHM.
[b] Acoustic variables on determined on first tonal signal.
[c] Data for these variables were unavailable for n= 5 DELAYED response lambs.
[d] Significance level determined by Fisher extract test.
[e] Data were log transformed to normalise distribution, back transformed means shown outside parenthesis.
na = Not available. NS = Not significant.

Direct linear relationships between acoustic parameters and latency to the first signal emitted and first tonal distress signal latency are indicated in Table 5.5. Amplitude intensity, *F*0 and both amplitude and *F*0 instability; and duration and proportion of mouth closure were significantly correlated with both first vocalisation and tonal distress signal latency. Rate of vocalisation was highly correlated with both types of signal response latency ($p<0.00001$). Maximum *F*0 and duration of signal demonstrated no relationship with either type of signal latency, and a relationship with *F*0Med was not strong ($p<0.09$).

Table 5.5: Correlation of acoustic measures with vocalisation responsiveness (Kendall's rank correlation). Bold type indicates significant ($p<0.05$) and marginally significant ($p<0.10$) correlations.

Variable	Latency to first vocal response		Latency to initiate a tonal distress signal	
	τ	n, p<	τ	n, p<
*F*0Max (Hz)	-0.11	35, 0.37	-0.11	35, 0.36
*F*0Med (Hz)	**-0.20**	**35, 0.09**	-0.18	35, 0.11
INTmax(dB)	**-0.34**	**34, 0.004**	**-0.27**	**34, 0.03**
INTmean (dB)	**-0.34**	**34, 0.004**	**-0.27**	**34, 0.02**
Jitter %	**0.35**	**35, 0.004**	**0.33**	**35, 0.006**
Shimmer %	**0.33**	**35, 0.005**	**0.25**	**35, 0.04**
HNR	**-0.25**	**35, 0.04**	**-0.20**	**35, 0.09**
DUR (s)	-0.06	35, 0.58	-0.05	35, 0.64
DURmc (s)	**0.27**	**35, 0.02**	**0.24**	**35, 0.04**
MCLOSE %	**0.31**	**35, 0.007**	**0.26**	**35, 0.03**
Bleat rate (bleats/s)	**-0.69**	**40, <0.00001**	**-0.67**	**40, <0.00001**

5.3.4 Sex, breed and birth weight associations

Average birth weight of the DELAYED response group was marginally heavier (4.52±0.22 kg, $p<0.08$) when adjusted for breed, with n=9 animals weighing >5.0 kg including n=3 lambs which did not make a tonal signal. RAPID group lambs weighed <5.2 kg (mean 4.24±0.17 kg). WSFxM lambs weighed significantly more than MxM lambs ($p<0.0001$) but the proportion of WSFxM lambs in either group was non significant (binomial tests $p>0.30$, refer to Table 5.1 and Figure 5.3).

For signals of the RAPID group, there were no differences in any of the acoustic parameters associated with sex (males = 10, female=11), birth weight or breed (WSFxM=

8, MxM=13), nor were there any correlations between birth weight and acoustic measures. In the DELAYED group, an effect of sex was not evident in any of the acoustic variables, nor breed following adjustment for birth weight. Where signals were available for analysis in the DELAYED group, heavier birth weight was correlated with signal amplitude and *F*0 instability, ie, higher shimmer (r=0.60, df=14, *p*=0.02), lower HNR (r= -0.65, df=14, *p*=0.006) and higher log jitter (r=0.57, df=14, *p*=0.03) as indicated in Figure 5.3. Associations between birth weight and *F*0Med (r= -0.31, df=14, *p*>0.10), and INTmean (r = -0.37, df=14 *p*>0.10), were not significant. There were no other significant associations between birth weight and other acoustic measures within the DELAYED response group.

Accurate interactions between effects in DELAYED response lambs were unable to be obtained because of low sample numbers. In DELAYED response lambs there appeared to be 2 clusters (Figure 5.3); at the lower birth weight range (birth weight <3.23 kg) indicators of signal perturbation were less evident, while in lambs of birth weight >4.25 kg variables associated with signal instability demonstrated higher values. Across all lambs (adjusted for birth weight, sex, breed and group) maximum and mean *F*0 was higher in MxM lambs but this was not significant (*F*0Max: mean 386.0±10.6; *F*0Med: 377.2±10.2, *p*>0.50 both measures) (refer to Figure 5.3).

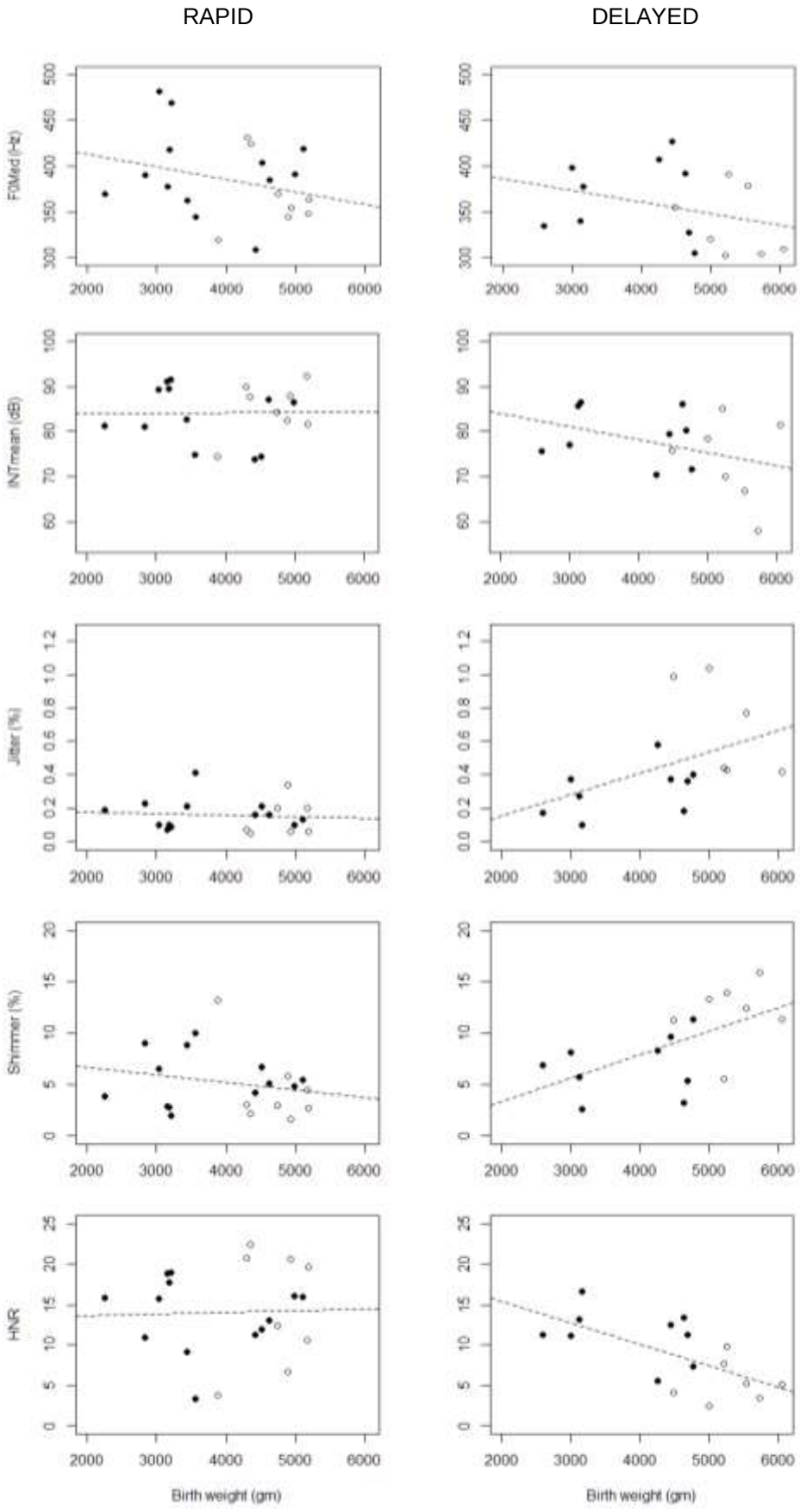


Figure 5.3: Relationship of birth weight, breed and acoustic parameters in RAPID and DELAYED group animals. MxM= ●, WSFxM= ○.

5.3.5 Correlation of acoustic variables with blood metabolite and physiological measures

Simple linear relationships between acoustic parameters and markers indicative of fetal distress are demonstrated in Table 5.6. Blood plasma glucose and lactate collected in the early postpartum period were correlated with a number of acoustic variables in this study (Table 5.6). As well as the association with first signal latency (LATB1) shown in Chapter 4, plasma glucose in this study was negatively correlated with other temporal parameters including bleat rate (r= -0.47, p<0.005) and latency to the first tonal distress signal (LATDS: r= -0.32, p<0.05). Plasma glucose was also associated with amplitude instability measurements (shimmer: r=0.44, p<0.01; HNR : r= -0.30, p=0.08) and lower intensity of amplitude (INTmean: r= -0.42, p<0.05). Plasma lactate was also negatively correlated with bleat rate (r= -0.39, p<0.05) and amplitude (r= -0.36, p<0.05). In this trial where cases were more disparate in terms of vocalisation latency (compared to Chapter 4) plasma lactate demonstrated a positive relationship with vocalisation latency (LATB1: r=0.54, p<0.005).

Longer labour duration (log transformed) was associated with *F*0 and amplitude instability indicated by higher jitter (r=0.56, p<0.05) and shimmer (r=0.55, p<0.05), and lower HNR (r= -0.48, p<0.05). Labour duration was also negatively correlated with amplitude intensity (INT mean: r= -0.66, p<0.005) and bleat rate (r= -0.51, p<0.05); and positively correlated with vocalisation latency LATB1 (r=0.70, p<0.005) as previously established in Chapters 3-4. Latency to the first tonal signal (LATDS) did not demonstrate as strong a relationship with labour duration (r=0.37, p<0.09) as LATB1. Birth weight associations were not as strongly correlated when compared across all lambs, but did demonstrate the expected trends indicated in Section 5.3.4 (levels of significance 0.05<p<0.09, Table 5.6) including an association with *F*0Med (r= -0.34, p<0.05).

Most of the physiological variables had a poor correlation with *F*0, but lower rectal temperature appeared to be associated with higher *F*0Med values when low birth weight animals were included in the data. As birth weight appeared to have a negative association with *F*0Med (r= -0.44, p<0.02) (which remained when low birth weight animals were excluded from the data, r= -0.30, p<0.07), and rectal temperature was highly correlated with birth weight (r=0.47, p<0.002), in summary there appeared to be a negative

relationship between birth weight (and associated rectal temperature) and median *F*0. Rectal temperature was only found to have an association with vocalisation latency and bleat rate when low birth weight animals were included in the data.

5.4 Discussion

The results of the experiment reported here indicate that lambs which respond to separation by emitting an immediate vocalisation are also more likely to emit an immediate signal of high frequency, amplitude and tonality associated with distress and signal communication over distance (Lingle et al., 2012; Owings & Morton, 1998; Ryan & Kime, 2003) than lambs that are slow to initiate a response. Lambs which have delayed separation responses were more likely to vocalise initially with a nasally-emitted signal, normally associated with close contact communication (de la Torre et al., 2015; Kiley, 1972; Sèbe et al., 2010; Teichroeb et al., 2013) and which, in the context of isolation and removal from the dam, is less likely to attract attention or immediate caregiver response (Lingle & Riede, 2014; Lingle et al., 2012). Such lambs also may not produce any tonal distress vocalisations during separation or following a stressful procedure involving pain. Delayed response animals were also shown to have greater *F*0 and amplitude instability (perturbation) signal characteristics than rapid responding lambs, which were linearly associated with increased birth weight. The finding that animals may not respond with a tonal signal when separated from their dam also necessitates redefinition of the classification of a lamb's isolation distress response as reported in Chapters 3-4, which was based on the first vocalisation initiated following the separation stimulus. By definition, a robust or appropriate distress vocalisation should not be a nasally-emitted signal associated with comfort and close contact communication in the *Ovis* species, so the description of the response measured in the previous chapters would be better defined as "the first vocalisation response" rather than "distress vocalisation".

Table 5.6: Pearson correlation coefficients describing relationship between acoustic and physiological variables. Bold type indicates significant (*p<0.05*) and marginally significant (*p<0.09*) correlations.

Variable	Plasma glucose [a]		Plasma lactate [a]		Labour length (log min) [ac]		Rectal temp °C [b]		Birth weight (kg) [a]	
	r	df, p<	r	df, p<	r	df, p<	r	df, p<	r	df, p<
*F*0Max (Hz)	0.12	33, 0.49	0.10	33, 0.60	-0.21	18, 0.37	**-0.40**	**35, 0.02**	-0.23	35, 0.17
*F*0Med (Hz)	0.09	33, 0.61	0.09	33, 0.62	**-0.45**	**18, 0.05**	**-0.44**	**35, 0.007**	**-0.34**	**35, 0.05**
INTmax (dB)	**-0.39**	**32, 0.03**	-0.28	32, 0.12	**-0.56**	**17, 0.002**	-0.26	34, 0.13	-0.26	34, 0.13
INTmean (dB)	**-0.42**	**32, 0.02**	**-0.36**	**32,0.04**	**-0.66**	**17, 0.003**	-0.23	34, 0.18	-0.29	34, 0.10
Jitter %	0.13	33, 0.47	0.009	33, 0.96	**0.56**	**18, 0.02**	-0.23	35, 0.18	**0.35**	**34, 0.04**
Shimmer %	**0.44**	**33, 0.009**	**0.30**	**33, 0.08**	**0.55**	**18, 0.02**	0.16	35, 0.34	**0.29**	**35, 0.09**
HNR	**-0.30**	**33, 0.08**	-0.15	33, 0.39	**-0.48**	**18, 0.04**	-0.19	35, 0.26	**-0.30**	**35, 0.07**
DUR (s)	-0.07	33, 0.70	0.02	33, 0.90	0.26	18, 0.27	-0.20	35, 0.25	-0.16	35, 0.32
DURmc (s)	0.13	33,0.45	**0.13**	**33,0.08**	0.30	18, 0.21	0.02	35, 0.90	0.02	35, 0.91
MCLOSE %	0.04	30, 0.84	0.29	30, 0.11	0.21	18, 0.37	0.02	32, 0.93	0.09	35, 0.58
LAT B1 (s)	**0.3**	**38, 0.04**	**0.54**	**38, 0.004**	**0.70**	**20, 0.0004**	**-0.34**	**40, 0.03**	0.03	40, 0.84
LAT DS (s)	**0.32**	**38, 0.05**	**0.31**	**38, 0.06**	**0.37**	**20, 0.09**	0.22	40, 0.18	**0.27**	**40, 0.08**
Bleat rate (bleats/s)	**-0.47**	**38, 0.003**	**-0.39**	**38, 0.02**	**-0.51**	**20, 0.02**	**-0.29**	**40, 0.07**	-0.14	40, 0.39

[a] Low birth weight animals (<2kg) excluded (n=2).
[b] Low birth weight animals (<2kg) included (n=2).
[c] Data transformed to natural log minutes.

If it is assumed that the control animals in this study are comparable to the reference or healthy populations in human neonate studies, tentative comparisons of the parallels between "healthy" and "abnormal" groups can be made between the two species. Differences between healthy and abnormal populations associated with vocalisation delay in human species were paralleled by the neonate lambs in this study in terms of signal amplitude and *F*0 instability or dysphonation (Golub & Corwin, 1982; Kheddache & Tadj, 2013; Lester et al., 1991; Michelsson, 1971; Zeskind & Lester, 1978; Zeskind et al., 2014), rate of vocalisation response (Lester et al., 1991; Rothenberg et al., 1995; Zeskind et al., 2014), and type of response - the number of non-tonal human infant signals are assessed by measurement of utterances <0.5 s (LaGasse et al., 2005). A higher stimulus threshold to elicit a response reported in delayed cry latency human neonates (Corwin et al., 1996; Zeskind & Lester, 1978; Zeskind et al., 1996), could also be interpreted to comprise the lambs in this trial which responded with a tonal vocalisation only after tagging, or by the longer latency to emit a true distress vocalisation, if longer time of separation is viewed as an increasing level of isolation stress. Duration of signal was slightly, but not significantly, shorter in delayed response lambs, as in the human model, although a difference in methodology may have influenced results as vocalisations >0.5 s only are measured for signal duration in current human infant cry studies (Golub & Corwin, 1985; LaGasse et al., 2005). Vocalisation latency was also positively correlated with the duration and proportion of mouth closure at the beginning of a signal, indicating that these lambs were slower to open their mouths to emit the tonal part of the signal. In rodent studies, parallel differences between healthy and abnormal groups have also been reported for vocalisation rate, signal duration, amplitude and frequency shift (Cox et al., 2012; Zeskind et al., 2014; Zeskind et al., 2011).

A lack of difference in breed or sex, independent of birth weight, in this trial corresponds to the reported lack of sex or race effects in the human neonate model (Corwin et al., 1996; Michelsson, 1971). Sex differences in human infants aged 30 days (Zeskind et al., 2014) and in rodents by postnatal day 14, equivalent to a 1 month old human (Cox et al., 2012), have been reported in vocalisation rate and other acoustic variables as a result of neurological impairment. The influence of gender specific associations on acoustic parameters appears not to be demonstrated in the early postnatal stage, but can be linked to biobehavioural change evident at equivalent developmental stages (Ehret, 1980; Lester & Boukydis, 1992). Studies which compare infants at close to the same age provide more

robust comparisons and as such the findings of Michelsson (1971), where infants were aged between 3 and 10 days, are more likely to correspond to the ontogenetic stage and age of lambs in this study.

A high pitched vocalisation (high *F*0) did not appear to be associated with delayed cry latency in this experiment, as is commonly reported in the human neonate (reviewed by Lester and Boukydis, 1992 and Corwin et al., 1996); but did appear to have an association with lower birth weight. It is difficult to ascertain whether the high *F*0 shown by underweight lambs with delayed responses in this trial could be linked to body size (Briefer & McElligott, 2011; Taylor & Reby, 2010) or delayed neurological development, as has been reported in premature (<2.48 kg) (Golub & Corwin, 1982; Michelsson, 1971; Rautava et al., 2007) and low birth weight infants exposed to prenatal cocaine exposure and subsequent restricted uterine blood flow (Lester et al., 1991). Mature but small-for-date human infants weighing less than 2.5 kg have been found to have significantly lower *F*0 frequencies than healthy infants weighing over 2.5 kg (Michelsson, 1971). In addition these infants did not demonstrate delayed cry latency or any abnormality of other acoustic parameters except signal duration. In comparing birth weights between the human and ovine neonates of Michelsson's and the current study, the effect of body weight alone as a causal factor for high *F*0 in low weight lambs seems doubtful, although caution must be applied to any generalisation because of the low sample number of this study. Fundamental frequency plasticity and lack of correlation with body size have also been reported in nonhuman studies including ungulate juveniles (Teichroeb et al., 2013) and adult animals (Riede & Titze, 2008; Sanvito et al., 2007). The effect of fetal growth retardation in both fetal lambs and human infants is known to result in similar clinical signs including lower birth weight, shorter body length and smaller head circumference (Dawes, 1976; Lester et al., 1991) as well as delayed neurodevelopment processes and an impact on brain neural connectivity (Louey, Cock, Stevenson, & Harding, 2000; Mallard, Rees, Stringer, Cock, & Harding, 1998). The underweight lambs in the present trial exhibited clinical signs of severe fetal growth restriction and therefore their vocalisation characteristics most likely reflected an associated neurodevelopment impact.

Unfortunately, measurement of *F*0 in more vocally unresponsive individuals, including the animal which had clearly suffered a significant degree of birth hypoxia, could not be determined accurately because of the lack of tonal vocalisation response in these animals.

Nasally-emitted signal *F*0 measures of these animals was within the median range of control animals, but is not comparable to tonal sound production because of nasal cavity resonance (Fitch, 2000; Fitch, 2006; Titze, 1994, 1994b).

Physiological markers indicative of some degree of hypoxia or birth trauma (elevated plasma glucose and lactate levels and prolonged labour, as described in Chapter 4) while not well correlated with *F*0, were associated with acoustic variables related to vocal instability, although caution should be applied to relationships which excluded the small number of animals with midrange vocalisation latency (>2 and <5 s). Low signal intensity and high signal perturbation appeared to have a consistent association with elevated glucose and lactate, and longer length of labour. Poor vocalisation rate, longer latencies to initiate a first vocalisation and appropriate distress vocalisations, were also correlated in varying levels with these same indicators. An association of these trends with birth weight known to increase risk of dystocia in Merino ewes (Alexander, 1984; Holst et al., 2002), is of specific interest and suggests that heavier birth weight animals with delayed responses may be exhibiting birth process-related effects on acoustic parameters. Of interest was the demonstration of a relationship of vocalisation latency with plasma lactate, which was not clearly apparent in the previous trial (Chapter 4) and possibly a reflection of the more disparate vocalisation delay samples sourced for this study.

A degree of fetal distress associated with either reduction of blood flow and oxygenation of the fetus (Comline & Silver, 1972; Gardner et al., 2002) or physical stress attributes (Dutra & Banchero, 2011) would not be unexpected in the lambs weighing over 5 kg in the current study, or even above 4 kg – a figure which has been suggested as the optimal lamb birth weight for reduced mortality (Hatcher et al., 2009). Such undetected impacts on the fetus as the ewe progresses through labour may have transitory or subclinical effects on the lamb's CNS as described in Chapter 4. While rectal temperature did not show a clear relationship with acoustic measures reflecting subclinical levels of fetal distress in this study, it may be that lamb body temperatures in this trial were not sufficiently impacted upon to demonstrate associations. Further research in this area, with animals demonstrating more severe effects of fetal distress and hypothermia, is warranted.

Dysphonation in particular has been reported as an indicator of pathology in clinical and automated analysis of human cry signals (Golub & Corwin, 1982; Kheddache & Tadj, 2013; Lester & Dreher, 1989; Wasz-Höckert et al., 1985) with healthy infant pain response cries being relatively noiseless (refer Table 2.1, pp 18-19) in comparison to those with a pathological condition (Hirschberg, 1999). Dysphonation, evident as glottal or vocal instability, or perturbation, can be indicated by high jitter, shimmer and low HNR measures in acoustic voice analysis (Little, Costello, & Harries, 2011). Glottal instability indicated by these indicators of perturbation can reflect immature innervation (Hirschberg, 1999) or poor neural control of the vocal folds, which is commonly associated with laryngeal nerve paralysis in the human neonate (Benjamin, Goldberg, & Malcolm, 2009; De Gaudemar, Roudaire, François, & Narcy, 1996; Miyamoto, Parikh, Gellad, & Licameli, 2005). Paralysis of the vocal folds can be bilateral or unilateral, cause respiratory and swallowing dysfunction, and is commonly associated with the physical trauma of birth, neonatal neurologic disease including anoxic brain injury (De Gaudemar et al., 1996) and heritable CNS neuropathy in juvenile dogs (Gabriel et al., 2006; Polizopoulou, Koutinas, Papadopoulos, & Saridomichelakis, 2003). Spontaneous recovery in human infants can occur over various periods between 6 months and a year depending on cause and degree of injury (Benjamin et al., 2009; De Gaudemar et al., 1996; Kaushal, Upadhyay, Aggarwal, & Deorari, 2005).

While the data provided here is preliminary, this is a possible physiological explanation for the acoustic results demonstrated in this experiment, and the transitory nature of delayed vocal behaviour reported in Chapter 3. Difficulties associated with accurate measurement of perturbation in the pathological human voice (Bielamowicz, Kreiman, Gerratt, Dauer, & Berke, 1996; Little et al., 2011) indicate that measures such as jitter, shimmer and HNR would require validation in the clinical setting. However as there are no known thresholds for non mammalian signals, the present results are of value primarily for inter animal and between group comparisons.

The findings reported here can also viewed as a reflection of the degree of stress reactivity or arousal capacity inherent to each animal. Individual response to a separation stimulus has been reported to reflect temperament or genetic characteristics associated with degree of stress reactivity (Beausoleil et al., 2012; Alain Boissy et al., 2005; Boissy, Fisher, Bouix, Hinch, & Le Neindre, 2005), which could impact upon tonal qualities of

vocalisation parameters, in particular perturbation measures (Fuller & Horii, 1986; Rothganger, Ludge, & Grauel, 1990). However as the arousal capacity of the human neonate is known to reflect integrity of the autonomic nervous system it is also plausible that delayed vocalisation responses in the early lamb neonate may also reflect poor coordination between the brainstem, midbrain and limbic systems (Zeskind, 2013; Zeskind et al., 1996).

Analysis associated with measurement of only one signal per animal could also be considered a limitation, as while many of the early human cry studies were based on sampling of a single cry (Michelsson, 1971), current studies may collect information from the average of between 1 and 3 cry samples (reviewed in LaGasse et al., 2005). In the experiment reported here statistical power and number of animals sampled would have been compromised if results had been standardised on analysis of more than one signal as many of the delayed response animals emitted only one tonal signal. Limitation of analysis to the first response may also have merits related to comparisons of initial neurobehavioural response to separation, and may more accurately reflect the ability of the lamb to be aroused. A decline in response following stimulus application has been shown to impact acoustic parameters of human and rodent infants (Cox Lippard et al., 2015; Prechtl et al., 1969; Runefors et al., 2000) although the distinct decline shown by Runefors et al. (2000) in human neonates following a pain stimulus may be different in lambs subjected to isolation stress.

In analysis of acoustic cry data it is also useful to have a blind analysis of results undertaken but this was not possible in this trial. A bias potentially associated with researcher assessment would be difficult to control as the temporal data relating to latency and all other acoustic parameters were available on the one Praat analysis screen. It was also not possible to limit analysis to all signals emitted prior to tagging as a small number of animals only made tonal responses following the additional stressor. In such cases, results were not compromised in terms of group allocation as all animals were classified as delayed based on poor response prior to tagging; and as discussed previously it is possible that, as in human infant studies, these individuals required a higher stimulus level to illicit a distress response (Zeskind & Lester, 1978; Zeskind et al., 1996). A potentially greater impact on study findings was that signals from animals not emitting tonal

vocalisations could not be analysed for acoustic parameters associated with glottal stability.

Suboptimal recording conditions associated with dam vocalisation and periods of windy weather were another limitation in this study but efforts were made to overcome contamination issues by selecting only high quality signals and audio files, and ensuring that direction of acoustic projection toward the recording device was standardised. Standardisation of subject age and Praat analysis settings should also have contributed toward optimal between-animal comparisons as neonate vocal parameters have been reported to change significantly in the days postpartum in goat kids (Terrazas et al., 2003); rodents (Ehret, 2005) and seal pups (Van Opzeeland, Van Parijs, Frickenhaus, Kreiss, & Boebel, 2011). A lack of prior acoustic technical knowledge and sophisticated recording equipment could also be seen to be a limitation in this experiment but the information gathered, especially related to temporal data, proved to be useful and is in line with equipment used to undertake human voice analysis (Gonzalez et al., 2003). Use of MP3 files at the higher bit rate recommended by Gonzalez et al. prevented data loss by enabling efficient data storage during test procedures via high compression rate. An advantage of lack of prior knowledge or expectation also meant that the researcher sought additional phonetic expertise and guidance rather than relying on currently reported bioacoustic analytical frameworks, and this input has contributed to the study rather than diminish it.

This study appears to be the first to describe in detail the phonetic and associated temporal structure of the predominant lamb vocalisation forms associated with relative proportions of nasal and oral signal production. Variants of these two forms have been documented by Kiley (1972) in other species, and may be age, sex or context dependent in the ovine species (Searby & Jouventin, 2003; Sèbe et al., 2007). Importantly, this study has demonstrated that all lamb vocalisations have an initial nasal component at the commencement of the vocalisation, and that *F*0 can vary significantly in both types of vocalisation production. The data of this study is consistent with a mean *F*0 of 345±49 Hz reported by Searby and Jouventin (2003) and range 278-358 Hz reported by Lingle et al. (2012) in neonate lambs (refer Chapter 2 pp 37), but also suggests that a slightly higher *F*0 may be evident in 3 hour old animals where analysis has been age standardised and measured on the tonal vocalisation segment only. While close-contact, nasally-emitted calls have previously been described as low frequency vocalisations (Briefer &

McElligott, 2011; de la Torre et al., 2015; Sèbe et al., 2010), the data reported here indicates that *F*0 of nasal signals can achieve frequencies of over 400 Hz. *F*0 measurement has also been recorded at frequencies above 400 Hz (peak *F*0) in the nasal signals of ungulate species with comparative neonate body weights including juvenile gazelles and neonate white-tailed deer (Atkeson et al., 1988; Richardson et al., 1983; Volodin et al., 2011). Thus in terms of signal depiction, "low frequency" is a misleading description for the nasal signals made by ruminant and possibly other neonates.

Conclusion

Vocalisation characteristics and behaviour of the neonate lamb may reflect a number of prenatal and birth related influences including immature development, obstetric difficulty or other neurological impacts including stress reactivity and arousal levels. A lack of reactivity, evident as delayed vocalisation responsiveness, has been shown to be potentially linked to fetal distress (Chapter 4), poor survival behaviour (Chapter 3) and greater risk of mortality (Brien et al., 2014), so it seems likely that lack of isolation distress or reactivity is a reflection of neurobehavioural deficit in the early lamb neonate rather than a genetic effect. Other acoustic measures including signal instability and vocalisation rate, also indicative of neurological deficit in the human and rat neonate, were found in this trial to be associated with delayed signal response in lambs, although high pitch as a marker of pathology does not seem to be a clear indicator in this study. Use of such acoustic traits as reported here may also have some limitations if lambs with neurobehavioural or other deficits do not make any signals at all. In such cases, latency of response would appear to be the overriding indicator of potential neurological impact. The findings of this study also contribute to the growing body of bioacoustic research regarding factors influencing acoustic and spectral analysis of mammalian vocalisations, and have implications for validity of automated vocalisation analysis especially where close range observation of vocal production and degree of mouth opening may not be possible. As far as is known this research has no precedent apart from the human studies on which it was based and the separation response studies of rodent neonates, and as such requires further verification. Nevertheless, as a preliminary experiment investigating acoustic properties associated with neurological deficit in the ovine neonate, this study highlights the potential of comparative studies of distress vocalisation characteristics across mammalian species.

Chapter 6

Maternal responsiveness to acoustic signals of the lamb neonate

6.1 Introduction

The distress vocalisation of the newborn of most species has a powerful and compelling effect on the maternal or care giving provider figure, stimulating release of oxytocin and promotion of maternal behaviour (Deis, 1968; Riem et al., 2011). The acoustic characteristics of this cry are also highly comparable across mammalian species (Lingle et al., 2012; Zeskind, 2013), and both the context of their occurrence and the neurobehavioural responses of animals hearing the signal (Lingle & Riede, 2014; Nelson & Panksepp, 1998) appear to have been conserved throughout mammalian evolution (Newman, 2007). The production of this particular type of vocalisation ensures that survival needs of the infant including retrieval, rescue, defence or feeding may be delivered in a timely manner, thereby increasing its chances of survival and the fitness of the attendant (Furlow, 1997; Newman, 2007; Zeskind, 2013).

While the infant distress vocalisation has been described as an honest signal reflecting need, it can also function to advertise health or viability status to the parent where other signs may not be obvious (Furlow, 1997; Soltis, 2004). The energy cost of signal production is likely to be higher for less vigorous infants (Grafen, 1990), so offspring with a greater chance of survival are most likely to emit comparatively stronger and more frequent signals and thus draw more attention from caregivers (Soltis, 2004). As such, disparity of infant signal production and parental discrimination associated with investment can play an important role in efficient resource allocation, particularly among species giving birth to larger litter sizes.

There are a number of species where parents use the vigour of infant signals as a reliable cue to indicate the viability and survival chances of the infant (Soltis, 2004). In birds preferential feeding behaviour to the most vigorous chicks is prompted by healthier

nestling chick gape colour (Saino et al., 2000) or condition of chick plumage (Hamilton, 1994). Female *Eptesicus* bats have been found to respond only to normal infant isolation calls but not acoustically abnormal vocalisations produced while the laryngeal nerve was compressed with cold forceps (Gould, 1975). Discrimination studies in rodents have also reported that maternal responses to pup acoustic signals only occur within very specific frequency, minimum duration and critical band ranges (Brudzynski et al., 1999; Ehret, 2005; Wöhr et al., 2008; Wöhr et al., 2010). In humans, parent-induced infanticide has been reported in African tribes where babies lacking a cry response at birth were traditionally abandoned because their chances of survival were poor (Basden, 1921). More recent studies of human parental response to high and variable pitch cries of unhealthy human infants have found that such cries can elicit strong negative emotions in individuals more likely to be abusive (Crowe & Zeskind, 1992; Frodi & Senchak, 1990). These studies provide evidence that cries more acoustically abnormal in terms of persistence and distance from the normal range, are more likely to be associated with withdrawal of parental care, abuse, abandonment or infanticide (Soltis, 2004).

Vocal communication of the infant can be a predominant stimulus for expression of maternal response, as is evident in temporal changes in maternal behaviour related to vocal development and ontogeny of the offspring (Ehret, 1980) and intensity of caregiver arousal (Lingle & Riede, 2014; Wiesenfeld & Malatesta, 1982), while recognition of the individual itself may be dependent on olfactory cues as in bats (Brown, 1976). In sheep multisensory cues involving olfactory, visual and acoustic stimuli are reported to be of great importance in maintenance of the mother-young attachment (Nowak, Keller, & Levy, 2011) but the degree to which these senses play a part in the immediate postnatal period has been inconsistently reported. Studies where olfactory and visual cues have been independently suppressed in this sensitive period (Poindron, Lévy, & Keller, 2007) demonstrate that maternal behaviour is not impeded by the loss of these senses in experienced ewes (Hernandez, Serafín, Vazquez, Delgadillo, & Poindron, 2001; Poindron et al., 2007; Poindron, Lévy, & Krehbiel, 1988). Visual clues have been reported to be more important than auditory cues in recognition of lambs aged older than 3 days old (Alexander & Shillito, 1977b; Shillito Walser & Alexander, 1980), but Keller et al. (2003) demonstrated that ewes were able to discriminate between lambs using both auditory and visual cues as early as 6 hours postpartum and that response to these clues was enhanced with prior maternal experience.

While many studies have explored the impact of the ewe's behaviour on maintenance and strength of the bond (reviewed by Dwyer, 2014), the role of the neonate lamb's behaviour in loss of attachment is not as well understood (Nowak, 1990b; Nowak & Lindsay, 1992; Stevens et al., 1982; Vince et al., 1985). Lamb vigour and behaviour has been shown to improve chances of survival through better following behaviour and suckling (Dwyer et al., 1996; Nowak et al., 1987; Stevens et al., 1987) and to stimulate greater maternal responsiveness (reviewed by Poindron et al., 2007) but few studies have focused on facilitation of maternal behaviour by active discrimination of lamb viability by the ewe. Poor acceptance and aggression towards lambs is known to occur in primiparous ewes (Dwyer & Lawrence, 1998b; Dwyer & Lawrence, 2005b) and has been attributed to the inexperience of the ewe (Nowak & Poindron, 2006; Poindron et al., 2007) but can also occur in experienced ewes (Dwyer, 2003; Dwyer, 2008; Putu, Poindron, & Lindsay, 1988). The question that still remains to be answered is whether abnormal lamb behaviour or signals given by the lamb are subsequently associated with rejection or abandonment by the dam, particularly in the case of twins (Alexander et al., 1983; Dwyer & Lawrence, 1999b; Lévy & Poindron, 1987; Nowak, 1996; Stevens et al., 1987).

In the previous chapters it was been shown that both latency to emit a distress signal; the rate and type of signal produced; and acoustic parameters related to the frequency and amplitude stability of the signal may be associated with impaired neurobehavioural potential on a scale that is determined by the degree of hypoxia or birth trauma experienced during birth processes, and subsequent impact on the CNS and functioning of the vocal folds. The study reported here aims to investigate the possibility that ewes demonstrate a preference towards individuals based on the acoustic qualities of the lamb's distress vocalisation. It was hypothesized that ewes would discriminate between lamb audio recordings in a two choice test based on preference towards lambs emitting signals associated with vigour including short initiation latency; higher fundamental frequency (*F*0), amplitude and signal intensity; and lack of dysphonation or signal instability. If possible within the range of animals studied it was also hypothesised that ewes would demonstrate poorer maternal responsiveness to atypical or deficient acoustic signals, and prefer tonal, open-mouthed signals over nasally-emitted close-contact signals.

6.2 Materials and method

6.2.1 Animals and maintenance

The experiment was conducted at the CSIRO FD McMaster Field Station, Armidale, NSW over the lambing period 8th-31st May 2014. Primiparous Merino x Border Leicester cross ewes were synchronised with a single subcutaneous implant of 18 mg melatonin (Regulin®, CEVA Australia) 40 days prior to natural joining with White Suffolk sires, and scanned to determine pregnant/non pregnant status at gestation day >80. Approximately five days before the expected lambing date, pregnant ewes were brought into a large yard adjacent to the animal house complex where they were familiarised with the testing arena and handlers, and monitored by camera for signs of impending birth. Ewes were initially fed in the testing arena, with mixture of 50% lucerne hay and 50% oaten chaff and grain (corn ration 100 gm/day) and then fed the same ration while penned for 12 hours or more following lambing. At the first sign of parturition behaviour ewes were moved into 3x2 m individual pens for closer observation and more accurate determination of time of birth. Immediately upon birth the first born twin was identified and marked with a coloured spray on the head. Testing as described below took place at specific times between 3 and 4 hours postpartum. On completion of all testing procedures, lambs were tagged, sexed, and weighed and a rectal temperature measured as described in Chapters 3-4, and then returned to their dam. Following monitoring for sufficient bonding, lamb and ewe units were released at 12 hours postpartum or older.

6.2.2 Experimental groups

Maternal preference for (a) vocalisation acoustic parameters and (b) type of signal (nasal or tonal) were tested in a two choice test. Ewe preference for acoustic parameters of distress vocalisation signals were tested on twin bearing ewes (n=13) comparing response to the recordings of distress signal stimuli from each co-twin. Response to nasal and tonal signals were compared in singleton bearing ewes (n=9), where nasal and tonal recorded distress vocalisation signals from the same lamb were used as stimuli. There were also n=2 twin bearing ewes included in this comparison because only nasally-emitted signals were able to be obtained from one of the lambs in each of these litters. This data is described separately because of the inadequate sample number.

6.2.3 Bleat recordings

At 3 hours after birth each lamb was taken individually to an adjacent sound recording building where a distress vocalisation was initiated by bleat test procedure as described previously (Chapters 3-5). While lambs were visually isolated during recording, and insulation provided some degree of acoustic isolation, some ewe vocalisations were still faintly audible to the subject. The vocalisation response was recorded over 90 seconds, or longer if latency to bleat was delayed, using a Marantz PMD661 solid state recorder (sample rate: 44.1 kHz , WAV file format 16 kbps,) connected to Sennheiser ME67shotgun microphone with Rycote softie windshield mounted on a rod placed approximately 1 m from the lamb's head. The average volume of the vocalisations were measured by a decibel meter (Digitech QM-1589) placed at 1 m from the lamb and the recording area was insulated with carpet to reduce echo. Sound files were transferred to a computer, assessed using Praat version 5.3.63 (Boersma and Weenink, 2009), and reconstructed into a 2-channel playback sequence using Audacity version 2.0.3. None of the co-twin signals were normalised or adjusted for amplitude prior to playback, but signals from each lamb for the signal-type comparison were normalised to 70-75 dB using the "Modify" and "Scale intensity" commands in Praat.

For the playback sequence, n=4 uncontaminated and clear open-mouthed distress vocalisations of highest amplitude intensity emitted over the 90 second recording period were selected as stimuli from the each lamb's vocalisation recording. A further n=4 uncontaminated nasal signals were also selected from those emitted by singletons. If nasal signals of singletons were not emitted during the recording session, nasal signals were recorded when the lamb was reunited with the mother using the same equipment and procedure. Lambs remained reunited with the mother until 4 hours postpartum when both ewe and lambs were taken together to the testing arena adjacent to the animal house.

6.2.4 Testing facility design

The test arena consisted of a 10x10x10 m triangular pen similar to that used for ewes described by Poindron, Gilling, Hernandez, Serafin, and Terrazas (2003) and Terrazas et al. (1999). The sides of the triangle were delimited by 1m high panels covered in black plastic, with two 1x1 m mesh pens in each corner encircled by hessian to prevent visibility but not impede sound. Contact zones and boundaries were delimited by a line of rice hull bedding material. The test procedure was similar to that used by Sèbe et al. (2011) but only recorded acoustic cues were available to the ewe and only audio recordings of the ewe's own lambs were used for the playback. Prior to the test the ewe was placed in the starting pen (zone A, Figure 5.1) with a mesh lift-up starting gate which allowed visibility of the test arena. The lambs were removed while ensuring that the ewe could see them being taken down the centre of the triangle so that they were last visualised by the ewe equidistant to both stimulus pens. The lambs were placed in a carry container behind the back triangle centre panel, quickly removed from the area, and a pretest bleat stimulus sequence commenced. In each of the stimulus pens (zones B, Figure 6.1) a powered loudspeaker (Advent AV570, 120V) connected to a computer was used for the playback. Each speaker was set at the same volume and equivalent to a normal lamb distress vocalisation level (average 70 dB SPL, at 1 m, measured at recording) checked by decibel meter (Digitech QM-1589). Each loudspeaker channelled the bleat signal sequence of one of the co-twins, and the side allocated to each twin and the sequence order of the pretest playback was randomised by birth order throughout the experiment.

The pretest playback, consisting of 4 consecutive but different signals of each lamb, separated by an interval of 2.5 seconds, was played over 30 seconds while the ewe was still in the starting pen. At the commencement of the discrimination test when the ewe was released, the playback sequence alternated (a) a single bleat from each lamb in the co-twin comparison, as per Sèbe et al. (2010); and (b) 4 consecutive bleats of each signal type in the signal-type comparison, as per Searby and Jouventin (2003). A total of 24 bleats were played consecutively during the test (12 bleats per lamb/signal from the allocated loud speaker, consisting of the 4 stimulus bleats repeated 3 times). In the twin playback an interval of 1.5 to 2 seconds in between each bleat was used to simulate the sound of two lambs bleating alternatively from each side of the arena. For the singleton signal type test, it was anticipated that the stimuli of signals from the same lamb could

promote confusion for the ewe if played from each speaker alternatively so the pretest stimulus sequence was repeated 3 times so that the ewe heard a 4-bleat stimulus of one type of signal and then the other.

The test period following release of the ewe was completed within 2 minutes by which time the ewe often displayed loss of interest in the stimulus pen areas and attempted to get out of the test arena (especially in the signal-type comparison test). At the end of each discrimination test, the ewe was returned to the starting pen and each lamb retrieved and tested for latency to return to the ewe in the starting pen over a distance of 5 m. While its co-twin was secured outside, each of the twins (randomly allocated by birth order) was placed on its side for 5 seconds on the side of the test arena (at point X, Figure 6.1), with its head facing away from the ewe's pen, released and timed to return to the ewe over the subsequent 3 minutes.

6.2.5 Measurement of ewe responses and lamb behaviour

All test arena behaviours were recorded by 3 strategically placed video cameras. The video recordings were visually analysed to determine latency and frequency of ewe and lamb behaviours (variables measured on the ewe are shown in Table 6.1). Rejection of, or obvious bias towards, lambs prior to or following testing was not observed as all ewes exhibited full maternal behaviour towards each/both lamb/s.

Arena test measurements of each lamb, conducted immediately after the ewe discrimination test, included latency to stand and vocalise (first vocal response), and return to the ewe contact zone (over a distance of 5 m) as described in Chapter 3. Measurement of lamb birth weight, rectal temperature and sex was also conducted immediately after the arena test as when the lamb was aged approximately 4 hours old. Missing data included n=4 lamb rectal temperature measures and n=1 video file of lamb arena behaviour. One co-twin was also excluded from latency to stand and return to ewe lamb data because of an apparent leg injury. One lamb did not make a tonal vocalisation for 10.7 s so this value was applied to the bleat latency dataset instead of an initial short nasally-emitted vocalisation made 0.85 s after release.

Table 6.1: Criteria of ewe response in the two-choice test.

Behaviour	Description
Exit zone	The side of the triangle entered when the ewe left the waiting pen.
First contact zone reached	The contact zone of the stimulus pen first reached after release.
Time spent in contact zone	Proportion of time spent in the contact zone of each stimulus pen. Time of entry or exit was recorded by positioning of the 2 front legs in the area.
Time spent seeking	Time spent trying to look into/ get into/ paw at the stimulus pen.
Response bleats to pretest stimuli	Number of response bleats produced by the ewe to pretest stimulus bleats (n=4 signals per lamb).

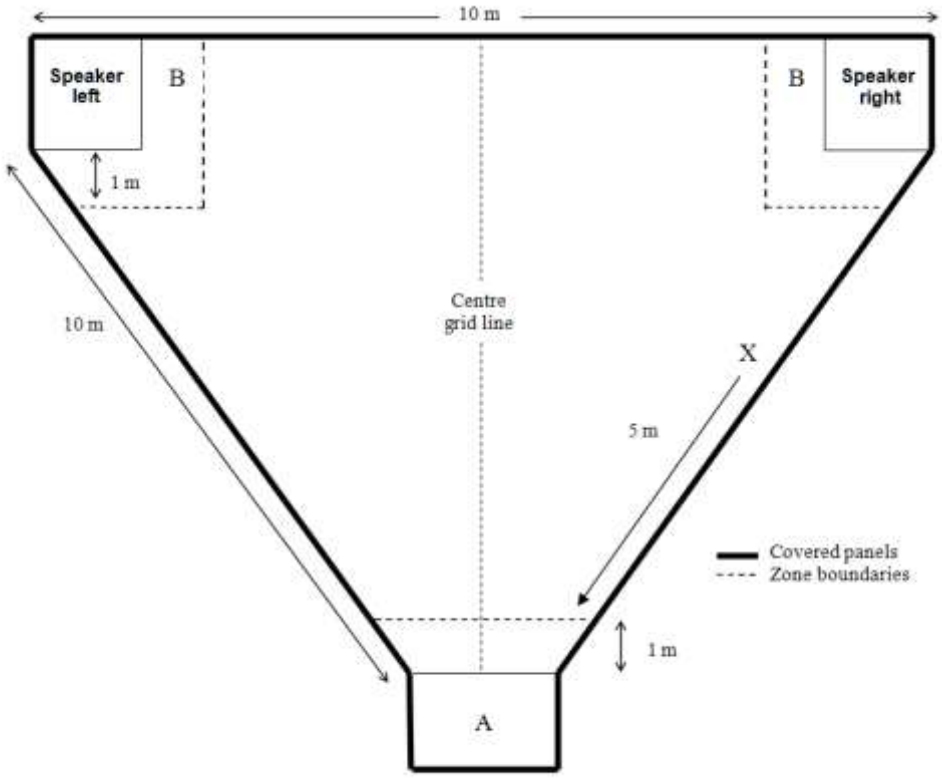


Figure 6.1: Schematic representation of test arena used for two-choice preference test of ewe response to lamb bleat stimuli. Zone A: starting pen to restrain ewe; Zones B: contact zones for each stimulus pen; position X: placement of lamb for return to ewe test.

6.2.6 Acoustic analysis

The 4 signals from each lamb used as playback stimuli were analysed using Praat linguistic software. Vocalisations were visualised as broadband spectrograms in Praat as shown in Figure 6.2 and the method of acoustic analysis was performed as described in Chapter 5, Section 5.2.3 pp 108-111. The segment length of the section analysed (where *F*0 and amplitude intensity were stable) for this trial was standardised at 0.5 s, a longer segment than described in Chapter 5, because all signals selected tended to be longer in duration as they were bleats consecutive to the initial vocal response. The same measurements were collected on the 4 signals of each animal as previously described (Table 5.2, Chapter 5 pp 111).

For purposes of comparisons, latency to the first vocal response (as described in the previous section, and measured previously in all Chapters), was not listed as an acoustic variable but as a vigour-related behaviour.

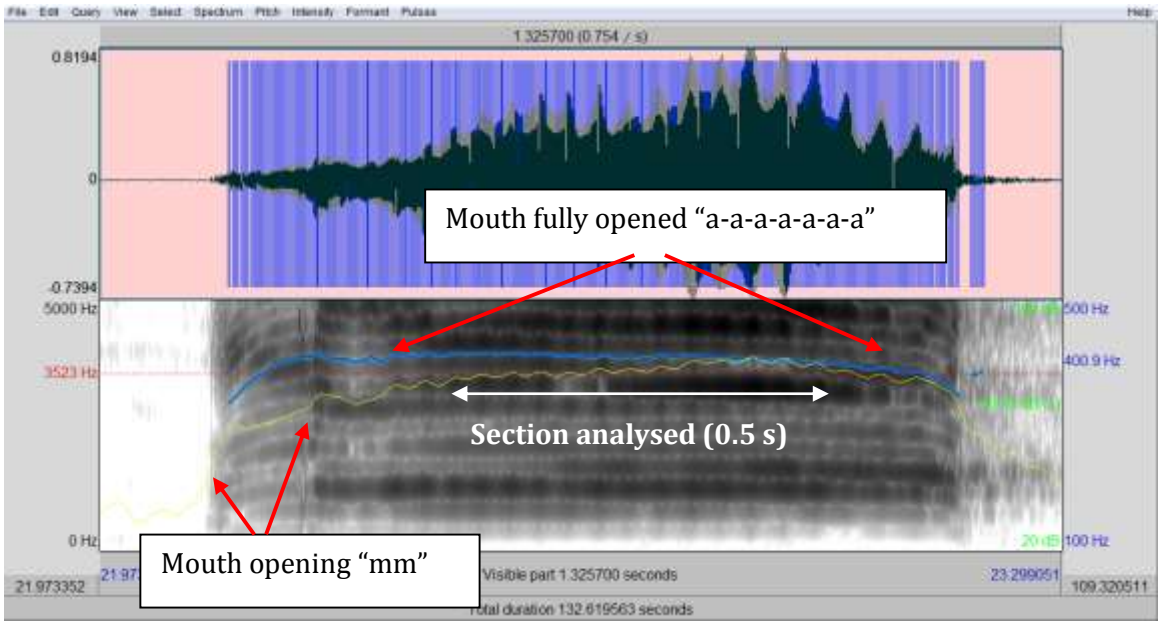


Figure 6.2: Praat spectrogram of lamb bleat showing vocal tract opening and vocal cord vibration and mid-section analysed. Pitch tracking line in blue, intensity in yellow. Formants (not analysed) are the darker shade bands of energy.

6.2.7 Statistical analysis

To calculate the preference towards one of the lamb playbacks, the proportion of time spent in each playback contact zone was calculated by the formula *Time in contact zone (%)= (time in contact zone A/B x100) ÷ (total time in both contact zones)*. Strength of response was also assessed by the proportion of time spent seeking the lamb (looking and trying to get into each stimulus pen) and ewe vocalisation response to each stimulus. Proportion of time spent seeking was calculated as *Time spent seeking (%)= (time spent looking into stimulus pen A/B x100) ÷ (total time spent looking into either stimulus pen)*. Non parametric statistical tests were used to analyse the two choice test results because distribution of data was non-normal. Evidence of positive discrimination against the probability of a random choice (50:50 side selection) for the proportion of time spent by each ewe in each stimulus contact zone and choice of starting gate exit side was compared in a binomial proportional test. Fisher exact probability tests were used to compare all other between-group proportions and Wilcoxon tests (paired and unpaired) were used for comparison of continuous variables within groups.

A linear mixed effect model *lme* (Pinheiro et al., 2011) was used to analyse the relationship between acoustic variables and ewe preference, and associated variance components. Ewe preference was entered into the model as a fixed effect, and a random effects hierarchical term with each of the 4 stimuli bleats nested within lamb identity, nested within ewe identity was used to identify between-signal (within-lamb), within-litter and between-litter variance. The effect of birth weight and sex were initially included in models but were removed as they were not significant. Cox PHM (*survival* package, Therneau and Grambsch, 2000) was used to investigate the relationship between latency variables and ewe preference, and data are presented as Kaplan-Meier distribution values. Linear relationships between acoustic variables and lamb vigour-related measurements (latency to bleat, stand and return to the ewe, and rectal temperature) were assessed by use of Pearson's correlation coefficient. Latency data and jitter were log transformed to normalise data for these comparisons.

Fisher's *F* test was used to test for homogeneity of variance of acoustic parameters related to preference and data was log transformed where required. P values for both linear and non parametric models were obtained by ANOVA, and Q-Q and model residual plots were inspected to ensure normality. All tests were carried out using R statistical package

(version 3.1.1, R Core Team, Vienna, Austria). Results are presented as median, 25-75th quantile, and range; or LSmean ±SE as specified. The proportion of variance attributed to between- and within-lamb and litter effects are expressed as percentages. To plot discrimination between co-twins based on acoustic parameters for individual ewes, a two-tailed student's *t* test was used to determine if significant differences existed between the 4 playback stimulus signals of each co-twin.

6.3 Results

6.3.1 Maternal preference within a litter

Test arena behaviour

All ewes demonstrated agitated behaviour while separated from their lambs in the starting pen (zone A), but began responding with vocalisations as soon as the pretest bleat-stimuli sequence commenced. Once the starting gate was raised, each ewe left the waiting pen immediately. Three ewes left the exit pen from the middle, and an equal proportion of ewes were orientated to the side where they last heard a pretest stimulus (n=5) or first heard a stimulus (n=5). There did not appear to be any significant exit bias associated with preferred lamb recording (Fisher exact probability $p>0.5$), or the last heard stimuli (binomial test $p>0.50$).

Of the 13 ewes used to compare acoustic preference between co-twins, the majority (n=11) demonstrated a clear preference towards the recording of one lamb as indicated by the proportion of time spent in the contact zone of the preferred stimulus pen (>80% of the time, Figure 6.3) and the time spent seeking (actively attempting to get into or look into) the lamb at the preferred stimulus pen. Ten ewes spent time seeking at the preferred stimulus pen only, and one ewe spent time seeking at both pens, but spent 88% of seeking time at the preferred stimulus pen. Two ewes did not show a clear preference with the proportion of time spent in each contact zone not differing from a random choice (11 vs 14 s and 9 vs 15 s, binomial tests $p>0.50$) so these animals were excluded from analysis related to preference (data discussed in next section). The proportion of ewes going to the preferred stimulus contact zone first following release was significant (10/11 ewes, Fisher exact probability $p=0.0001$). The proportion of ewes which preferred a recording based

on the side of the test arena did not differ from random (4 vs 7, Fisher exact probability $p>0.10$). The last heard pretest stimulus did not appear to affect preference for lamb recordings (7/11 ewes heard the pretest stimulus of the preferred lamb last, Fisher exact probability $p>0.30$), nor the time spent in the contact zone of each stimulus pen (Wilcoxon test: n=13, W=63, $p>0.20$).

Preference associated with signal acoustic variables

Mean values of acoustic variables associated with preferred and non preferred lamb recordings are shown in Table 6.2. In addition the orientation of co-twin preference by each ewe is shown in Figure 6.3, where statistical difference between acoustic parameters within a litter is indicated. There were significant differences related to preference of distress signals in frequency and amplitude variables. Ewes demonstrated a preference towards the signals of lambs with higher *F*0, maximum intensity and HNR; and lower modulation of amplitude and frequency (refer to Table 6.2). However at intensity values of >70 dB, preference of signals did not appear to be associated with higher mean amplitude as ewes did not prefer recordings with the loudest signals, and 64% of ewes (n=7) exhibited preference for non-statistically different, or less loud, stimuli (see Figure 6.3). Mean signal duration (DUR) was longer (0.835±0.06 vs 0.688±0.09, $p>0.10$) and proportion of mouth closure (MCLOSE) was less (12.76% vs 15.15%, $p>0.10$) in preferred recordings but these differences were also not significant.

The signals of the lambs where ewes did not show a clear preference demonstrated relatively little difference between all acoustic measures except *F*0. Vocalisation latency measures for these lambs were all <2.3 s; signal intensity and HNR values were high; and jitter and shimmer values were low. The lambs of one ewe had signal *F*0 values over 350 Hz; and the lambs of the other ewe had maximum signal *F*0 values under 340 Hz (mean *F*0: Lamb A=335 Hz, Lamb B=260 Hz).

Preference associated with lamb physiological variables

Lamb weights ranged from 3.3-5.2 kg, and the overall male:female ratio was 1:1. There was no preference towards co-twin signals associated with birth order (5 first born vs 6 second born, Fisher exact probability $p=1.00$) or birth weight (Wilcoxon test: n=11, V=43, $p=0.37$). Preference associated with sex could not be determined as only 3 of the

11 litters were of mixed gender. Median values and range for vigour-related measures are shown in Table 6.3. Lamb behavioural latencies were significantly shorter in preferred lambs for vocalisation response (median latency:1.60 vs 2.60 s, χ^2=5.6, df=1, p<0.05) but not for return to the ewe or time to stand latencies (p>0.10). However the maximum latencies for time to stand and return to the ewe recorded were associated with non preferred lambs. Three lambs from different litters (IDs 1, 2 and 3; Figures 6.3 and 6.4) had significantly delayed vocalisation responses (latency >10 s) and these animals were all non preferred by their dam. Two of these animals (latency >12 s) also recorded stand and return latencies higher than the 75th percentile of non preferred lambs (refer to Table 6.3), and the largest rectal temperature difference between lambs within a litter. There was no effect of birth weight or sex on any of the vigour-related measures. Preference associated with higher rectal temperature was only evident in litters with a rectal temperature difference between co-twins of >0.1°C (Wilcoxon test: n=5, V=20, p=0.02) (Figure 6.3).

Table 6.2: Comparison of acoustic parameters of lamb distress vocalisations associated with maternal preference within a litter. Lambs aged 3 hours old. Data shown as LSmean±SE [ab] and range.

Acoustic parameters	Preferred co-twin signals (n=11) mean±SE	range	Non preferred co-twin signals (n=11) mean±SE	range	*p* value
F0Max (Hz)	376.4 ± 13.14	[329.4 - 438.0]	341.5 ± 13.27	[258.8 - 434.1]	0.07
F0Med (Hz)	367.2 ± 12.93	[319.9 - 424.7]	338.0 ± 12.60	[248.3 - 424.2]	0.04
INTmax (dB)	81.06 ± 2.54	[67.35 - 88.96]	74.86 ± 3.75	[54.70 - 85.97]	0.05
INTmean (dB)	78.23 ± 2.73	[64.05 - 86.87]	71.72 ± 3.10	[50.20 - 83.82]	0.06
Jitter (%) [b]	0.23 (-0.45 ± 0.26)	[0.07 - 0.45]	0.48 (-0.74 ± 0.33)	[0.17 - 8.02]	0.06
Shimmer (%) [b]	4.28 (1.45 ± 0.14)	[2.50 - 8.85]	6.80 (1.93 ± 0.17)	[3.17 - 15.45]	0.02
HNR	16.41 ± 1.41	[7.87 - 24.13]	12.03 ± 1.83	[2.51 - 19.70]	0.04
DUR (s)	0.835 ± 0.06	[0.674 - 1.315]	0.688 ± 0.09	[0.298 - 1.042]	NS
MCLOSE (%) [b]	12.76 (2.55 ± 0.17)	[3.7 – 47.5]	15.15 (2.72±0.18)	[3.3 – 68.1]	NS

[a] n= 4 stimulus signals nested within lamb identity nested within ewe identity.
[b] Data were log transformed to normalise distribution, transformed means shown in parenthesis.

Table 6.3: Vigour-related measures of co-twins within a litter shown as median (25–75th percentile), and range.

Vigour-related measures	Preferred co-twin [a] (n=11) median	range	Non preferred co-twin [a] (n=11) median	range	*p* value
Latency to vocalise (s)	1.60 (0.69-2.37)	[0.40-2.51]	2.60 (0.90-10.70)	[0.08-38.06]	0.02
Latency to stand (s) [b]	2.31 (1.73-4.90)	[1.04-16.82]	3.50 (2.06-17.43)	[1.31-19.82]	NS
Latency to return (s) [b]	12.81 (10.80-13.90)	[8.90-15.86]	11.70 (11.5-20.83)	[9.05-192]	NS
Rectal temperature (°C) [c]	39.9 (na)	[39.5-40.5]	39.6 (na)	[39.5-39.9]	NS

[a] Preference based on recorded distress vocalisation signals of each co-twin.
[b] n= 10, [c] n=9, na = Insufficient data. NS = Not significant.

6.3.2 Maternal preference for vocalisation type

Ten ewes with singleton, and two ewes with twin lambs, were used to compare ewe response to nasally-emitted (MCLOSE=100%) and tonal (mouth-open) stimuli. Two of the 10 singleton ewes engaged solely in attempted escape behaviour from the test arena and did not go near the stimulus pen contact zones so were discarded from discrimination data. Two other singleton ewes did not show a clear preference (17 vs 18 s and 6 vs 9 s, binomial tests: $p>0.50$) although both animals spent slightly more time at the pen corresponding to the side of the last stimulus sequence. The last heard stimuli did not appear to influence the time spent by singleton ewes at each stimulus pen (Wilcoxon test: n=8, V=114, $p>0.90$), but did influence the amount of time spent at the first pen reached (Wilcoxon test: n=8, W=2, $p=0.05$). Where there was a clear preference toward recordings (ewes spent 100% of time in contact zone), 3/4 singleton ewes preferred the tonal distress stimulus although this preference, nor time spent in the tonal contact zone, was not significant (binomial test: n=4, $p>0.30$; Wilcoxon test: n=4, V=7, $p>0.20$). Time spent at the tonal stimuli pen was also not significant across ewes showing a less obvious preference (<100% of time in contact zone) (Wilcoxon test: n=4, V=2, $p>0.70$).

Both twin bearing ewes displayed less interest in the nasally-emitted signal stimuli, making no attempt to seek the lamb emitting this signal, although one of the ewes spent a small amount of time (<5 s) in the nasal stimuli contact zone. In both cases there was a

preference by the ewe towards the tonal distress signal, with ewes spending significantly more time in the contact zone of the tonal stimuli (>85%; binomial test p=0.0005). Vocal response rates of singleton and twin ewes to the pretest stimuli indicated that ewes responded more frequently to tonal, open-mouthed distress signals (mean response rate tonal stimuli 90.0% vs 67.5% nasal stimuli, Wilcoxon test, n= 0, V=0 , p=0.005).

6.3.3 Correlation of lamb acoustic characteristics with vigour-related measures

Latency to vocalise was strongly correlated with the time it took the lamb to stand in the test area (r=0.65, p<0.0001) and negatively correlated with amplitude (r= -0.47, p=0.02). There was also a weak relationship of vocalisation initiation with log jitter (r=0.37, p=0.07) (Table 6.4). Latency to stand also tended to have similar associations. Latency to return to the ewe was not correlated with any of the acoustic parameters or latency to vocalise, and was only correlated with latency to stand (r=0.42, p=0.05). Birth weight and rectal temperature were not correlated with any of the acoustic measures in this trial.

Table 6.4: Correlation of acoustic measures with lamb vigour-related measures (latency to vocalise and stand). Bold type indicates significant (p<0.05) and marginal (p<0.09) correlations.

	Latency to vocalise log(s) (n=26) [a]		Latency to stand log(s) (n=24) [a]	
	r	p value	*r*	p value
Latency to vocalise (s)	-	-	**0.65**	**<0.0001**
*F*0Max (Hz)	-0.10	NS	-0.05	NS
*F*0Med (Hz)	-0.12	NS	-0.07	NS
INTmax (dB)	**-0.47**	**0.02**	**-0.38**	**0.07**
INTmean (dB)	**-0.47**	**0.02**	**-0.35**	**0.09**
Log Jitter (%)	**0.37**	**0.07**	**0.37**	**0.07**
Shimmer (%)	0.28	NS	0.28	NS
HNR	-0.25	NS	-0.24	NS
DUR (s)	-0.32	NS	-0.35	0.10
MCLOSE (%)	0.30	NS	0.12	NS

[a] Data also includes litters of ewes not demonstrating a clear preference (n=2).

Ewe ID	1	2	3	4	5	6	7	8	9	10	11
Time in contact zone of preferred lamb (%)	100	84	86	100	100	100	100	100	86	82	100
Sex (preferred/ less preferred)	m/m	m/f	f/f	f/f	m/m	m/f	m/f	m/m	m/m	f/f	f/f
Higher rectal temperature	●	na	●	•	na	●	•	•	●	●	na
Shorter vocalisation latency (s)	●	●	●	●	•	•	•	•	•	•	•
Acoustic variables											
Higher *F*0Max (Hz)	●	•	●	○	•	○	●	●	●	•	●
Higher *F*0Med (Hz)	●	•	●	•	•	○	●	●	●	•	●
Higher INTmax (dB)	●	●	●	•	•	•	○	●	●	●	•
Higher INTmean (dB)	●	●	●	•	•	•	○	•	•	●	•
Lower jitter (%)	●	●	●	•	●	•	•	●	○	●	●
Lower shimmer (%)	●	●	●	•	●	•	•	●	○	●	•
Higher HNR	●	●	●	•	•	•	•	●	○	●	●
Longer duration (s)	•	●	●	•	•	○	•	●	•	●	•
Smaller MCLOSE (%)	●	●	•	•	•	○	•	●	○	●	•

Figure 6.3: Ewe preference [a] associated with acoustic and vigour-related variables within a litter (n=11).
Preferred lamb = ●; Non preferred lamb = ○; No significant difference [b] = •.
[a] Preference based on recorded distress signals of each co-twin.
[b] Student t-test $p>0.05$, n=4 distress signals/lamb.
na = Not available.

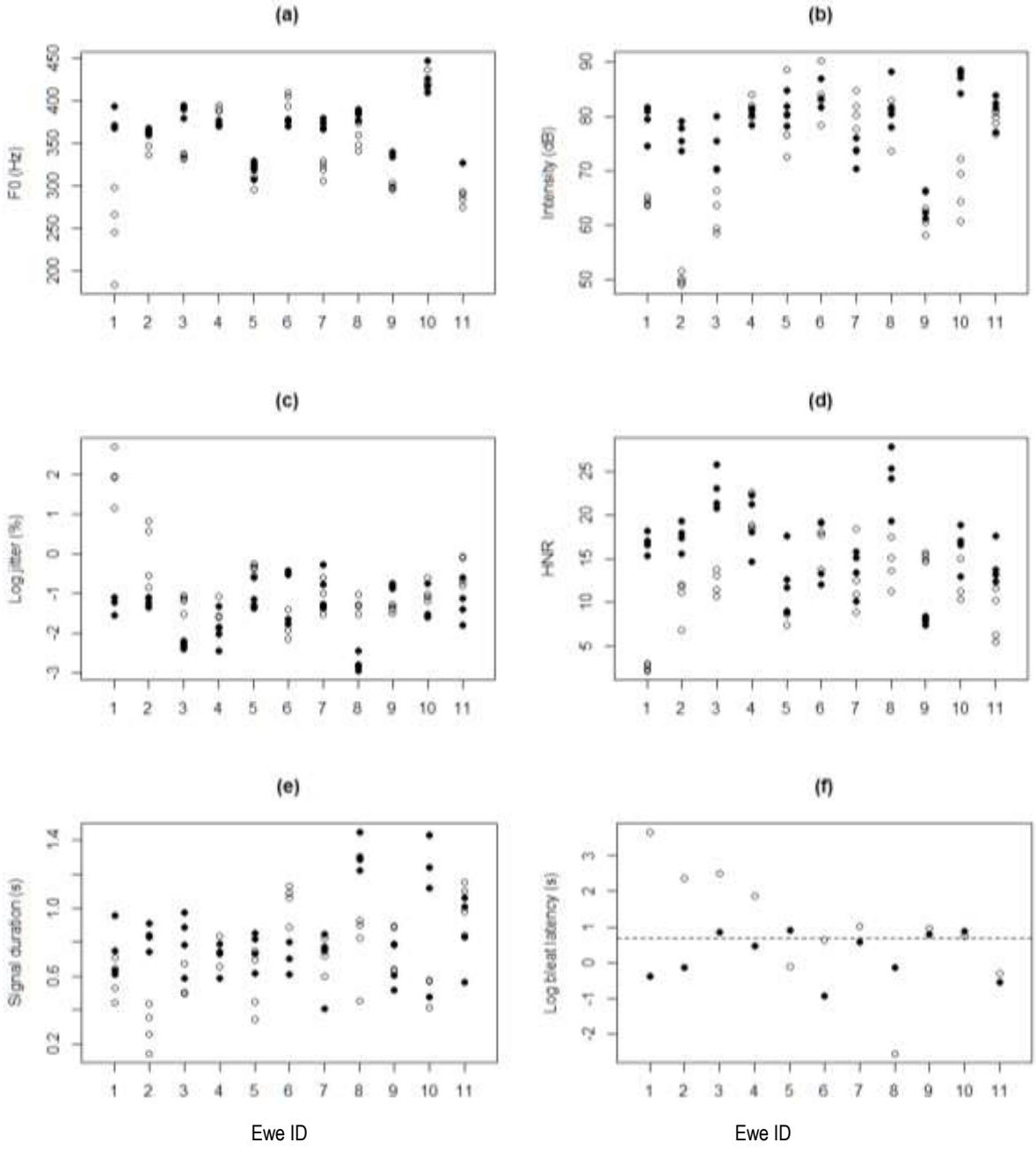


Figure 6.4: Difference in lamb acoustic [a] and signal latency measures, between and within litters (n=11). (a) Median *F*0, (b) Mean Intensity, (c) Log jitter, (d) HNR, (e) Signal duration and (f) Log vocalisation latency (- - - data <log 0.69=<2 s).
Preferred lamb signals = ●; Non preferred lamb signals = ○.
[a] Data is n=4 signals/ lamb.

6.3.4 Within and between-litter variance components

The proportion of variation contributed by differences in acoustic parameters between the selected stimuli signals, lambs and litters is shown in Table 6.5, and can be visualised in Figure 6.4 (for some variables only). Differences in *F*0 and amplitude intensity measurements between the 4 stimulus bleats of individual lambs accounted for less than 10% of the random variation. A relatively higher proportion of variability was evident between lamb signals for amplitude perturbation parameters (shimmer: 31.6%; HNR: 20.5%). Temporal measurements were also variable within vocal signals of lambs (28-40% of variance). However, by comparison the greatest source of variation was demonstrated between lambs within a litter for all acoustic measures (>57% depending on acoustic parameter). *F*0 and amplitude measures contributed the least proportion of variation between litters. The proportion of variance associated with vocalisation latency was primarily associated with lamb difference with a litter (98.68%, based on one vocalisation per lamb). Data was not analysed for variance associated with repeated vocalisation latency or rectal temperature measures between each lamb.

Table 6.5: Variance of acoustic and vigour-related parameters between the distress signals of each lamb (n=4); between different lambs within a litter (n=2); and between litters (n=11).

	Proportion of variation (%)			
Variable	Between bleats of each lamb	Between lambs within a litter	Between litters	Residual
*F*0Max (Hz)	3.39	57.63	37.04	1.94
*F*0Med (Hz)	9.37	59.05	27.98	3.60
INTmax (dB)	6.69	65.05	25.31	2.95
INTmean(dB)	7.97	68.15	20.59	3.28
Log jitter (%	13.08	78.71	4.68	3.52
Log shimmer (%)	31.55	59.82	3.32	5.32
HNR	20.52	79.37	<0.01	0.11
DUR (s)	39.36	60.57	<0.01	0.07
MCLOSE %	27.59	44.91	20.99	6.51
Bleat latency	na	98.68	0.17	1.15
Rectal temp	na	99.99	<0.005	<0.005

na = Not available.

6.4 Discussion

The aim of the experiment reported here was to test the influence of acoustic parameters on maternal preference towards lambs, using the difference between non manipulated distress call signatures of lambs within a litter as stimuli for choice tests. The results indicated that ewes clearly responded to these acoustic differences and exhibited a subsequent preference based on these cues. In cases where a demonstrated difference between co-twin recordings was evident ewes showed a greater response towards, or preferred the signals of, lambs associated with greater tonality and reduced signal perturbation. These signal parameters are known to be the most effective for signal transmission over distance and to maximise probability of detection (Endler, 1992; Ryan & Kime, 2003). There was also a preference favouring higher fundamental frequency (*F*0), which would appear to confirm the results reported in Chapter 5, i.e. that high *F*0 in the ovine neonate model may not be one of the prime acoustic indicators of pathology as it is in the human model (Corwin et al., 1996). Preference was also associated with faster vocalisation responsiveness, although ewes were not specifically tested for their response to this measure during this experiment. This is not surprising, however, given that the results of the previous experiment (Chapter 5) have indicated that the same acoustic parameters associated with preference in the current trial were correlated to vocalisation responsiveness.

Comparison of maternal response to altered acoustic parameters or interspecies vocalisations have shown that deer mothers respond to infant distress signals with a *F*0 remaining within the species-specific range of deer (Lingle & Riede, 2014; Teichroeb et al., 2013), as has also been reported in rodents - especially if given a choice between pups (Ehret, 2005; Ehret & Haack, 1982; Ehret & Haack, 1984). If sheep dams have a similarly specific "frequency response range" (Teichroeb et al., 2013) it is possible that some of the non-preferred lambs in this study did not meet this criteria. Previous findings demonstrated that the lowest *F*0 measure of vocally responsive lambs was 308 Hz (302 Hz for delayed response lambs - see Chapter 5), which is similar to the lower limit of frequency response range for white tailed deer (300 Hz), a species of comparable weight for age as an ovine neonate - approximate birth weight 3-4 kg (Lingle et al., 2007). A total of 4 non preferred lambs in the current trial had median *F*0 measures close to or below 300 Hz, including one co-twin which belonged to a ewe demonstrating lack of

clear preference. Other acoustic traits, or combination of traits, may also be required to elicit optimal maternal response as shown by deer mothers who do not respond to signals with the same *F*0 but different structure (Lingle & Riede, 2014). Duration of the signal may also play a role in the degree of maternal response in deer (Lingle & Riede, 2014) but the results reported here with a small number of animals did not clearly indicate this, nor was reported as a preference-related feature in a rodent maternal preference study (Cox Lippard et al., 2015).

In rodents, acoustic cues relating to maternal response have been studied in greater detail than in any other species (Ehret & Haack, 1982; Zeskind et al., 2011), and a number of acoustic parameters including repetition rate, sound pressure level, bout structure and frequency modulation are assumed to be important for maternal arousal control (Ehret, 2005). Signal duration, as well as arousal level, *F*0, and vocalisation rate have been shown to be highly variable in rodent studies related to age and environmental conditions (Ehret, 2005; Hahn & Lavooy, 2005). Nonetheless, the considerable disparity between ultrasonic vocalisation and ungulate signal form, including identification of different types of context related calls, make it difficult to directly compare species. However, few rodent studies have investigated the degree to which maternal response may be influenced by deficient infant neurobehavioural responses and associated vocalisation parameters. In a two-choice test using alien pups exposed to prenatal cocaine, rat mothers also treated with cocaine were found to exhibit more preference-behaviours toward untreated pups that had significantly higher vocalisation rates than cocaine-exposed pups (Cox Lippard et al., 2015). Untreated mothers did not show a difference in pup preference associated with vocalisation rate and pup treatment, which was possibly confounded by a lack of somatosensory stimuli necessary for rat nesting behaviour as dams could not touch the pups (Cox Lippard et al., 2015; Stern, 1997). Other acoustic parameters including *F*0, amplitude, and signal duration measures were not reported to be related to dam preference in that study, and differences were only shown on the first postnatal day (Cox Lippard et al., 2015). A difference between the rodent preference test and our study was the use of own progeny for stimuli in a species proposed to have an earlier discriminative ability (Nowak et al. 2000), which may have stimulated greater maternal response in the animals of this study. The use of recorded signals for the stimuli in the current study would also have eliminated any confounds associated with infant response behaviour or other sensory variables.

During this experiment, lamb vocal stimuli during the test were played back at the same rate in the current trial for both choice options, so it was assumed that ewes were responding to signal acoustic characteristics rather than signalling traits such as bout rate. However, given that the lambs had been separated from their mothers once previously for the recording session, it is possible that ewes had recalled the rate of vocalisation of each lamb and were associating each signal with intensity of bleating expressed by each lamb at that time. If this was a factor, it would imply that ewes are making preference judgments about lamb fitness based on temporal rather than, or in addition to, spectral features such as vocalisation rate and response signal latency early in the postnatal period. These traits are highly correlated in both the ovine neonate (demonstrated in Chapter 5) and human and rodent neonates (Corwin et al., 1996; Lester & Boukydis, 1992; Venerosi et al., 2009; Wellmann et al., 2010), but also with other acoustic measures (Lingle & Riede, 2014) as was shown in Chapter 5. In rodents, high pup vocalisation rates have been shown to be more effective in eliciting maternal response than low rates (Brunelli, Shair, & Hofer, 1994; Cox Lippard et al., 2015; Farrell & Alberts, 2002; Zimmerberg, Kim, Davidson, & Rosenthal, 2003) and in the ewe, maternal response to vocalisation rate of rapidly bleating mothered lambs is similarly reported to be more intense (Nowak, 1990b).

Ewes may also have been associating acoustic variables with physical attributes of the lamb and basing preference of animals on physical fitness as has been shown in rodent mothers (Deviterne, Desor, & Krafft, 1990). In the current study the physical attributes of lambs, apart from vocalisation responsiveness, did not appear to influence preference as ewes did not base their preference on birth weight or vigour-related behaviours. Mean latency to stand was not significantly shorter in preferred lambs, although interestingly one co-twin with slow standing latency due to a leg injury handicap, who responded quickly vocally was the preferred animal. It would also be difficult to envisage ewes in the current study discriminating lambs on following behaviour as they had not been placed in a position to observe this; and as primiparous ewes they would also not be basing judgement of behavioural or other cues on previous experience (Dwyer & Lawrence, 1998b; Dwyer & Lawrence, 2005b).

Amplitude was not adjusted in the co-twin choice test, and ewes did not always prefer the signal sequence with higher mean or maximum intensity, with half of the litters demonstrating a lack of statistical difference in signal intensity between co-twin

vocalisations used as stimuli. There was no evidence that ewe hearing was compromised, as they responded to both stimulus signals during the pretest and usually looked towards and explored both stimulus environs during trials. Confounds associated with test arena side or stimulus sequence bias were also not apparent, and the significantly higher proportion of time, both spent near each zone and involved in seeking behaviours, did appear to capture distinct preferences of each dam. A difference in maternal tendency could also be considered as a factor associated with the degree of maternal response (Bickell et al., 2009; Nowak & Poindron, 2006; Rosenblatt et al., 1985), but it was not an obvious confound in these results, nor expected with the breed of ewe used in the trial. By disregarding data where lack of distinct preference was evident, and using a maternal breed reported to demonstrate stronger maternal behaviour than Merino ewes (Nowak & Poindron, 2006), it was hoped that differences associated with varying maternal response levels would be limited.

Single-bearing dams given a choice of signal from their own lamb appeared to have more difficulty making a clear choice regarding signal preference. A greater number of ewes demonstrated escape and non-preference behaviours in the singleton signal-type test (moving to the alternate stimulus pen during gaps in stimuli playback). It was highly probable that when the ewes reached a stimulus more recently heard or localised, which they recognised as belonging to their lamb, they would spend considerable time in this location before searching elsewhere. This was indicated by the fact that singleton ewes were more likely to spend a greater period of time at the first stimulus pen reached, especially if this signal was the last heard in the pretest sequence, independent of signal type. Additionally, maternal response has been reported to vary in response to littersize and associated acoustic cues (Elwood & Broom, 1978) although how this might impact on sheep dams is unknown.

The results of the singleton ewe response suggest that the ewes in this experiment may have been able to recognise both nasal as well as tonal vocalisations of their own lamb, because they remained in the contact zone of the nasal signal rather than move away immediately to the tonal signal. While most studies have identified that ewes recognise the open-mouthed bleats of their own lamb at older ages (Poindron & Carrick, 1976; Searby & Jouventin, 2003; Shillito Walser, 1980; Shillito Walser, Hague, & E., 1981) none have demonstrated recognition associated with nasally-emitted vocalisation at this

early stage postpartum. In the current study only auditory senses would have been employed by the ewes so this corroborates other research stressing the importance of auditory cues (Keller et al., 2003) at an even earlier age. Ewes are known to respond to signals given by other lambs (Levy, Gervais, Kindermann, Orgeur, & Piketty, 1990; Shillito Walser et al., 1981; Shillito Walser et al., 1982; Shillito Walser et al., 1983) as do rodent dams and deer mothers (Cox Lippard et al., 2015; Ehret, 2005; Lingle & Riede, 2014), so the results reported here are not demonstrating discrimination of own lamb among alien lamb signals *per se*. While the preference behaviour and proportion of time spent by singleton mothers near each stimulus did not reach statistical significance, possibly due to the confounds of dual signal stimuli from the same lamb or consequences of artificial amplitude adjustment, the results clearly indicated that tonal signals elicited a higher rate of vocal response from the ewe. This was particularly salient in the preference-behaviour of the twin-bearing ewes where co-twins were available for signal-type comparisons, although sample numbers were insufficient to be conclusive and further research in this area is warranted.

What is also of interest is the portioning of within- and between-litter variance related to differences in acoustic characteristics between co-twins in this study. It would be assumed that the greatest difference would be between litters and that acoustic signatures between closely-related co-twins would be highly similar, especially within same-sex litters (Debruyne, Decoster, Van Gijsel, & Vercammen, 2002; Przybyla, Horii, & Crawford, 1992; Van Lierde, Vinck, De Ley, Clement, & Van Cauwenberge). However the results of this study demonstrate that the greatest difference for all acoustic measures was between co-twins. While not able to determine monozygotic or dizygotic status of the co-twins in this trial, it is reported in human vocal twin studies that dizygotic twins are more genetically similar than non-related individuals (Debruyne et al., 2002; Przybyla et al., 1992). Little information is available regarding vocal signature difference in lamb neonates, but these findings suggest that the acoustic characteristics measured here, and in particular stress responsiveness indicated by distress vocalisation initiation, may not be associated with genetically linked temperament or behavioural traits. Distinctive characteristics involved in recognition of monozygotic/dizygotic twin lambs by ewes has similarly been proposed to be related to an interaction between genetic and environmental factors by Romeyer et al. (1993). The degree of variance associated with between-signal variation for each lamb indicates that the signals selected for the stimuli were relatively

uniform, especially in relation to *F*0, a measure commonly reported to remain consistent between intra-individual vocal signals in other species (Charrier, Mathevon, & Jouventin, 2003; Lenhardt, 1977; Maciej et al., 2011; Terrazas et al., 2003).

Conclusion

The data reported here indicate that ewes will demonstrate preference behaviour when they are given a choice between differentiated acoustic signals of their own lambs, if it is assumed that they are responding to signal characteristics rather than recall of associated attributes such as bleat rate, vocal responsiveness or other sensory stimuli. A particular frequency response range relevant to ovine maternal response may be one of the factors influencing maternal behaviour and arousal, promoting specific physiological and hormonal reactions (Rosenblatt et al., 1985; Stern, 1997; Wiesenfeld & Malatesta, 1982), although a suite of acoustic parameters is more likely to be involved. Lack of signal perturbation or glottal instability (reflected by preference toward high HNR, for example) may be a feature preferred also by sheep dams, and could reflect a discrimination of fitness by the ewe. It would appear that these results also corroborate the suggestion in Chapter 5 that lower *F*0 in the ovine species may be an indicator of abnormality, or a parameter associated with discernment of lamb attractiveness or fitness by ewes. Further validation of these results would provide valuable information regarding the critical role of acoustic stimuli in eliciting optimal maternal response, and the importance of lamb behaviour in facilitating acceptance by the dam. In particular, studies testing for the upper and lower limits of the frequency response range in sheep dams, as has been undertaken in deer, may confirm threshold levels associated with neurobehavioural deficit and rejection of lambs by ewes.

Chapter 7

Conclusions and future directions

A lack of significant improvement in lamb survival rates in Australia suggests that alternative approaches are required to facilitate adoption of strategies which utilise superior genetic material for both lamb and maternal-based traits. Identification of lambs with improved vigour and dams with better mothering capacity are potential areas where improvement in genetic selection may contribute towards reduced lamb mortality.

While an extensive body of research has already been undertaken on this topic, there are some areas in which information regarding maternal-young behavioural cues and physiological responses of the lamb itself remain unidentified or unknown. In particular the impact of auditory cues emitted by the lamb on the ewe, and early neonate behaviours associated with neurological deficit as a result of intrapartum processes, have not been investigated as a greater research focus has been directed toward the ewe and her role in maintaining a successful maternal-young attachment. Furthermore the significance and underlying physiological mechanisms of vocalisation behaviour as a marker for neurological status in the lamb has not been reported, although a number of studies have highlighted the high incidence and possible behavioural and survival consequences for neonate lambs associated with neurological damage occurring during birth.

In the human neonate, these very issues have been investigated in great detail. Physiological and behavioural indices determined by observational studies in the human neonate have been assessed by more invasive research in animal models to test effects on the fetus and the neonate. Both the sheep and rodent species have been commonly utilised for these purposes and these studies have provided valuable data in order to better understand human and nonhuman fetal/neonate physiology. In particular the effect of fetal distress and damage to the central nervous system occurring before and during birth are known to impact on vocalisation characteristics of the human and neonate rodent, but this has not been applied to the lamb or many other mammalian species.

The objectives of this thesis were therefore to investigate the possibility that characteristics of vocal signals or vocal behaviour may reflect neurological deficit or other viability indicators in the lamb neonate. This translational approach, where comparative neurophysiology is assumed because of common evolutionary origins, has been shown to be valid in studies of interspecies maternal response and neurological impact in rodent pups, using a separation stress stimulus to elicit distress vocalisations.

The results of each experiment reported in this thesis have consistently indicated that this model can also be applied to the lamb neonate. The neonate distress vocalisation, which functions as biological siren, can be elicited by pain or isolation depending on the species. In the *Ovis* neonate the separation of the lamb a short distance from the dam was able to elicit an immediate reaction indicated by a distress vocalisation, reflecting high arousal and innate awareness of separation and vulnerability. Latency related to emission of this signal was found to be delayed in some animals, and associated with factors known to be linked to poor lamb survival including longer and more difficult parturition; male sex; high birth weight or associated larger thoracic circumference; low rectal temperature; poor behavioural indices and biochemical markers indicative of fetal distress.

In the initial experiment (Chapter 3) it was hypothesised that an isolation distress stimulus could be applied to the neonate lamb to elicit vocalisation responsiveness, a measurable and simple behavioural indicator, which would also reflect other behavioural indices of vigour. This indicator appeared to be well correlated with behavioural markers reflecting capacity of following behaviour and viability. The timing of early lamb milestone behaviours utilised by some other researchers did not accurately reflect potential vigour differences in this study, possibly because lamb deficits were not varied enough to clearly delineate differences as has also been reported by other authors. This study also identified that first born co-twins were at greater risk of both delayed vocalisation and test arena behaviours, which was further validated in following twin testing procedure refinement (Chapter 4), suggesting the possibility that vocalisation latency could indicate subtle within litter differences in fetal distress. Furthermore this supposition was supported by measurement of significantly higher plasma glucose levels in first born co-twins.

In the subsequent experiments (Chapters 4 and 5), blood assay results indicative of fetal distress and hypoxemia were found to be correlated with vocalisation latency. These

experiments were designed to assess the relationship between vocalisation delay and hypoxic effects during birth by studying lambs of a breed cross associated with heavier lamb birth weight and greater risk of intrapartum fetal distress. It was found that larger lambs, of both Merino and Merino x White Suffolk cross weighing over 5 kg, demonstrated longer delays in vocalisation, lower blood oxygenation and higher levels of plasma glucose - markers known to indicate fetal distress in neonate lambs, humans and rodents. Variation between co-twins of the same litter further verified that male lambs were also at greater risk of fetal distress indicated by both higher plasma glucose and lactate levels.

Furthermore, application of acoustic methodology modelled on human neonate cry analysis revealed that acoustic and temporal parameters of the lamb distress vocalisation demonstrated features which could be compared to those shown by neurologically compromised human infants, in particular those associated with poor neural control of the vocal folds. Blood parameters associated with fetal distress were also correlated with these acoustic variables. These results were postulated to be associated with a number of causal agents including mild to severe hypoxic effects within the brain itself, or traumatic damage during birth to the laryngeal nerve. What was interesting was the finding that not only did some lambs exhibit delayed or even non-existent vocalisation responses, but that others emitted an entirely inadequate and inappropriate signal in the context of separation, implying poor cognitive processing in these animals.

The final experiment (Chapter 6) tested the effect of acoustic cues on maternal response by comparing maternal preference to acoustic signals emitted by their own lamb/s. Because of within-litter difference this study was successful in demonstrating that ewes do show a preference for certain traits of the signal which reflect efficient vocal cord functioning, such as higher amplitude and higher harmonic to noise ratio, which may also convey an impression of infant vigour. High-pitched signals, an indicator of pathology in the human neonate, appeared to be preferred by ewes in comparison to distress signals of a lower *F*0. The frequency response range of sheep dams has not previously been documented but these results also implied that *F*0 below 300 Hz may not elicit strong maternal response in ewes, and that sheep dams could have a lower threshold in their frequency response range similar to certain deer species.

Appropriate distress vocalisation behaviour, particularly in neonates of ungulate "follower" species, is such a critical survival trait it would appear that delayed vocalisation latency in neonate lambs almost certainly reflects poor cognitive capacity and vocalisation characteristics indicative of neurological insult as demonstrated in the human and rat neurobehavioural models. At the very least, an inability to elicit an immediate and positive maternal response is likely to result in compromised survival in young of precocious species dependent on effective maternal-young attachment and predator protection. These deficits may occur as a result of prenatal effects including fetal growth restriction; or intrapartum trauma leading to neurological insult associated with varying levels of brain oxygen deprivation. Following birth, and depending on the extent of this damage, regenerative processes within the rapidly developing neonate lamb brain would facilitate repair and normalisation of neurobehavioral function, as has been indicated in Chapter 3 by improvement of vocal responsiveness in less responsive animals over a 12 hour period. There may also be feral-type breeds of sheep exhibiting characteristics similar to "hider" species, like the Soay (Shillito Walser et al., 1984), in which vocalisation behaviour elicited by a separation stimulus may reflect somewhat different trends, but these breeds were not studied in this thesis.

The significance of these findings, as found in human studies, is that the acoustic and temporal characteristics of the neonate lamb distress signal may be one way in which the lamb contributes to failures in maternal-young interaction. While human studies have found that in a supportive environment the "frail" and "urgent" high pitched cry of at-risk infants may elicit caregiver behaviour facilitating recovery and extra care of such infants; in a non-supportive environment where maternal predisposition or resources may be limited, such signals are more likely to result in neglect or abandonment. In free ranging sheep, it is far more likely that the later would apply and in particular where a choice of signals, such as between progeny or within the flock, may be available.

Contributions of this research

A number of significant findings have emerged from the research reported in this dissertation. Firstly, the finding that vocalisation behaviour may reflect compromised neurobehavioural status in the lamb neonate suggests that this indicator may be a useful and practical tool for on-farm use in order to assess vigour and the incidence of sub-

clinical dystocia thus aiding genetic selection for breeding programs. An attempt to assess lamb survival risk by use of bleat scores has already been attempted in Australian field studies, however as recovery appears to be relatively rapid in the postpartum period, valid vocalisation comparisons should be undertaken at standardised time periods. As has been indicated by this study, only more severely impacted animals may be detected in periods later than 12 hours postpartum. The correct partitioning of birth order is also necessary to allow for within-litter difference which is difficult to determine in unobserved births in the field.

Vocalisation features such as latency and vocalisation rate could also be applied to APGAR-type assessments of viability and neurological status in other mammalian neonates, especially in settings where time of birth is known and where a stimulus can elicit a distress vocalisation. In other "follower" ungulate species where isolation is a stressful state a similar method as reported here could be applied, but in more vocally pain-responsive animals, such as piglets, a pain stimulus could be applied to identify at-risk individuals. Such assessment may also provide useful information to determine neurobehavioural status of neonate or young animals used in behavioural tests requiring standardised cognitive ability; or intensive production systems where at-risk infants need to be identified. This study also reports on lamb blood assay values which may be associated with subclinical or graded hypoxemia which have not previously been associated with neurological impact.

This research has important implications for understanding failed maternal-young interactions in ungulate and other species, and contributes to knowledge regarding the hitherto suspected, but non validated, importance of acoustic cues given by the infant. In particular, genetic selection of animals based on maternal behaviour exhibited in the postpartum period may be misdirected if the infant itself, for whatever reason, may be contributing to deficient bonding processes and behaviour. This thesis has demonstrated that overt signs and symptoms normally associated with dystocia may not be present in cognitively deficient animals; and an easily utilised marker of neurobehavioural deficit (as opposed to post mortem results or other behavioural tests), which could confirm the source of deficiency, may greatly assist breeding selection. In addition the phonetically based analysis of lamb vocalisation types and the reporting of the degree of oral opening and nasal resonance in lamb signals has relevance for measurement of mammalian

acoustic parameters. This information will add to the growing body of mammalian bioacoustic literature currently available.

Finally the implications of this research for phylogenetic studies and the field of human neonate medical research are substantial. While it has been hypothesised that commonality of vocal neurobiology exists among mammalian species, few studies have indicated this implicitly. Expressions of the potential for comparisons between species have previously been guarded because of different physiological mechanisms between the species currently studied, however the lamb neonate has a very similar mode of vocal production and acoustic characteristics (as well as body size, metabolic rate and other physical traits) to that of the human newborn. Such comparisons could contribute significantly to the study of prenatal effects on early neurobehavioural development.

Further directions

Any studies investigating the physiological status and behaviour of neonates associated with parturition are likely to be subject to huge variation because the maternal uterine, and genetic, environment of the fetus cannot be controlled or standardised. Most of the experiments conducted in this thesis were dependant on enough difference reflecting variation in fetal distress (especially for blood assay results); but because of available animals or other study constraints, animals clearly demonstrating hypoxic effects associated with intrapartum processes were limited. Some of the experiments reported in this thesis were conducted without other experimental precedent in the *Ovis* species, and time constraints and resources made it difficult to run larger pilot studies than those conducted, for example in the final trial (Chapter 6). Therefore, further validation of these results is required as the data and assumptions made in this dissertation are quite novel.

In particular, further studies to quantify asphyxia thresholds associated with vocalisation deficit in the *Ovis* neonate are needed - investigation of cerebral hypoxia models in the sheep neonate, similar to those based on measurement of rodent USV parameters, could also provide valuable modelling for human neonate disorder. Other biomarkers of fetal distress and brain injury (including glucocorticoids and their metabolites, S100B or Activin A) may also be useful in comparison of results but unless a greater variation in hypoxic-ischemic damage is evident, this data may not provide much additional

information. Studies where fetal distress could be deliberately controlled in ewes; in a temperature-regulated environment with access to immediate blood monitoring, as has conducted in many fetal lamb studies, could verify vocalisation associations with hypoxic brain injury more clearly. Modelling such trials on those already conducted in rodent species would be of value.

A number of studies applying other findings of this thesis could also be conducted and would provide additional confirmation of the results presented here. Further research to determine longitudinal and ontogenic-related vocalisation change associated with gender, developmental physiology and a number of causal factors is required. Investigation of the impact of traumatic birth processes on the functioning of the laryngeal nerve in the lamb neonate, for example, may clarify the difference in temporal and acoustic parameters identified in this study. Validation of results studying other breeds of sheep, including those reported to have different behavioural and vocalisation attributes in older lambs, should also be undertaken to investigate the degree of variability in responsiveness and acoustic measures of the early neonate. Any such comparisons should consider the conformational differences and degree of fetal distress risk associated with those breeds or bloodlines and the effects of birth order in twin studies.

More accurate determination of neonate age is required in the first day of life to enable standardised age-determined comparisons for practical field application under extensive systems. Previously determined age estimates based on lamb coat dampness, hoof status and activity may be unreliable if activity of the lamb is impacted by neurobehavioural and cognitive condition. Development of models for field studies which account for within-litter difference related to likelihood of greater neurobehavioural impacts on the first born could also be calculated to account for birth weight-related and developmental variability.

Finally, testing for thresholds of the sheep dam frequency (*F*0) response range, as has been conducted in deer, would determine the importance of this acoustic parameter and its relevance in facilitating optimal maternal response levels. Further clarification of the upper and lower range of pitch indicative of neurobehavioural deficit in the lamb is inherent in these determinations. It is envisaged that selection for genetically determined traits where ewes express a greater tolerance for a wider range of signal *F*0 or a capacity

to react more strongly to this, or other, acoustic signal parameter/s of the lamb may contribute to development of more gainful reproductive breeding objectives.

Other acoustic parameters not reported here could also be of relevance regarding lamb neonate cues involved in maternal-young communication, for example signalling of identify or other attributes in formant frequencies. This is a developing area of potential bioacoustic research where validation and standardisation of analytical frameworks is required. Exploration of the potential acoustic descriptors based on principals of human phonetics, as conducted in this study, is an area where these frameworks could be applied to relate acoustic and articulatory parameters of other mammalian signals more accurately.

In summary and at its simplest, vocalisation responsiveness reflects a propensity for arousal which can only be an advantageous attribute in the young of a highly mobile and gregarious "follower" species. As such, vocalisation rate, latency and signal characteristics which promote the highest state of arousal and associated maternal behaviour in the dam, who is biologically tuned to respond, are valid indicators of viability or survival potential in the neonate lamb. This thesis has identified the importance of this behavioural indicator, its association with neurological deficit and the need for further research to confirm and extend the documented results and conclusions.

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Appendix 1:

Tables of Cox PHM hazard ratios and latency to perform vocalisation, early and test arena behaviours by sire.

Table A1.1: Model hazard ratios for early behavioural latencies of singleton lambs by sire. Data are presented as hazard ratio [95% confidence interval] and median (25-75th percentile).

Variable	S1 [b] n=25	S2 n=25	S3 n=19	S4 n=25	S5 n=32	S6 n=25
First stand						
Hazard ratio	ref [b]	1.19 [0.66-2.13]	0.96 [0.51-1.80]	0.98 [0.55-1.72]	1.59 [0.91-2.77]	0.87 [0.48-1.57]
Median (min) [a]	35.0 (20.0-42.0)	22.50 (15.0-44.50)	29.00 (23.0-49.0)	22.00 (14.0-56.0)	18.00 (10.0-36.0)	33.00 (17.0-57.0)
Suckle						
Hazard ratio	ref [b]	1.38 [0.78-2.45]	0.93 [0.51-1.72]	1.07 [0.60-1.90]	1.47 [0.86-2.51]	1.11 [0.61-2.02]
Median (min) [a]	51.0 (36.0-85.0)	45.00 (32.0-77.0)	61.00 (34.0-106.0)	49.00 (36.0-79.0)	38.5 (30.5-59.5)	44.0 (36.0-68.0)

[a] Kaplan Meier estimate, unadjusted for sex and parity.
[b] Reference level for each sire comparison.
* $p<0.05$ significantly different to the reference sire.
** $p<0.01$
*** $p<0.001$
**** $p<0.0001$.

Table A1.2: Model hazard ratios for vocalisation and test arena return latencies of singleton lambs by sire. Data are presented as hazard ratio [95% confidence interval], median (25-75th percentile).

Variable	S1 [b] n=28	S2 n=30	S3 n=20	S4 n=29	S5 n=35	S6 n=27
Bleat 3 hrs [c]						
Hazard ratio	ref [b]	**2.26 [1.24-4.15] ****	1.52 [0.78-2.95]	1.40 [0.80-2.44]	1.51 [0.82-2.76]	0.81 [0.43-1.52]
Median (s) [a]	7.02 (1.27-16.44)	2.89 (0.75-6.40)	3.67 (1.49-10.06)	4.48 (2.12-8.60)	3.97 (1.65-8.0)	6.00 (2.72-39.4)
Bleat 4 hrs						
Hazard ratio	ref [b]	**1.64 [0.93-2.87]** [t]	1.44 [0.78-2.65]	1.38 [0.77-2.47]	1.44 [0.84-2.49]	1.03 [0.57-1.84]
Median (s) [a]	2.32 (1.19-4.75)	1.64 (1.02-2.94)	1.32 (0.83-4.69)	1.81 (0.80-3.97)	1.75 (0.80-3.35)	2.10 (1.25-4.96)
Bleat 8 hrs [c]						
Hazard ratio	ref [b]	**2.09 [1.14-3.82] ***	1.44 [0.75-2.77]	1.00 [0.59-1.68]	1.52 [0.88-2.61]	0.78 [0.44-1.35]
Median (s) [a]	2.31 (0.81-3.69)	1.13 (0.57-1.72	1.31 (0.83-2.17)	2.00 (1.56-3.69)	1.59 (0.71-2.66)	2.58 (1.53-6.53)
Bleat 12 hrs [d]						
Hazard ratio	ref [b]	**2.76 [1.56-4.89] *****	1.46 [0.78-2.72]	1.29 [0.73-2.29]	**1.58 [0.92-2.70]** [t]	1.12 [0.64-1.97]
Median (s) [a]	2.19 (1.03-4.83)	0.91 (0.69-1.88)	1.44 (0.75-3.17)	1.89 (0.93-3.88)	1.70 (0.91-3.03)	2.47 (1.10-3.05)
Return to ewe 4 hrs [a]						
Hazard ratio	ref [b]	**3.05 [1.42-6.56] ****	**2.37 [1.07-5.26] ***	**2.53 [1.16-5.52] ***	**2.40 [1.17-4.93] ***	**2.54 [1.20-5.38] ***
Median (s) [a]	114.00 (37.0-na)	24.00 (11.5-37)	26.00 (17.0-61)	19.00 (8.5-120.0)	25.00 (11.5-118.0)	30.00 (11.0-78.0)
Return to ewe 8 hrs [c]						
Hazard ratio	ref [b]	**3.19 [1.59-6.41] ****	**3.66 [1.71-7.80] *****	**2.15 [1.07-4.32] ***	**2.27 [1.16-4.42] ***	**2.21 [1.10-4.44] ***
Median (s) [a]	40.00 (18.0-na)	9.50 (4.0-42.0)	11.50 (5.0-21.0)	12.50 (6.0-56.0)	13.50 (6.0-5.48)	10.00 (5.0-60.0)
Return to ewe 12 hrs [d]						
Hazard ratio	ref [b]	**3.09 [1.55-6.17] ****	**2.21 [1.04-4.69] ***	**2.12 [1.07-4.18] ***	**2.03 [1.05-3.89] ***	1.23 [0.62-2.42]
Median (s) [a]	16.00 (11.5-55.5)	7.00 (3.0-15.0)	7.00 5.0-20.5)	8.50 (4.0-30.0)	8.00 (4.0-28.0)	25.00 (6.0-49.0)

[a] Kaplan Meier estimate, unadjusted for sex and parity. [b] Reference level for each sire comparison.

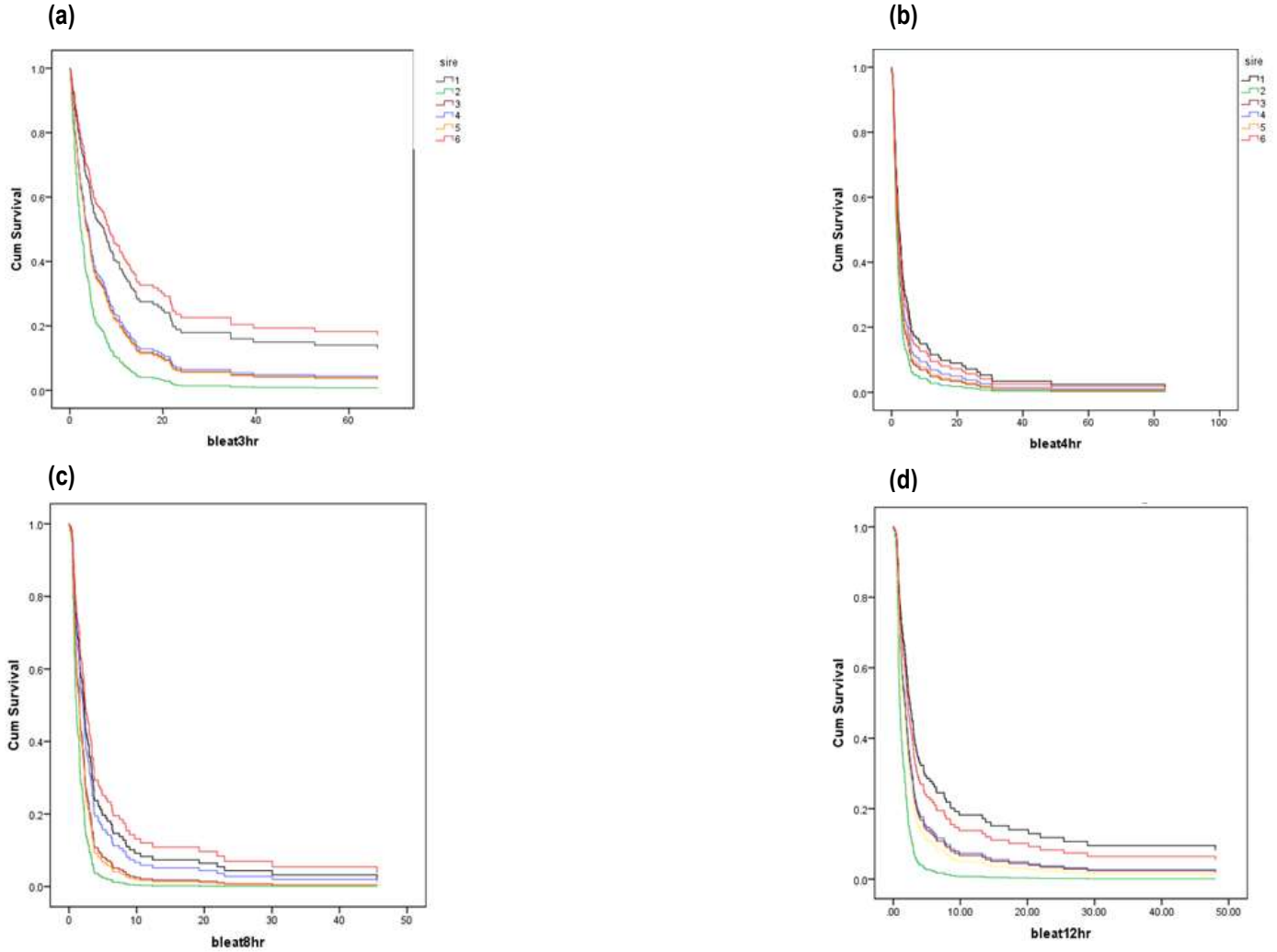
[c] $p<0.05$ significance of sire effect in model with sex and parity as strata, birth weight as a covariate.

[d] $p<0.01$ significance of sire effect in model with sex and parity as strata, birth weight as a covariate.

* $p<0.05$; **$p<0.01$; ***$p<0.001$; ****$p<0.0001$, [t] $p<0.08$ for significantly different to the reference sire.

Appendix 2:

Cox PHM survival distribution curves for vocalisation and early behavioural latencies.



Y-axis equates to proportion of lambs not initiating a response at each second.

X-axis is time from application of stimuli (seconds).

Figure A2.1: Survival distributions by sire for Cox PHM models of singleton lamb latency to vocalise at (a) 3 hours (b) 4 hours (c) 8 hours and (d) 12 hours postpartum. Black=S1 (reference) Green=S2, Maroon=S3, Blue=S4, Yellow=S5, Red=S6. Variables fitted include sex and parity.

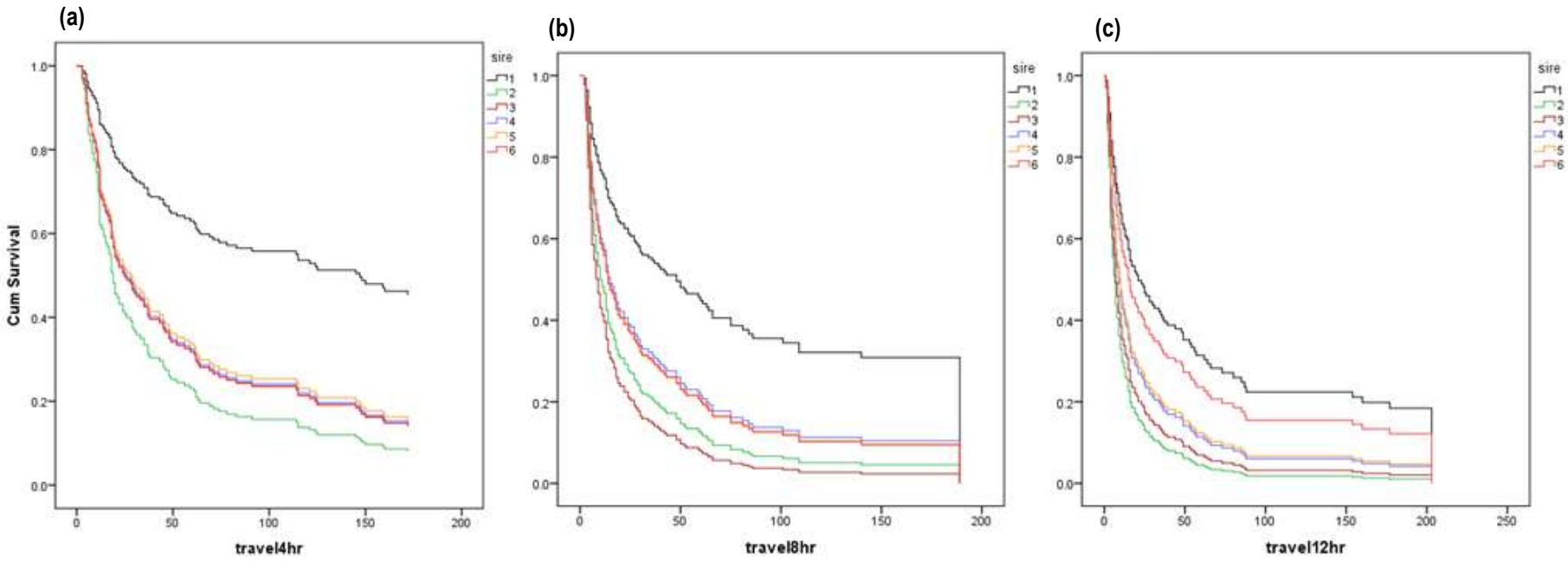


Figure A2.2: Survival distributions by sire for Cox PHM models of singleton lamb latency to return to ewe at (a) 4 hours (b) 8 hours and (c) 12 hours postpartum.
Black=S1 (reference), Green=S2, Maroon=S3, Blue=S4, Yellow=S5, Red=S6.
Variables fitted include sex and parity.
Y-axis equates to proportion of lambs not reaching the dam at each second.
X-axis is time to return to dam from standing position (seconds).