Some characteristics of brain electrical activity in the domestic chicken

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Abstract

Studies in mammals have used measures of brain activity, such as the electroencephalogram (EEG), to assess animal welfare. The aim here was to determine whether the EEG could be used in a similar way in birds.

The effects of anaesthesia on the chicken EEG were relevant for later studies on anaesthetised chickens. Therefore the effects of different concentrations of four anaesthetics were recorded. The EEG was also used to examine the development of brain activity in chicks with relevance to the onset of consciousness. The EEG has been used to record responses to noxious stimulation in anaesthetised mammals (i.e. without the animals experiencing pain). To test this model in birds, anaesthetised chickens were exposed to noxious stimuli as their EEG was recorded. The EEG response to decapitation was also recorded in anaesthetised chickens to assess whether this is a suitable method to use for killing chickens.

Halothane caused less suppression of brain activity than some of the other agents, which suggests halothane would be a suitable agent to use in chicken EEG studies. EEG first appeared on day 13 of the chick's incubation, frequency and amplitude increased until day 17. EEG activity decreased before hatching, possibly due to an oxygen shortage in the egg. Consciousness seems unlikely until after hatching. There did not seem to be any consistent change in the EEG after noxious stimulation. This result contrasts with that found in mammals, and may be due to differences in brain anatomy between the two species. It would be worthwhile conducting further research to explore generators of the EEG and pain processing in birds. After decapitation there were significant changes in the EEG. The EEG also persisted for around 35 seconds following decapitation. This may indicate that consciousness is not lost instantly at the time of decapitation and that decapitation is an unsuitable method of killing chickens.

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Commonly used acronyms

Acronym	Meaning
ANOVA	Analysis of variance
BSR	Burst suppression ratio
ECG	Electrocardiogram
EEG	Electroencephalogram/electroencephalographic
MAC	Minimum anaesthetic/alveolar concentration
SEM	Standard error of the mean

Chapter 1. General introduction

1.1 This thesis

This thesis explored some of the characteristics of brain electrical activity in the domestic chicken (*Gallus domesticus*). The brain electrical activity was examined by use of an electroencephalogram (EEG), which recorded the electrical activity using superficial needle electrodes placed on the head of the chicken. There were four main experiments that made up this thesis. These experiments studied: 1. the EEG responses to changes in anaesthetic concentration using 4 different anaesthetics; 2. the development of the EEG of the chick during incubation and shortly after hatching; 3. the EEG responses to noxious stimuli in anaesthetised chickens; and 4. the EEG responses to decapitation of anaesthetised chickens.

The EEG was chosen as a method for recording because it reflects brain activity that is strongly linked to consciousness. This is important in animal welfare studies as will be discussed shortly.

1.2 Animal welfare

Animal welfare is a concept that has evolved over the years and now encompasses multiple dimensions (Duncan, 2002; Mellor et al., 2009). Most people intuitively have some idea about what animal welfare is, however definitions of animal welfare may differ slightly between groups of people. Yet, many of the overarching ideas about animal welfare are likely to align very closely with each other (Duncan, 2002). For the purposes of making a quantitative assessment of animal welfare, however, a more refined

description/definition of animal welfare is needed (Duncan, 2002; Mellor et al., 2009).

1.2.1 Three orientations on animal welfare

There are three major view points underlying evaluations of animal welfare (Fraser, 2003). The first of these viewpoints is based on biological/physiological functioning. According to this viewpoint, an animal is believed to have good welfare when they have good health and their biological systems are functioning. Welfare assessments are based on criteria such as the animal's health, body condition, productivity (for farm animals) and reproductive success (Fraser, 2003).

A second view on welfare assessment is based on the animal's natural state. According to this orientation, an animal's welfare is good when their experiences and environment resembles that which would occur in the animal's natural/wild state, i.e. how the animal would exist in the wild (Fraser et al., 1997; Fraser, 2003).

The third view on animal welfare is based on the animal's affective (emotional) state. According to this viewpoint, an animal is said to have good welfare when it is in a positive affective state i.e. the animal feels good emotionally for the majority of the time (Fraser, 2003; Dawkins, 2006).

These three orientations on welfare are not independent of each other. In many cases the three overlap (Fraser et al., 1997; Fraser, 2003). For example, according to the biological functioning view, an animal would experience poor welfare when they were ill. Similarly, illness is also accompanied by negative affective

states i.e. pain, malaise etc (Duncan, 2002; Mellor et al., 2009). Thus both views see the animal as having poor welfare.

The natural state view aligns with some aspects of the health and affective state orientations. For example, an animal in an environment that resembles its natural habitat is likely to be able to express natural behaviours (e.g. for chickens foraging, dust-bathing, flapping wings etc). The ability to perform natural behaviours would promote a positive affective state in the animal (Fraser et al., 1997). However, there are natural experiences which do not equate to positive welfare states according to either the affective state or biological functioning viewpoints (Sandoe et al., 2004). For example, predation and subsequent injury, and states of disease, exposure to temperature extremes or malnutrition are all natural occurrences in the wild. However, these experiences would cause poor welfare for the animal in terms of its affective state and biology (Fraser et al., 1997).

Aside from some areas of complementary overlap, such as those mentioned above, assessments of animal welfare will vary depending on which of these three orientations is adopted (Fraser, 2003). Recently scientific welfare assessments have become increasing focused on the animal's affective state (Fraser and Duncan, 1998). Many animals (especially vertebrates) are largely accepted as being conscious. That is, they are sensitive to their surroundings and capable of experiencing affective states/emotions (Dawkins, 2006). Such affective states are elicited by the influences of the environment weighted against the animal's ability to cope with that environment (Broom, 1991; Mellor and Stafford, 2001). Therefore, welfare has been loosely defined as the state of an animal which occurs as a result of its environment and its ability to cope with that

environment. This state includes both physiological and affective components (Broom, 1991).

1.2.2 Five domains of potential animal welfare compromise

A useful way to evaluate animal welfare is using the five domains of potential welfare compromise (Mellor and Reid, 1994). These five domains represent aspects that are important to the welfare of the animal. The domains are: the nutritional state of the animal; the environmental conditions to which the animal is subjected; the health and physical function of the animal; the ability of the animal to perform natural behaviours; and the psychological (mental) state of the animal (Mellor and Reid, 1994).

The first four domains (nutrition, health, environment and behaviour) reflect physical impacts, and these influence the fifth (mental) domain (Mellor and Stafford, 2001). Therefore, compromise in each of the first four domains can cause a negative effect on the animal's mental state. For example, deficiencies in the nutritional domain lead to mental experiences or feelings of thirst or hunger, poor health can result in the animal feeling sick or nauseous, an inadequate environment can cause the animal to suffer during temperature extremes and bad weather, and failing to meet an animal's behavioural needs can result in them experiencing boredom, frustration and loneliness (Mellor et al., 2009). Ultimately, it is the animal's mental experience or feelings that determine its welfare state, not the biological/behavioural deficiency per se (Duncan, 2002).

1.2.3 Relevance of consciousness to welfare

An animal's mental state/feelings are a major component of its welfare. It is the negative emotional component of welfare compromise that makes the experience unpleasant for the animal (Duncan, 2002). However, experiences such as pain or illness must be perceived consciously by the animal in order to affect its welfare. An unconscious animal does not have the capacity to experience positive or negative states that would otherwise affect its welfare (Mellor and Reid, 1994). Consciousness is an experience that is apparently generated by the transfer of neural signals within complex nervous networks (Seth et al., 2005). Thus in order for an animal to be conscious they need to have a sufficiently complex and functional nervous system (Mellor and Diesch, 2006).

Part of welfare assessments therefore involve examining whether or not the animal is conscious (Velarde et al., 2002; Raj and O'Callaghan, 2004; Dawkins, 2006; Mellor and Diesch, 2006). These welfare studies can involve assessing the criteria for consciousness in the animal. For example, examining whether the animal has the neural structure necessary to support consciousness, and determining whether those structures are functional (Mellor and Diesch, 2006).

Recent animal welfare studies have investigated the properties of brain activity in animals in different situations. Notably, researchers have used measures of brain activity to assess the potential for consciousness in animals (Velarde et al., 2002; Raj and O'Callaghan, 2004; Diesch et al., 2009a, 2009b). Such studies have also examined whether certain noxious sensory inputs are processed at higher levels of the brain; which would indicate pain perception in a conscious animal (Johnson et al., 2005b; Murrell and Johnson, 2006; Gibson et al., 2007; Murrell et al., 2007; Gibson et al., 2009a).

1.2.4 Brain activity and animal welfare

Brain electrical activity, hereafter referred to as brain activity, and its relationship to pain perception and consciousness have been studied in several different mammalian species. Methods measuring brain electrical activity have been developed to examine the onset of consciousness in mammals (Diesch et al., 2009a, 2009b) and to assess responses to noxious stimuli in anaesthetised mammals (Murrell et al., 2003; Murrell and Johnson, 2006; Gibson et al., 2007; Murrell et al., 2007; Gibson et al., 2009b). These studies have provided useful insights relevant to animal welfare, as will be discussed later in this introduction as well as in Chapters 3-5.

The focus of this thesis is avian species. Previous studies have explored some aspects of brain activity in birds. For example, the development of brain activity in the domestic chicken was explored in multiple experiments conducted 10-50 years ago (e.g. Garcia-Austt Jr, 1954; Tuge et al., 1960; Peters et al., 1965; Ellingson and Rose, 1970; Cusick and Peters, 1973; Hunter et al., 1999)). These studies will be discussed in detail in Chapter 3. The findings of these experiments were recently reviewed with relation to the onset of consciousness in chicks (Mellor and Diesch, 2007).

Other experiments have explored the chicken's brain activity in response to noxious stimulation (Woolley and Gentle, 1987; Gentle and Hunter, 1990) and slaughter (Gregory and Wotton, 1986), as will be discussed in Chapter 4 and 5. This thesis expands on this previous work, by conducting studies to further understand the properties of brain electrical activity in chickens. The results of

the experiments outlined in this thesis are also used to explore how brain activity might be used to assess welfare in chickens.

1.3 Consciousness in mammals and birds

The ranging levels of neural organisation and complexity in different species may result in diversity in conscious experience among those animals. Thus a bird's experience of consciousness may be different to a mammalian experience of consciousness (Edelman et al., 2005).

The avian brain is structurally very different from the mammalian brain. However, the two have recently been carefully compared and, despite differences in gross anatomy, it appears that the structures which generate consciousness in the mammalian brain have analogous counterparts in the avian brain (Butler et al., 2005; Butler and Cotterill, 2006). So, although the avian brain differs structurally from the mammalian brain, it has regions which are able to perform functions similar to the conscious processing that seems to occur in the different brain regions of mammals. Birds are also capable of performing complex behaviours, which in mammals would be associated with conscious thought processes, including memory and recall (Butler et al., 2005).

1.4 Electroencephalogram as a measure of brain function

The electrical activity of the brain, especially the cerebral cortex in mammals, can be monitored by use of an electroencephalogram (EEG). This electrical activity is generated by the summation of the electrical potentials that are produced by groups of neurons in the cerebral cortex. An EEG records the electrical potential difference between two points on an animal's head (Murrell and Johnson, 2006).

This potential difference (voltage) fluctuates as the electrical activity changes at either site, and a randomly oscillating waveform is generated (Klem, 1969).

The EEG signal represents the combined electrical potentials of many cortical neurons. When groups of dendrites depolarise at the same frequency (have a common rhythm) they are described as being synchronised. Unconscious states such as sleep, vegetative states or anaesthesia are often characterised by slow, high amplitude regular waves (Baars et al., 2003). During sleep there are large numbers of neurons depolarising in synchrony (Steriade et al., 1993). Less information processing and greater synchronisation occurs during rest e.g. eyes closed and/or sleep. This EEG state is also seen during hypoxia and brain cooling (Simons et al., 1989). In contrast, during information processing there is less synchronisation and rhythmic potentials in the waveform are reduced (Simons et al., 1989). Consciousness involves widespread, high frequency, low-amplitude interactions in the thalamocortical region of the brain (Seth et al., 2005). This activity is often distinguishable in the EEG as low-amplitude, high frequency irregular waveforms (Baars et al., 2003).

Synchronisation is therefore recognised as high amplitude low frequency activity occurring during rest or sleep, whereas de-synchronisation is represented by increased high frequency activity, usually occurring during arousal or consciousness (Murrell and Johnson, 2006). The frequency of the EEG is proportional to cortical metabolism, which can be slowed by low oxygen availability, reduced blood flow, low temperatures and sedatives or anaesthesia (Boveroux et al., 2008). Alterations in the EEG occur within seconds of the change in cortical metabolism, making the EEG a useful monitor of brain function (Simons et al., 1989; Velarde et al., 2002; Raj and O'Callaghan, 2004).

1.5 Application of the electroencephalogram

Brain electrical activity recorded using the EEG has been used in several mammalian species to explore questions related to consciousness and animal welfare issues such as pain perception (Johnson et al., 2005b; Murrell and Johnson, 2006; Gibson et al., 2007; Johnson et al., 2009). The general consensus is that EEG predominantly records the electrical activity of the cerebral hemispheres (Murrell and Johnson, 2006). The cerebral hemispheres are a major component in generating consciousness in mammals (Baars, 2001); therefore the EEG recording has been used to obtain information related to consciousness. For example, the EEG has been used to distinguish between states of sleep, unconsciousness, brain death and consciousness (Velarde et al., 2002; Baars et al., 2003; Raj and O'Callaghan, 2004).

1.5.1 Onset of consciousness and the EEG

At the time the egg is laid, the domestic chick embryo consists of a relatively small number of undifferentiated cells (Romanoff, 1960). At this stage, there would be insufficient nervous tissue to support consciousness. However, newly hatched chicks are precocial; at the time of hatching they are anatomically well developed and show signs of conscious awareness, interacting with their surroundings and other chicks within minutes to hours of hatching (Cusick and Peters, 1973; Rogers, 1995). The question is therefore, when, during incubation, might the chick develop the neural capacity for consciousness? It is likely that the structures necessary for consciousness are present before hatching. But at what point does the nervous system become functional and capable of supporting consciousness?

Factors which may influence the timing of the onset of consciousness in chicks are reviewed by Mellor and Diesch (2007), and will be discussed in Chapter 2. The EEG has been used to investigate the onset of consciousness in rat pups and wallaby joeys (Diesch et al., 2009a, 2009b). Similarly, the EEG can be used to assess brain activity in chicks at different stages of development. The objective of the study in Chapter 2 was to examine the development of brain activity in chicks before and after hatching and relate this to the potential for consciousness at each stage.

1.6 Pain

An important component of measuring animal welfare is pain assessment. Pain in animals has been defined as "an aversive sensory and emotional experience representing awareness by the animal of damage or threat to the integrity of its tissues" (Molony and Kent, 1997). Pain therefore includes a subjective experience. It can be difficult to quantify pain in humans because each individual can have a different perception of the same stimulus (Craig et al., 1996). Humans however, can use language to describe their perception of pain. Pain assessment in other animals is more complicated (Gentle, 1992).

Quantitative pain assessments in animals include measures such as heart rate, hormone levels, respiratory patterns and specific behaviours– which vary according to species (Morton and Griffiths, 1985; Mellor et al., 2000). These various approaches to pain assessment have strengths and weaknesses. For example, measures such as heart rates and hormone levels are relatively straight forward to record, and certain behaviours can provide specific information on

pain location (e.g. elevation of an injured limb) (Morton and Griffiths, 1985). However, some of the physiological parameters, such as heart and respiratory rate, can either increase or decrease in response to pain (Morton and Griffiths, 1985). Likewise, some hormone levels can increase in situations that are either stressful or pleasurable (Broom, 1988; Dawkins, 2006). Furthermore, the experimenter must have a thorough knowledge of the animal's 'normal' behaviour before they can accurately assess behavioural indicators of pain (Morton and Griffiths, 1985).

1.6.1 EEG responses to noxious stimuli

During noxious stimulation in mammals there are changes in brain activity as the nervous system processes the neural signals relating to pain. Therefore, changes in the EEG have been used as a method of pain assessment (Murrell and Johnson, 2006).

Electrophysiology has recently been used to infer subjective experience (i.e. pain) during noxious, invasive procedures conducted on anaesthetised animals (Murrell and Johnson, 2006). This methodology, referred to as the minimal anaesthesia model, has been applied to the assessment of: velvet antler removal in deer (Johnson et al., 2005b), castration in horses (Murrell et al., 2003), castration in lambs of different ages (Johnson et al., 2005a; Johnson et al., 2009), dehorning in cattle (Gibson et al., 2007), different noxious stimuli in rats (Murrell et al., 2007) and slaughter in cattle (Gibson et al., 2009a; Gibson et al., 2009c). One of the aims of this thesis was to explore whether similar studies could be conducted in birds.

There are now quantitative measures which can be used to more objectively describe changes in the EEG. The EEG is made up of many frequencies superimposed on each other (Cantor, 1999; Murrell and Johnson, 2006). The Fast Fourier Transformation (FFT) is a mathematical tool that calculates how much power each frequency band contributes to the overall EEG waveform. The FFT generates a frequency spectrum, which is a graphical representation showing the power contribution made by each frequency (Murrell and Johnson, 2006). Variables derived from the frequency spectrum and describing the EEG include: the total power, which is the total area underneath the frequency spectrum curve; the median frequency, which is the frequency below which half the total power is located; and the 95% spectral edge frequency, which is the frequency below which 95% of the total power is located. These three variables are an effective quantitative tool for assessing changes in the EEG activity (Murrell and Johnson, 2006).

Higher frequency activity in the EEG is usually associated with arousal and increased neural processing, whereas decreases in the EEG frequency are often associated with sleep, loss of brain function or anaesthesia (Simons et al., 1989; Steriade et al., 1994; Bates, 2010). EEG responses to noxious inputs have varied between studies, but often there is an increase in the high frequency activity of the EEG. This EEG change is referred to as an activated EEG, or de-synchronisation (Murrell and Johnson, 2006). During de-synchronisation the brain neurons are in an activated state and fewer of them are firing in synchrony. The result of this neural firing pattern is that the amplitude (voltage) of the EEG is reduced, but there is an increase in the EEG frequency (Bates, 2010).

When a noxious stimulus is applied to an animal, inducing activation of brain and more high frequency EEG activity, there is an increase in the median frequency and the 95% spectral edge frequency (Gibson et al., 2007). The total power is strongly influenced by the low frequency waves, because of the large contribution they make to the power spectrum. Therefore when there is an increase in EEG frequency, the total power can decrease as the frequency spectrum curve becomes skewed more towards the mid to high frequency range instead of the low frequency range (Gibson et al., 2009a).

So far, little is known about EEG responses to noxious stimulation in birds. Two previous studies failed to find any changes in the chicken EEG following noxious stimulation (Woolley and Gentle, 1987; Gentle and Hunter, 1990). The experiments in Chapter 3 aimed to quantify, using FFT variables, any EEG changes occurring after noxious stimuli were applied.

1.7 Anaesthesia

Anaesthesia has been an important component of mammalian studies during which EEG responses to noxious stimulation were recorded. Both the depth of anaesthesia and the type of anaesthetic used will affect whether an EEG response to noxious stimulation is detected (Murrell and Johnson, 2006). Previous anaesthesia studies in mammals revealed that halothane is the most appropriate anaesthetic to use during recording of EEG responses to noxious stimulation. Halothane causes less suppression of brain activity than some of the other inhalation agents (Johnson et al., 1994; Johnson and Taylor, 1998; Murrell et al., 2008). When the animal is on a light plane of anaesthesia the brain activity

remains responsive to external sensory inputs (Murrell and Johnson, 2006; Gibson et al., 2007; Murrell et al., 2007).

As part of this research, it was necessary to explore how the avian EEG responded to anaesthesia and to determine whether a minimal anaesthesia model similar to that used in mammals, could be used in birds. Two components were addressed in the anaesthesia study in Chapter 2. Firstly, we aimed to determine how the chicken's brain responded to changes in anaesthetic depth with different anaesthetics and, secondly, we examined how those different anaesthetics compared to each other in terms of their effect on the EEG. The results from the anaesthesia experiment were used to interpret other studies within the thesis.

1.8 Decapitation

Slaughter methods can vary greatly in their welfare impacts. Ideally the killing method should render the animal insensible (unconscious) instantaneously without the potential for pain or suffering. However, not all slaughter methods accomplish this and, in such cases, it is recommended that head stunning be used to render the animal unconscious before slaughter (Gibson et al., 2009b).

Decapitation is a killing method that has been used in research studies when tissues are to be harvested without chemical contamination or the extensive damage that is often caused by percussive stunning (Holson, 1992). However, concerns have been raised about how long the animal may be conscious and capable of pain perception following decapitation (Mikeska and Klem, 1975; Tidswell et al., 1987; Bates, 2010). The study described in Chapter 4 explored

EEG responses to decapitation in chickens and aimed to examine the duration of potential pain perception and consciousness after decapitation.

1.9 Thesis structure

Four main aspects relating to brain activity in chickens are covered in this thesis. These areas have been studied in mammals; the objective of this thesis was to explore the situation in birds. Firstly, the effects of anaesthesia on the chicken EEG were examined; information gained in this study provided background information for designing and interpreting the later experiments and also provided the opportunity to compare avian and mammalian responses to anaesthesia (Chapter 2). Secondly, the development of brain activity was examined relative to hatching. As well as enabling further comparison with mammals, this experiment was valuable for exploring the question of the onset of consciousness in the domestic chicken (Chapter 3).

The third experiment was designed to examine how the chicken EEG changed in response to noxious sensory input (Chapter 4). The findings of this study could be applied in future bird husbandry and analgesia studies designed to assess pain in birds. It was also anticipated that this information could be useful for future developmental studies. For example, studies could examine EEG responses to noxious stimulation in chicks of different ages to explore the extent of sensory processing at different stages of development. The fourth experiment aimed to determine whether decapitation was an appropriate method for killing chickens as it is one of the methods in current use for this species (Chapter 5).

Each chapter contains a review of the relevant literature (introduction), Methods, Results and Discussion sections. The final chapter (Chapter 6) is a General Discussion which links the results of the various studies and presents discussion of the overall conclusions and implications of the findings for animal welfare.

1.10 References

Baars, B. J., 2001: There are no known differences in brain mechanisms of consciousness between humans and other mammals. *Animal Welfare,* 10: S31-S40.

Baars, B. J., Ramsoy, T. Z., and Laureys, S., 2003: Brain, conscious experience and the observing self. *Trends in Neurosciences,* 26: 671-675.

Bates, G., 2010: Humane issues surrounding decapitation reconsidered. *Journal of the American Veterinary Medical Association,* 237: 1024-1026.

Boveroux, P., Bonhomme, V., Boly, M., Vanhaudenhuyse, A., Maquet, P., and Laureys, S., 2008: Brain function in physiologically, pharmacologically and pathologically altered states of consciousness. *International Anesthesiology Clinics,* 46: 131-146.

Broom, D. M., 1988: The scientific assessment of animal welfare. *Applied Animal Behaviour Science,* 20: 5-19.

Broom, D. M., 1991: Animal welfare: concepts and measurement. *Journal of Animal Science,* 69: 4167-4175.

Butler, A. B., Manger, P. R., Lindahl, B. I. B., and Arhem, P., 2005: Evolution of the neural basis of consciousness: a bird-mammal comparison. *BioEssays,* 27: 923-936.

Butler, A. B. and Cotterill, R. M. J., 2006: Mammalian and avian neuroanatomy and the question of consciousness in birds. *Biological Bulletin,* 211: 106-127.

Cantor, D. S., 1999: An overview of quantitative EEG and its applications to neurofeedback. *In* Evans, J. R. and Abarbanel, A. (eds.), *Introduction to Quantitative EEG and Neurofeedback*. San Diego: Academic Press, 3-27.

Craig, A. D., Reiman, E. M., Evans, A., and Bushnell, M. C., 1996: Functional imaging of an illusion of pain. *Nature,* 384: 258-260.

Cusick, C. J. and Peters, J. J., 1973: Electromyographic, electroencephalographic, and behavioural changes during the onset of erect posture in newly hatched chicks. *Poultry Science,* 53: 1456-1462.

Dawkins, M. S., 2006: A user's guide to animal welfare science. *Trends in Ecology and Evolution,* 21: 77-82.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009a: Electroencephalographic responses to tail clamping in anaesthetized rat pups. *Laboratory Animals,* 43: 224-231.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009b: Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Laboratory Animals,* 00: 1-9.

Duncan, I. J. H., 2002: Poultry welfare: science or subjectivity. *British Poultry Science,* 43: 643-652.

Edelman, D. B., Baars, B. J., and Seth, A. K., 2005: Identifying hallmarks of consciousness in non-mammalian species. *Consciousness and Cognition,* 14: 169-187.

Ellingson, R. J. and Rose, G. H., 1970: Ontogenesis of the electroencephalogram. *In* Himwich, W. A. (ed.), *Developmental Neurobiology*. Illinois: Charles C Thomas Publisher, 441-474.

Fraser, D., Weary, D. M., Pajor, E. A., and Milligan, B. N., 1997: A scientific conception of animal welfare that reflects ethical concerns. *Animal Welfare,* 6: 187-205.

Fraser, D. and Duncan, I. J. H., 1998: 'Pleasures', 'pains' and animal welfare: towards a natural history of affect. *Animal Welfare,* 7: 383-396.

Fraser, D., 2003: Assessing animal welfare at the farm and group level: the interplay of science and values. *Animal Welfare,* 12: 433-443.

Garcia-Austt Jr, E., 1954: Development of electrical activity in cerebral hemispheres of the chick embryo. *Proceedings of the Society for Experimental Biology and Medicine,* 86: 348-352.

Gentle, M. J. and Hunter, L. N., 1990: Physiological and behavioural responses associated with feather removal in *Gallus* var *domesticus*. *Research in Veterinary Science,* 50: 95-101.

Gentle, M. J., 1992: Pain in birds. *Animal Welfare,* 1: 235-247.

Gibson, T. J., Johnson, C. B., Stafford, K. J., Mitchinson, S. L., and Mellor, D. J., 2007: Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal,* 55: 152-157.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Hulls, C. M., Mitchinson, S. L., Stafford, K. J., Johnstone, A. C., and Mellor, D. J., 2009a: Electroencephalographic responses of halothane-anaesthetised calves to slaughter by ventral-neck incision without prior stunning *New Zealand Veterinary Journal,* 57: 77-83.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Mitchinson, S. L., Stafford, K. J., and Mellor, D. J., 2009b: Amelioration of electroencephalographic responses to slaughter by non-penetrative captive-bolt stunning after ventral-neck incision in halothane-anaesthetised calves. *New Zealand Veterinary Journal,* 57: 96-101.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Mitchinson, S. L., Stafford, K. J., and Mellor, D. J., 2009c: Electroencephalographic responses to concussive non-penetrative captive-bolt stunning in halothane-anaesthetised calves. *New Zealand Veterinary Journal,* 57: 90-95.

Gregory, N. G. and Wotton, S. B., 1986: Effect of slaughter on the spontaneous and evoked activity of the brain. *British Poultry Science,* 27: 195-205.

Holson, R. R., 1992: Euthanasia by decapitation: evidence that this technique produces prompt, painless unconsciousness in laboratory rodents. *Neurotoxicology and Teratology,* 14: 253-257.

Hunter, M., Battilana, M., Bragg, T., and Rostas, J. A. P., 1999: EEG as a measure of developmental changes in the chicken brain. *Developmental Psychobiology,* 36: 23-28.

Johnson, C. B., Young, S. S., and Taylor, P. M., 1994: Analysis of the frequency spectrum of the equine electroencephalogram during halothane anaesthesia. *Research in Veterinary Science,* 56: 373-378.

Johnson, C. B. and Taylor, P. M., 1998: Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. *British Journal of Anaesthesia,* 81: 748-753.

Johnson, C. B., Stafford, K. J., Sylvester, S. P., Ward, R. N., Mitchinson, S. L., and Mellor, D. J., 2005a: Effects of age on the electroencephalographic response to castration in lambs anaesthetised using halothane in oxygen. *New Zealand Veterinary Journal,* 53: 433-437.

Johnson, C. B., Wilson, P. R., Woodbury, M. R., and Caulkett, N. A., 2005b: Comparison of analgesic techniques for antler removal in halothane-anaesthetised red deer (*Cervus elaphus*): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia,* 32: 61-71.

Johnson, C. B., Sylvester, S. P., Stafford, K. J., Mitchinson, S. L., Ward, R. N., and Mellor, D. J., 2009: Effect of age on the electroencephalographic response to castration in lambs anaesthetised with halothane in oxygen from birth to 6 weeks old. *Veterinary Anaesthesia and Analgesia,* 36: 273-279.

Klem, W. R., 1969: *Animal electroencephalography*. London: Academic Press Inc.

Mellor, D. J. and Reid, C. S. W., 1994: Concepts of animal well-being and predicting the impact of procedures on experimental animals. *In* Baker, R. M., Jenkin, G., and Mellor, D. J. (eds.), *Improving the well-being of animals in the research environment*. Glen Osmond, South Australia: Australian and New Zealand Council for the Care of Animals in Research and Teaching, 3-18.

Mellor, D. J., Cook, C. J., and Stafford, K. J., 2000: Quantifying some responses to pain as a stressor. *In* Moberg, G. P. and Mench, J. A. (eds.), *The Biology of Animal Stress: basic principles and implications for welfare*. Wallingford, UK: CAB International, 171-198.

Mellor, D. J. and Stafford, K. J., 2001: Integrating practical, regulatory and ethical strategies for enhancing farm animal welfare. *Australian Veterinary Journal,* 79: 762-768.

Mellor, D. J. and Diesch, T. J., 2006: Onset of sentience: the potential for suffering in fetal and newborn farm animals. *Applied Animal Behaviour Science, 100*: 48-57.

Mellor, D. J. and Diesch, T. J., 2007: Birth and hatching: key events in the onset of awareness in the lamb and chick. *New Zealand Veterinary Journal,* 55: 51-60.

Mellor, D. J., Patterson-Kane, E., and Stafford, K. J., 2009: Focus of animal welfare, *The Sciences of Animal Welfare*. Oxford, UK: Wiley-Blackwell, 1-12.

Mikeska, J. A. and Klem, W. R., 1975: EEG evaluation of the humaneness of asphyxia and decapitation euthanasia of the laboratory rat. *Laboratory Animal Science,* 25: 175-179.

Molony, V. and Kent, J. E., 1997: Assessment of acute pain in farm animals using behavioral and physiological measurements. *Journal of Animal Science,* 75: 266-272.

Morton, D. B. and Griffiths, P. H. M., 1985: Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *The Veterinary Record,* 116: 431-436.

Murrell, J. C., Johnson, C. B., White, K. L., Taylor, P. M., Haberham, Z. L., and Waterman-Pearson, A. E., 2003: Changes in the EEG during castration in horses and ponies anaesthetized with halothane. *Veterinary Anaesthesai and Analgesia,* 30: 138-146.

Murrell, J. C. and Johnson, C. B., 2006: Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29: 325-335.

Murrell, J. C., Mitchinson, S. L., Waters, D., and Johnson, C. B., 2007: Comparative effect of thermal, mechanical, and electrical noxious stimuli on the electroencephalogram of the rat. *British Journal of Anaesthesia,* 98: 366-371.

Murrell, J. C., Waters, D., and Johnson, C. B., 2008: Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. *Laboratory Animals,* 42: 161-170.

Peters, J., Vonderahe, A., and Schmid, D., 1965: Onset of cerebral electrical activity associated with behavioural sleep and attention in the developing chick. *Journal of Experimental Zoology,* 160: 255-262.

Raj, A. B. M. and O'Callaghan, M., 2004: Effect of amount and frequency of head-only stunning currents on the electroencephalogram and somatosensory evoked potentials in broilers. *Animal Welfare,* 13: 159-170.

Rogers, L. J., 1995: *The Development of Brain and Behaviour in the Chicken*. Cambridge: Cab International, 273 pp.

Romanoff, A. L., 1960: *The Avian Embryo*. New York: The Macmillan Company, 1305 pp.

Sandoe, P., Forkman, B., and Christensen, S. B., 2004: Scientific uncertainty - how should it be handled in relation to scientific advice regarding animal welfare issues? *Animal Welfare,* 13: S121-126.

Seth, A. K., Baars, B. J., and Edelman, D. B., 2005: Criteria for consciousness in humans and other mammals. *Consciousness and Cognition,* 14: 119-139.

Simons, A. J. R., Boezeman, E. H. J. F., and Pronk, R. A. F., 1989: Automatic EEG monitoring of anaesthesia. *In* Jones, J. G. (ed.), *Bailliere's Clinical Anaesthesiology: International Practice and Research, Vol.3 number 3*. London: Bailliere Tindall, 623-646.

Steriade, M., McCormick, D. A., and Sejnowski, T. J., 1993: Thalamocortical oscillations in the sleeping and aroused brain. *Science,* 262: 679-685.

Steriade, M., Amzica, F., and Contreras, D., 1994: Cortical and thalamic cellular correlates of electroencephalographic burst-suppression. *Electroencephalography and clinical neurophysiology,* 90: 1-16.

Tidswell, S. J., Blackmore, D. K., and Newhook, J. C., 1987: Slaughter methods: electroencephalographic (EEG) studies on spinal cord section, decapitation and gross trauma of the brain in lambs. *New Zealand Veterinary Journal,* 35: 46-49.

Tuge, H., Kanayama, Y., and Yueh, C. H., 1960: Comparative studies on the development of EEG. *Japanese Journal of Physiology,* 10: 211-220.

Velarde, A., Ruiz-de-la-Torre, J. L., Rosello, C., Fabrega, E., Diestre, A., and Manteca, X., 2002: Assessment of return to consciousness after electrical stunning in lambs. *Animal Welfare,* 11: 333-341.

Woolley, S. C. and Gentle, M. J., 1987: Physiological and behavioural responses in the hen (*Gallus domesticus*) to nociceptive stimulation. *Comparative Biochemistry and Physiology A-Physiology,* 88A: 27-31.

Chapter 2. Effects of halothane, methoxyflurane, isoflurane and sevoflurane on the electroencephalogram of the chicken

"Jimmy" Photo by L A Mowbray

The findings from this chapter were presented at the Association for Veterinary Anaesthetists Conference in Santorini, Greece in 2010.

McIlhone, A. E., Beausoleil, N. J., Mellor, D. J., Mitchinson, S. L., and Johnson, C. B., 2010: Effects of halothane, isoflurane, sevoflurane and methoxyflurane on the chicken electroencephalogram. *Association of Veterinary Anaesthetists Autumn Meeting*: p55 (Abstract).

2.1 Abstract

This study explored how anaesthetics affect the chicken electroencephalogram (EEG). The findings could be used as a framework for further investigations into the character of brain electrical activity in birds.

Chickens were anaesthetised with halothane, sevoflurane, isoflurane and methoxyflurane (n=12). Anaesthesia was adjusted to 1, 1.5 and 2x MAC for each agent. EEG was collected after a baseline at each concentration. EEG variables median frequency, 95% spectral edge frequency and total power were calculated, as was the burst suppression ratio (BSR).

High concentrations of methoxyflurane and halothane reduced median frequency ($P<0.05$). 95% spectral edge frequency was lower with high concentrations of methoxyflurane. The effect of halothane was biphasic ($P<0.05$). Total power decreased with high concentrations of methoxyflurane and increased with halothane ($P<0.05$). Burst suppression was rare with halothane and methoxyflurane but present throughout isoflurane and sevoflurane increasing at deeper concentrations.

Halothane caused less suppression of brain activity than some other agents, suggesting that halothane may be a suitable anaesthetic agent to use in future studies that explore EEG activity in anaesthetised birds.

2.2 Introduction

Anaesthetics are used to induce unconsciousness, amnesia and loss of muscle tone during surgical or experimental procedures (Antognini and Carstens, 2002). Anaesthesia has been studied extensively in mammals. For example, examining sites of action of anaesthesia (Antognini and Schwartz, 1993) and examining anaesthesia's effect on the EEG (Johnson et al., 1994; Johnson and Taylor, 1998; Murrell et al., 2008). In situations of controlled anaesthesia, the EEG can be used to record brain electrical responses to noxious stimulation. However, both the anaesthetic agent and the depth of anaesthesia are variables that need to be carefully controlled. The methodology of recording EEG responses to noxious stimulation has proved useful in many experiments in mammals. However, little is known about anaesthesia, or the EEG responses to anaesthesia in birds. Future studies discussed in this thesis will be examining the EEG of chickens that have been anaesthetised. These experiments rely on some background understanding of how anaesthetics affect the chicken EEG i.e. is the situation the same or different to what occurs in mammals? The present study was undertaken to compare the effects of different anaesthetic agents on the EEG of the chicken. The information gained from this study will be applied in future experiments in this thesis.

There are a range of different anaesthetic drugs; those examined in this study were the inhalation anaesthetics halothane, methoxyflurane, isoflurane and sevoflurane. Comparisons of the effects of these anaesthetics were made based on multiples of the *minimum alveolar concentration* (MAC – explained below) for each anaesthetic.

2.2.1 Measuring anaesthetic depth

During surgical or experimental procedures, the depth of anaesthesia needs to be carefully monitored. Overdosing anaesthetics can be harmful to the animal, but there is also a need to ensure the animal does not move or regain consciousness during such procedures. There are various methods for assessing depth of anaesthesia. These include tests such as limb-withdrawal in response to a toe-squeeze, judging muscle tone, eye blink reflexes (palpebral and/or corneal) and measures of blood pressure and heart rate. However, these assessments can be difficult because muscle tone, withdrawal reflexes and haemodynamic responses can be affected by other drugs such as muscle relaxants and analgesics that are used during surgery (Antunes et al., 2003).

2.2.2 Caution in using anaesthesia in experimental situations

Knowing the effect of anaesthetics on the EEG is important in experimental situations. If animals are anaesthetised during EEG studies, the effect of the anaesthetic will add a source of variation to the results. It is important to know how the anaesthetic affects the EEG, and also to carefully monitor and control anaesthesia during these experiments.

2.2.3 Minimum anaesthetic concentration (MAC) as a measure of anaesthetic potency

Controlling the depth of anaesthesia is vital in all uses of anaesthetics, whether clinical or experimental. Some anaesthetic agents are stronger (i.e. more potent) than others. That is, for some anaesthetics a greater concentration of the anaesthetic needs to be inhaled in order to reach a suitable depth of anaesthesia (Dugdale, 2010). The *minimum alveolar concentration* (MAC) is a well-known

gauge of anaesthetic potency in mammals. MAC is referred to as *minimum anaesthetic concentration* in birds, which have parabronchi instead of alveoli as the site of gas exchange. MAC is defined as the minimum anaesthetic concentration that is required to prevent a movement response to noxious stimulation in 50% of subjects (Ludders et al., 1988). MAC values are measured as percentages, i.e. the percent of end tidal gas expired into the anaesthetic monitor. They are specified for each anaesthetic and are also species-specific (Steffey, 1996).

While the loss of movement is a desirable outcome of anaesthesia, it is important to consider other endpoints when estimating anaesthetic requirements. Loss of consciousness occurs before loss of movement e.g. at about 0.45 MAC for isoflurane (Dwyer et al., 1992). Therefore, individuals that are anaesthetised to the point of loss of movement will also have lost consciousness. The amount of anaesthetic that is required to induce unconsciousness, relative to the amount required for MAC, is different for various anaesthetic agents (Dwyer et al., 1992).

Because MAC is a standard gauge for anaesthetic potency, multiples of MAC were used in this study to select the concentrations of anaesthetic that were being compared. However, there are limitations to MAC in terms of what it reveals about the state of the anaesthetised animal.

2.2.4 Site of action of anaesthetics

In mammals, the primary site of action for inhalation anaesthetic is on the spinal cord (Antognini and Schwartz, 1993). Anaesthetics seem to disrupt neural signal transfer, which in turn blocks or alters signal transfer through neural circuits (Alkire et al., 2000; Dilger, 2002; Arhem et al., 2003). The value of MAC reflects

spinal anaesthesia (Rampil et al., 1993). When anaesthesia is isolated to the spinal cord, the amount of anaesthetic required remains the same as for standard anaesthetic delivery (Antognini and Schwartz, 1993).

In contrast other endpoints of anaesthesia, i.e. unconsciousness and amnesia, are mediated by the brain (Antognini and Schwartz, 1993). It is likely that some inhalation agents (e.g. isoflurane) affect cortical EEG activity indirectly via actions on the spinal cord (Antognini et al., 2000a). When isoflurane anaesthesia is isolated to the animal's head and brain, the anaesthetic requirements increase (Antognini and Schwartz, 1993). Therefore, MAC is not as strongly related to cerebral anaesthesia as it is to spinal anaesthesia (Rampil et al., 1993).

In line with this, previous studies have found a lack of correlation between MAC anaesthetic requirements and measures of anaesthetic depth based on brain activity (EEG recordings) (Rampil and Laster, 1992; Murrell et al., 2008). This inconsistency is a result of the primary site of action of the anaesthetic (spinal cord) being different from what is being measured (brain activity). MAC is therefore limited in its application as a measure of anaesthetic requirements because it does not correlate well with consciousness or brain activity. In this study MAC will be used to calculate a set of anaesthetic concentrations. The EEG will then record brain electrical activity at these different concentrations to determine how brain activity, changes at different multiples of MAC.

2.2.5 The electroencephalogram (EEG) as a measure of brain activity

The EEG represents global brain activity, particularly the activity of the cerebral cortical cells which is correlated with conscious brain activity (Baars et al., 2003;

Murrell and Johnson, 2006). Therefore the EEG provides an indication of brain activity that is related to brain function and state of consciousness as opposed to simply the loss of movement response indicated by MAC. This property of the EEG has been applied in studies that aim to estimate the potential for conscious brain activity in mammals and birds (Velarde et al., 2002; Raj and O'Callaghan, 2004).

2.2.6 Effects of anaesthetics on the EEG in mammals

The EEG has been investigated as a method of monitoring anaesthetic depth in mammals (Simons et al., 1989; Murrell et al., 2008; Otto, 2008). For example, increasing the depth of anaesthesia often results in more low frequency activity and less high frequency activity in the EEG waveform. These changes in the EEG frequency can be quantified, for example by calculating variables such as the median frequency, spectral edge frequency and total power of the EEG. These quantitative EEG variables (defined below in the Materials and Methods section) enable the use of statistical tests to examine how the EEG waveform changes (Murrell et al., 2008).

Anaesthesia also causes an increase in wave amplitude and some anaesthetic agents can result in the appearance of burst suppression (Simons et al., 1989; Steriade et al., 1994). Burst suppression is identified as periods of isoelectric EEG, interspersed with bursts of EEG activity (Steriade et al., 1994). Burst suppression represents depression of cortical electrical activity for example by drugs, trauma or hypothermia (Otto, 2008). At deeper concentrations of some anaesthetics the periods of burst suppression become longer and occur more frequently (Steriade et al., 1994). To quantify the burst suppression, a burst

suppression ratio (BSR) is calculated. This ratio is a percentage of the recording that is isoelectric over a set period of time (e.g. 60s) (Otto, 2008).

A limitation of the EEG for evaluating depth of anaesthesia is that it is not directly related to loss of movement, which is one of the desired endpoints of anaesthesia. In some cases an animal can have an EEG with burst suppression, yet still move in response to a noxious stimulus (Rampil and Laster, 1992).

2.2.7 EEG responses to noxious stimulation can be measured in anaesthetised mammals

In situations of controlled anaesthesia the EEG can record responses to noxious stimulation, and because the animal is anaesthetised this can be done with minimal compromise to the animal's welfare. Recent experiments have recorded EEG changes that occur when a lightly anaesthetised mammal is subjected to a noxious stimulus (Johnson et al., 2005b; Murrell and Johnson, 2006; Gibson et al., 2007; Murrell et al., 2007; Gibson et al., 2009). These EEG changes can be used to estimate the intensity or severity of a noxious stimulus. The animal is anaesthetised and therefore not consciously perceiving pain, yet the EEG provides a measurable response to potentially painful events. This protocol makes it possible to run experiments with a humane negative control group (i.e. a group exposed to a noxious stimulus with no analgesia) when testing analgesics or different methods of potentially painful husbandry practices (Murrell and Johnson, 2006; Gibson et al., 2007).

2.2.8 Effects of anaesthetic agents on responses to noxious stimulation

In addition to changes in baseline EEG, anaesthetics may alter EEG responses to noxious stimuli. The anaesthetic agent used and the depth of anaesthesia will both influence how the EEG responds to noxious stimulation. There is evidence that suggests that the anaesthetic agent used is important. For example, EEG responses to noxious stimulation are more detectable in animals anaesthetised with halothane than in those anaesthetised with propofol (Antognini et al., 2006). Changes in cerebral activity in response to noxious stimulation are less likely to occur during deep anaesthesia (Antognini and Carstens, 1999; Antognini et al., 2000a). Anaesthesia therefore must be controlled and monitored throughout EEG studies, and prior knowledge of the effects of the chosen anaesthetic on the EEG is necessary for interpreting results.

Currently, there are no quantitative studies examining the effects of different anaesthetic agents on the chicken EEG, there are also no known studies that examine how anaesthetic concentration affects the chicken EEG.

2.2.9 Study objective

The objective of this study was to measure the effects of different concentrations of four anaesthetics on the chicken's EEG. This was undertaken to provide background information for planning further studies and for interpreting the results of those studies, for example recording EEG responses to noxious stimuli in anaesthetised chickens. The results may also be useful clinically, in showing how the avian EEG responds to anaesthesia.

2.3 Methods

The four anaesthetics used were halothane (Nicholas piramal India limited, Ennore, Chennai), methoxyflurane (Medical Developmenta International Ltd, Smith Road, Springvale, Australia), isoflurane (Biomac Laboratories Ltd., Manakau City, Auckland, New Zealand) and sevoflurane (Abbott Laboratories Ltd., Nae Nae, New Zealand). All procedures were approved by the Massey University Animal Ethics Committee, protocol numbers: 09/09 and 09/85

2.3.1 Chickens

Two groups of chickens were used, 12 for testing halothane, and another 12 for methoxyflurane, isoflurane and sevoflurane. The chickens were females of the Hyline Brown layer strain and were sourced from a commercial caged layer farm at least 2 days before the experiment started. The chickens in the halothane trial were 8-10 weeks old during testing and those that received the other three anaesthetics were aged between 6 and 11 weeks.

Once at Massey University, the chickens were kept in groups of 5-7 under controlled temperature and lighting conditions (20°C, 12hr dark/light cycle). Food (Ingham chick starter crumbles, Levin, New Zealand) and fresh water were available *ad-libitum*. The chickens were kept on wood-shavings substrate with access to perches and small huts which they used as refuges during their early adjustment to the enclosure. Several days after recovery from anaesthesia the chickens were re-homed to free-range lifestyle blocks.

2.3.2 Anaesthesia

Anaesthesia was induced using either a chamber or a face mask. Generally, an induction chamber was used for the first 3 or 4 chickens with each anaesthetic agent, then, as the operators became familiar with the action of the anaesthetic and handling the chickens, the face mask was used instead of the chamber. The anaesthetic agent in oxygen was delivered through the mask/chamber until the chicken lost its righting reflex, which was the primary criterion of unconsciousness used in this study.

Once the chicken was unconscious it was intubated. First, lignocaine local anaesthetic (0.1 – 0.2mL, Nopaine; Phoenix Pharm Distributers Ltd, Auckland, New Zealand) was applied to the back of the chicken's throat to desensitise the larynx, then a 2.5mm non-cuffed endotracheal tube was inserted down the chicken's trachea. The chicken was maintained under anaesthesia using a t-piece non-rebreathing anaesthetic circuit and was ventilated using an intermittent positive pressure ventilation (IPPV) system (V-valve ventilator, Vetronics, Bioanalytical Systems Inc, W La Fayette, IN, USA).

An anaesthetic monitor (Hewlett Packard M1025B; Hewlett Packard, Hamburg, Germany) recorded respiration rate, inspired and end-tidal anaesthetic and CO_2 concentrations. The chicken was placed on a water-heated blanket set to approximately 37°C and covered with a polypropylene blanket to reduce heat loss. Heart rate and temperature were monitored throughout the experiment (Figure 2.1).

2.3.3 EEG and electrocardiogram (ECG) recording

The EEG was recorded using four 27-gauge subcutaneous, stainless-steel needle electrodes (Viasys Healthcare, Surrey, England). These were positioned to record the EEG from the left and right sides of the brain using two channels on the chart recorder. The electrode positions were based on the montage for horses, described by Mayhew and Washbourne (1990). The electrode sites on the chickens were: lateral to the comb (non-inverting electrodes) and caudal to the external auditory meatus (inverting electrodes).

The chicken's ECG was recorded with three electrode needles placed medio-cranially to the keel bone, caudal to the sternum and lateral to the pelvis (earth). This last electrode served as a common earth for both ECG and EEG recording.

The EEG and ECG electrode cables fed into three identical break-out boxes, one for each channel, with each break-out box plugged into a physiological signal amplifier (Iso-Dam isolated physiological signal amplifier, World Precision Instruments, Sarasota FL, USA). The amplifiers provided a signal gain of 1000 and recorded frequencies between 0.1 Hz and 0.1 kHz, i.e. high-pass filters were set at 0.1 Hz and low-pass filters were set at 0.1 kHz. Each amplifier fed into an analogue-to-digital converter (Powerlab, ADInstruments Ltd, Sydney, Australia), which digitised the input signals at 1000 points/second and displayed and stored them on an Apple personal computer using Chart 5.5.6 (ADInstruments Ltd) recording software (Figure 2.1).

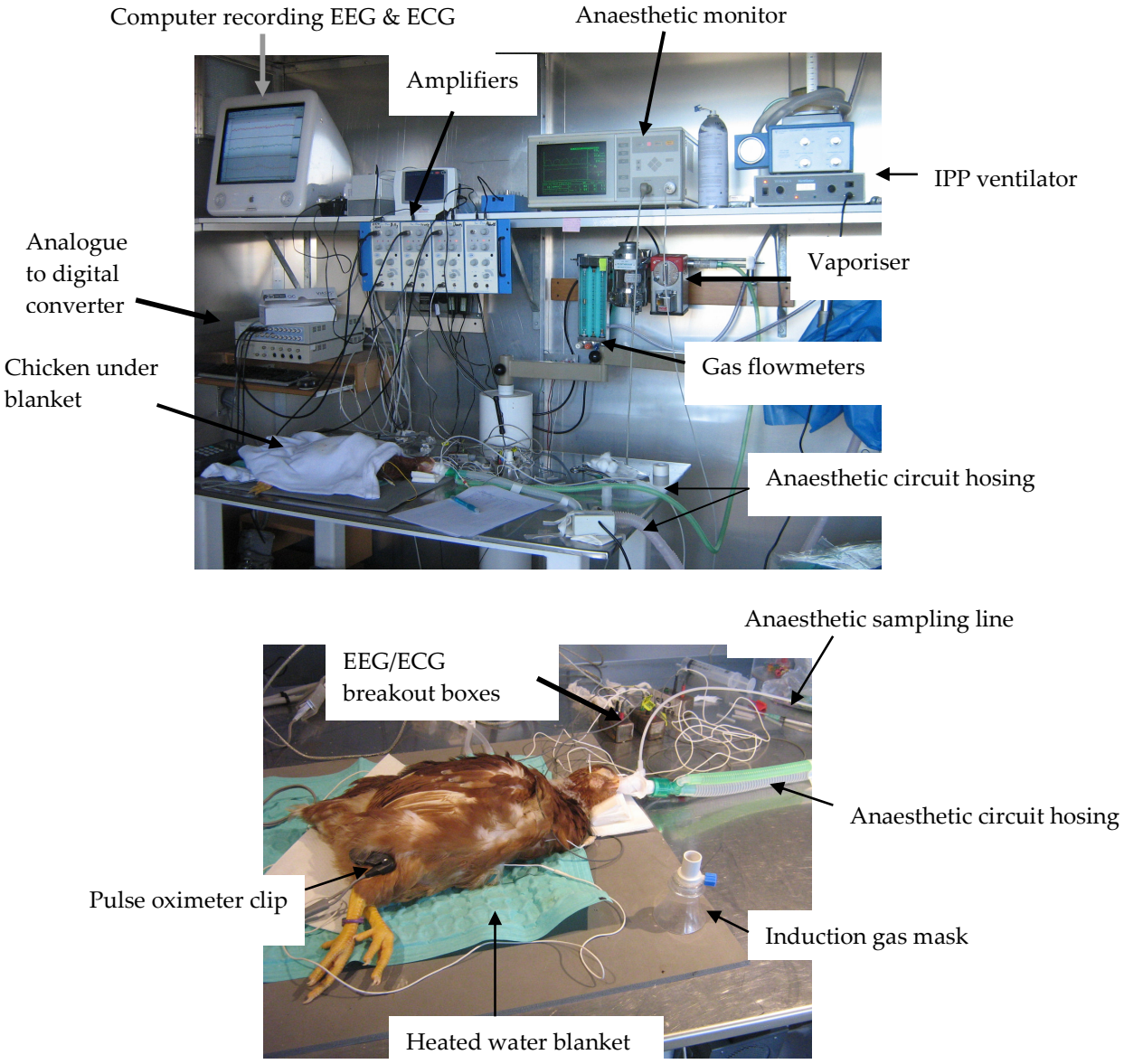


Figure 2.1 Experimental setup for recording the chicken's EEG at different anaesthetic concentrations

2.3.4 Experimental procedure

Each anaesthetic was delivered at three end-tidal concentrations based on 1, 1.5 and 2 times MAC for each agent. The sequence of delivery for each agent is discussed below and presented in Table 2.1.

Anaesthesia was held stable at each concentration before the sampling period started. During these stabilisation periods, the end-tidal agent concentration was monitored closely to ensure it remained within the desired range (see below for range descriptions). If concentration drift occurred, the vaporiser was adjusted and the stabilisation time was restarted. Once stability had been achieved, the EEG was recorded at the set depth of anaesthesia. The vaporiser was then adjusted to change the anaesthetic concentration to the next level. The details for each agent were as follows, and summarised in Table 2.1.

Table 2.1 Stabilisation and EEG recording times for each anaesthetic at the three multiples of MAC. 1 MACa and 1.5 MACa were the values administered as the concentration was being sequentially increased; whereas 1 MACb and 1.5 MACb were the values administered as the concentration was being decreased.

Anaesthetic	1 MACa	1.5 MACa	2 MAC	1.5 MACb	1 MACb
Halothane	10min stable	10min stable	10min stable	10min stable	10min stable
	15min EEG	15min EEG	15min EEG	15min EEG	15min EEG
Methoxyflurane	---	---	10min stable	10min stable	10min stable
			15min EEG	15min EEG	15min EEG
Isoflurane	10min stable	10min stable	10min stable	10min stable	10min stable
	15min EEG	15min EEG	5min EEG	15min EEG	15min EEG
Sevoflurane	2min stable	2min stable	2min stable	2min stable	2min stable
	5min EEG	5min EEG	2min EEG	5min EEG	5min EEG

2.3.4.1 Group 1: Halothane

The MAC value for halothane used in this study was derived from Ludders et al (1988). The authors determined a MAC value of 0.8% halothane for chickens. The criterion used was a purposeful movement or response to a pair of haemostats applied to a "claw" for 60 seconds.

In the current study, the multiples of 1, 1.5 and 2 MAC for halothane were 0.8, 1.2 and 1.6% (Ludders et al., 1988). Anaesthesia was held stable at 0.8% (± 0.05%) for 10 minutes. The EEG was then recorded for 15 min, after which the vaporiser was turned up and the anaesthesia was stabilised at the new level, 1.2% (± 0.05%). This concentration was held stable for 10 minutes and then the EEG was recorded for a second 15 minute period. This procedure was repeated for 1.6% (± 0.05%) halothane and then again as the anaesthetic concentration was reduced to 1.2% and finally 0.8% (Table 1). All of the chickens recovered well from halothane anaesthesia.

2.3.4.2 Group 2: Methoxyflurane, isoflurane and sevoflurane

Order of receiving anaesthetics

All of the Group 2 chickens received methoxyflurane, isoflurane and sevoflurane, with at least seven days between each anaesthetic. Methoxyflurane was always the last anaesthetic to be given, due to a delay in the supply. The order of isoflurane and sevoflurane was alternated. Thus, six chickens received isoflurane, sevoflurane and then methoxyflurane; the remaining six chickens received sevoflurane, isoflurane and then methoxyflurane

Methoxyflurane

The MAC value for methoxyflurane in chickens was unknown, so the value used was that reported for rats (Waizer et al., 1973). This MAC value was established by Waizer et al. using a pair of haemostats applied to the rat's tail for 30 seconds and the authors were looking for movement of head or limbs. Use of this MAC value was justified because the MAC values for other anaesthetics appeared to be similar between rats and chickens (Steffey, 1996; Naganobu et al., 2000; Martin-Jurado et al., 2008).

The anaesthetic monitor could not measure methoxyflurane concentration directly. Instead the monitor was set to detect desflurane and the values were adjusted using a two-point calibration. To calibrate the anaesthetic monitor for methoxyflurane, the vaporiser was set at 1%; the monitor gave a reading of 2.8% desflurane. Using this calibration (the values 0 and 2.8% desflurane), the end-tidal multiples of MAC for methoxyflurane were converted to the appropriate readings for the desflurane setting on the anaesthetic monitor. Therefore the values of 0.27, 0.405 and 0.54% methoxyflurane became 0.80, 1.10 and 1.50% desflurane respectively (all ± 0.05%).

At 1 MAC the chickens were found to be at a very light level of anaesthesia, indicated by behavioural responses to noise; they appeared to be much more lightly anaesthetised than with the other agents at 1 MAC. Anaesthetic induction with methoxyflurane also took longer than with the other anaesthetics. Therefore the first EEG recording was done at the highest concentration, 2 MAC, and this was followed by 1.5 and then 1 MAC, before the chickens were allowed

to recover (the anaesthesia was not returned to 2 MAC). A 10 minute stabilisation period was used at each anaesthetic concentration before the EEG was recorded for 15 minutes (Table 1).

Isoflurane

The isoflurane MAC for chickens was derived from the study by Martin-Jurado et al (2008). The authors applied a pair of haemostats fully closed on "interphalangeal fold" and maintained the pressure for 60 seconds. Slight retraction of the leg was considered a positive response.

The sequence of concentrations for isoflurane was the same as described for halothane. The end-tidal concentrations of isoflurane for 1, 1.5 and 2 MAC were 1.15, 1.70, and 2.3% (all ± 0.05%) (Martin-Jurado et al., 2008). Anaesthesia was held stable for 10 minutes at each level before the EEG recordings were started. The recordings at 1.15 and 1.70% were both made for 15 minutes (Table 1). The recording at 2.3% was limited to 5 minutes because the isoelectric nature of the EEG at this level suggested that longer recording intervals were not necessary.

Sevoflurane

The sevoflurane MAC value for chickens was calculated by Naganobu et al (2000). The authors applied a "toe" clamp for 60 seconds using a pair of haemostats. A positive response was gross purposeful movement.

The sequence of concentrations for sevoflurane was the same as described for halothane and isoflurane. The end tidal concentrations of sevoflurane for 1, 1.5 and 2 MAC were 2.2, 3.3 and 4.4% (all ± 0.1%) (Naganobu et al., 2000).

Sevoflurane stabilised at an end tidal concentration more quickly than the other agents, so the recording periods were adjusted and made shorter. At the start of the experiment with sevoflurane each chicken had 15 minutes for the EEG and the end-tidal CO_2 and anaesthetic concentrations to stabilise. Thereafter, at each end-tidal concentration, the anaesthesia was held stable for 2 minutes, followed by EEG recording for 5 minutes at 2.2 and 3.3% and for 2 minutes at 4.4% (Table 1).

2.3.5 Analysis of the EEG

The EEG results were stored on an Apple personal computer and were analysed after the recordings had been completed. There were two components in the analysis: 1. determination of the burst suppression ratio (BSR), i.e. the ratio of the time occupied by isoelectric EEG (defined below), as opposed to active EEG during a particular time period; and 2. quantitative analysis of the EEG itself in terms of the changes in frequencies of the EEG waveform.

2.3.5.1 Burst suppression

The burst suppression ratio (BSR) was calculated over a continuous 2-minute period close to the middle of the EEG recording at each anaesthetic concentration. The BSR, expressed as a percentage, represented the number of seconds of isoelectric EEG, divided by the total number of seconds (120), and then multiplied by 100.

Isoelectric EEG is identified as flat or virtually flat EEG. It has been defined as EEG that had an amplitude 1/8 or less of the active EEG amplitude (Gibson et al., 2009). A period of burst suppression was defined as a segment of isoelectric EEG

equal to or greater than 0.5 s in duration. Similarly, to be counted as active EEG or burst activity, the EEG waveform needed to last for 0.5 s or more. Single or small bursts of spike activity were occasionally present during burst suppression. If these groups of spikes were less than 0.5 s in duration they were not considered to be EEG activity and were treated as being continuous with the burst suppression.

The burst suppression results were tested to ensure they met the assumptions for parametric analysis. BSR results were analysed using a two-way repeated measures ANOVA which tested whether there was a significant difference between different concentrations of the same anaesthetic and between anaesthetics at the same concentration. If significant effects of concentration or anaesthetic were found, the ANOVA was followed by post-hoc testing using a Bonferroni adjustment for multiple comparisons. The methoxyflurane results were not included in the ANOVA analysis because the sample sizes were not the same. Methoxyflurane was only tested at progressively decreasing concentrations; therefore there were only three test concentrations rather than the five with the other agents.

2.3.5.2 EEG frequency analysis

The EEG was analysed by Fast Fourier Transformation (FFT) using a purpose-written software program (spectral analyser, C.B. Johnson, 2002), which calculated the FFT for each second of EEG. The FFT generates a frequency spectrum, which is a graphical representation of the contribution that each frequency makes to the power of the EEG waveform. Analysis of the EEG is based on the area under this frequency spectrum graph (Figure 2.2). Variables

derived from the frequency spectrum included the following: the *total power* of the EEG, which is measured as the total area under the frequency spectrum; the *median frequency,* which is the frequency below which half the total power is located; and the *95% spectral edge frequency,* which is the frequency below which 95% of the total power is located (Murrell and Johnson, 2006).

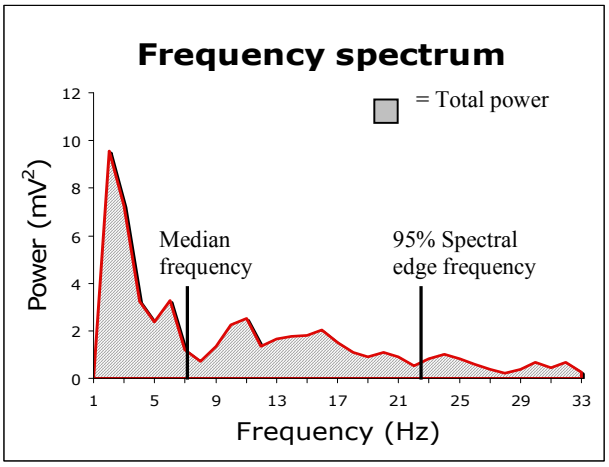


Figure 2.2 An example of a frequency spectrum from 1 second of EEG recorded from a single 1-day-old chick. The different frequencies present in the EEG waveform are plotted along the x-axis and the contribution that each of these frequencies makes to the EEG power is plotted up the y-axis in mV^2 (figure modified from Murrell and Johnson (2006)). The shaded area under the curve is the total power of the EEG.

Where possible, 5 minutes of EEG were collected from the middle of each recording and used in the FFT analysis. Segments containing burst suppression were excluded from this analysis.

All of the results were tested for normal distribution. A mixed model could not be used for this analysis because the sample sizes were different for

methoxyflurane compared to the other three anaesthetics. In addition, extensive burst suppression at 1.5 and 2 MAC compromised the FFT analysis for both Isoflurane and sevoflurane. Instead, for each anaesthetic, a repeated-measures ANOVA was used to test for an overall effect of concentration on median frequency, 95% spectral edge frequency and total power. If a significant concentration effect was found, the ANOVA was followed by post-hoc testing with a Bonferroni adjustment for multiple comparisons. The results for total power using halothane were not normally distributed. The analysis was therefore conducted using the non-parametric Friedman's test followed by the post-hoc test using Dunn's adjustment for multiple comparisons. All results were analysed using Graphpad Prism 5.01 for Windows (GraphPad Software, San Diego California, USA). Differences were considered significant at $P<0.05$.

2.4 Results

The following figures show segments of EEG from four different chickens that received varying concentrations of one of the four anaesthetics: halothane (Figure 2.3), methoxyflurane (Figure 2.4), isoflurane (Figure 2.5) or sevoflurane (Figure 2.6). These segments illustrate the difference in the EEG for the four anaesthetic agents at different concentration.

1 MAC halothane

1.5 MAC halothane

2 MAC halothane

Figure 2.3. Typical EEG segments from one chicken at 1, 1.5 and 2 MAC using halothane. The L-shape indicates 40 μV on the vertical and 0.5 sec on the horizontal axis. Each segment is 6 seconds long.

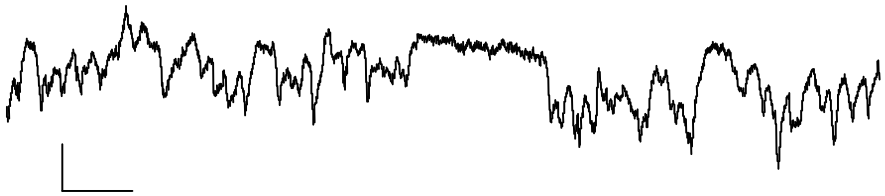
1 MAC methoxyflurane

1.5 MAC methoxyflurane

2 MAC methoxyflurane

Figure 2.4. Typical EEG segments from one chicken at 1, 1.5 and 2 MAC using methoxyflurane. The L-shape indicates 40 μV on the vertical and 0.5 sec on the horizontal axis. Each segment is 6 seconds long.

1 MAC isoflurane

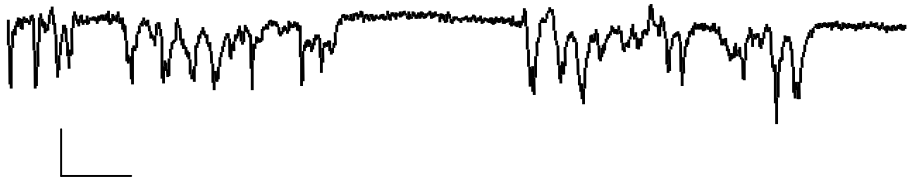


1.5 MAC isoflurane

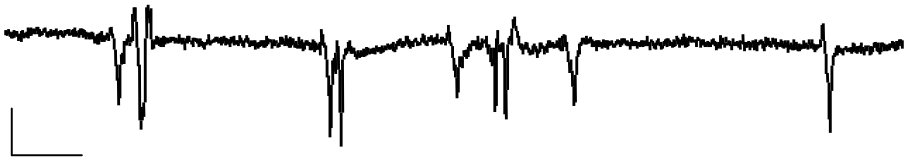
2 MAC isoflurane

Figure 2.5. Typical EEG segments from one chicken at 1, 1.5 and 2 MAC using isoflurane. The L-shape indicates 40 μV on the vertical and 0.5 sec on the horizontal axis. Each segment is 6 seconds long. **Note:** at 2 MAC the trace is predominantly isoelectric. Although there are spikes present, the groups of spikes are both less than 0.5 sec in duration and are separated by more than 0.5 sec of isoelectric EEG. Therefore this particular segment of EEG would have been classed as exhibiting 100% burst suppression.

1 MAC sevoflurane



1.5 MAC sevoflurane



2 MAC sevoflurane

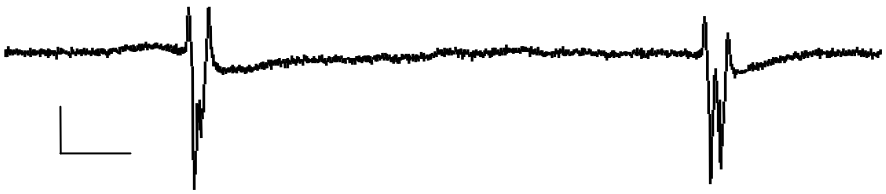


Figure 2.6. Typical EEG segments from one chicken at 1, 1.5 and 2 MAC using sevoflurane. The L-shape indicates 40 μV on the vertical and 0.5 sec on the horizontal axis. Each segment is 6 seconds long.

2.4.1 EEG frequency analysis

Most of the results were normally distributed and met the assumptions required for parametric analysis. The results from halothane total power did not meet the parametric assumptions; therefore the results shown for this variable for halothane come from non-parametric analysis.

The results reported here are from channel one (left side of the brain). The EEG from channel two (right side of the brain) was also analysed. The results from channels one and two were equivalent in both the EEG frequency analysis and in the burst suppression ratios; therefore only the results from channel one are included here. The results from channel two are presented in Appendix A.

Halothane

There was a significant effect of halothane concentration on median frequency ($F(4,44) = 8.5$, $P<0.05$). As halothane concentration increased, median frequency tended to decrease, with the median frequency at 2 MAC being significantly lower than at 1 MAC. In agreement with this, the median frequency tended to increase as the halothane concentrations was decreased. The median frequency was approximately 0.6Hz lower with the halothane concentration at 2 MAC compared to 1 MAC. However, the change in median frequency was not significant as the concentration changed between MAC multiples 1 and 1.5 or 1.5 and 2 (Figure 2.7).

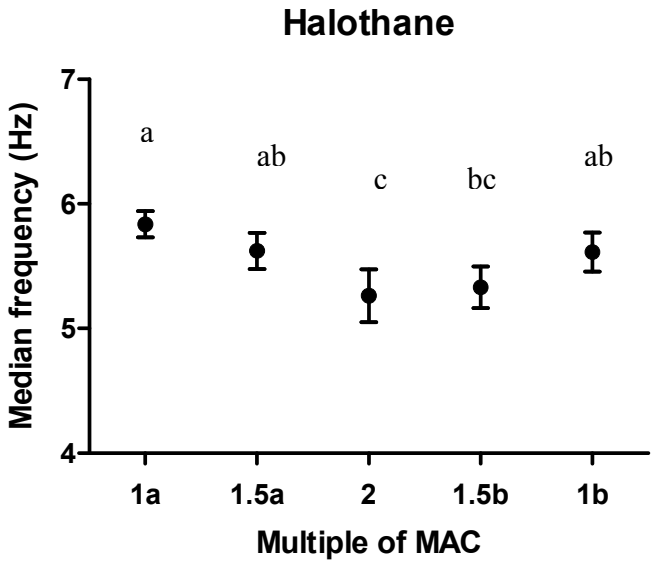


Figure 2.7. Changes in the mean median frequency in response to altered concentration of halothane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $P<0.05$.

Halothane concentration also had a significant effect on 95% spectral edge frequency ($F(4,44) = 7.5$, $P<0.05$). The 95% spectral edge frequency was lower at halothane concentrations of 1.5 MAC than at 1 or 2 MAC. This difference was not statistically significant when the halothane concentration was increased from 1 to 1.5 MAC. However, there was a significant 0.6 Hz increase in 95% spectral edge frequency as the halothane concentration was reduced from 1.5 to 1 MAC. At 2 MAC the mean spectral edge frequency was 0.6 Hz higher than at 1.5 MAC (Figure 2.8).

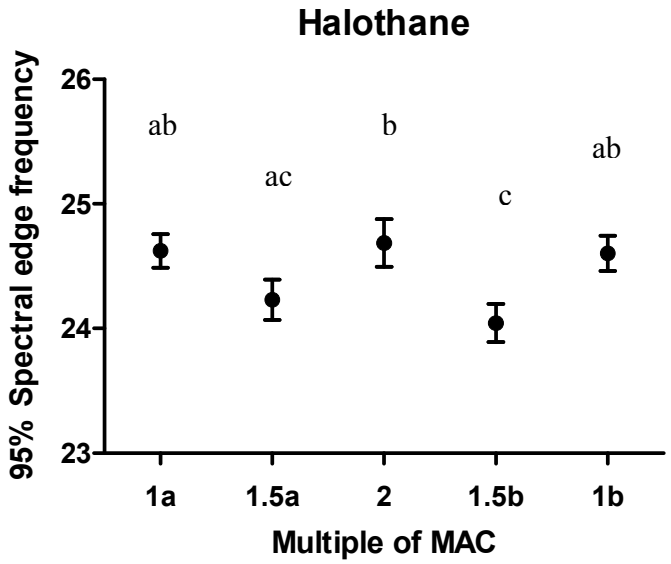


Figure 2.8. Changes in the mean 95% spectral edge frequency in response to altered concentration of halothane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $P<0.05$.

Total power was significantly affected by changes in halothane concentration ($F(4,44) = 50.6$, $P<0.05$). There was a trend for total power to increase as the concentration of halothane increased and for total power to decrease as the halothane concentration decreased. Total power was significantly higher at halothane concentrations of 1.5 and 2 MAC than at 1 MAC in both directions. Total power increased by an average of 5 μV^2 as the halothane concentration increased from 1 to 2 MAC (Figure 2.9).

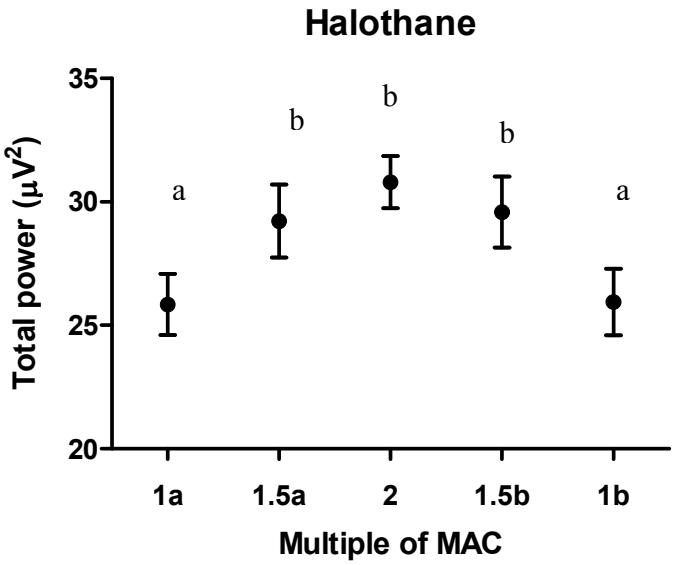


Figure 2.9. Changes in the mean total power in response to altered concentration of halothane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $P<0.05$.

Methoxyflurane

One chicken died during induction with methoxyflurane. A necropsy was performed but the cause of death was not determined. There were therefore EEG recordings from 11 chickens included in the methoxyflurane study. The methoxyflurane concentration had a significant effect on median frequency ($F(2,20) = 3.8$, $P<0.05$), 95% spectral edge frequency ($F(2,20) = 4.0$, $P<0.05$) and total power ($F(2,20) = 5.2$, $P<0.05$). A decrease in methoxyflurane concentration from 2 to 1 MAC caused a significant increase of 0.7 Hz in median frequency (Figure 2.10) and a significant increase of 0.4 Hz increase in 95% spectral edge frequency

(Figure 2.11). Total power increased significantly when methoxyflurane concentration decreased from 1.5 to 1 MAC (Figure 2.12).

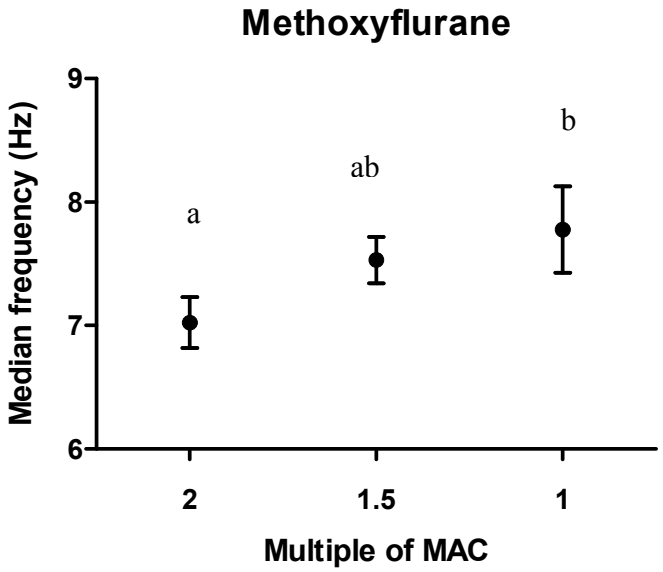


Figure 2.10. Changes in the mean median frequency in response to altered concentration of methoxyflurane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $P<0.05$.

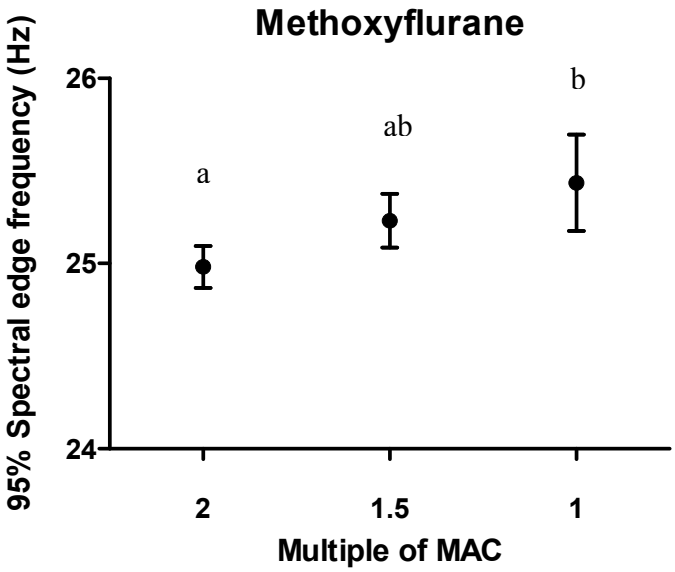


Figure 2.11. Changes in the mean 95% spectral edge frequency in response to altered methoxyflurane concentrations. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $P<0.05$.

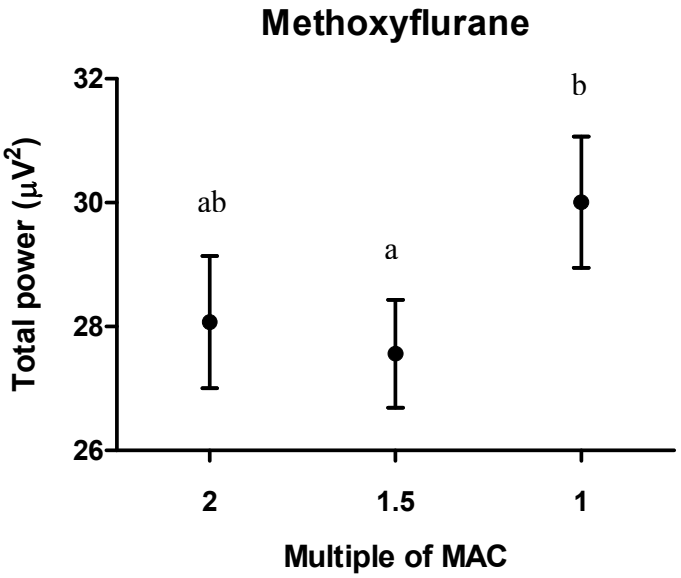


Figure 2.12. Changes in the mean total power in response to altered methoxyflurane concentrations. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $P<0.05$.

Isoflurane

Isoflurane caused burst suppression at 1 MAC. At 1.5 and 2 MAC the high levels of burst suppression meant that FFT analysis could not be used on the EEG. Therefore a comparison of these variables between the concentrations was not possible.

Sevoflurane

Sevoflurane caused burst suppression at 1 MAC. As with isoflurane, at 1.5 and 2 MAC the high levels of burst suppression meant that the FFT analysis could not

be used on the EEG and evaluation of the effects of concentration on these variables was not possible.

2.4.2 Burst suppression

There was a significant interaction between anaesthetic and concentration on BSR ($F(8,33) = 16.36$, $p<0.05$). This interaction shows that the BSR changed with anaesthetic concentration in a way that differed between the different anaesthetics. There was virtually no burst suppression detected during halothane or methoxyflurane anaesthesia (Table 2.2). Methoxyflurane was not included in the statistical analysis because the administration of concentrations was incomplete. Qualitative comparisons suggest that methoxyflurane and halothane caused similar quantities of burst suppression at each concentration.

For isoflurane and sevoflurane, BSR increased significantly with increasing anaesthetic concentration. The BSR was lower at 1b MAC than at 1a MAC for isoflurane. The BSR was higher at 1.5b MAC than 1.5a MAC for sevoflurane. The BSR caused by halothane was significantly lower than that caused by isoflurane and sevoflurane at all concentrations. There was no significant difference between isoflurane and sevoflurane BSR at 1a or 2 MAC. However, isoflurane caused less burst suppression than sevoflurane at 1.5 MAC (both before and after 2 MAC) and at 1b MAC.

Table 2.2 Mean (SEM) burst suppression ratios for the four anaesthetic agents at different concentrations, all values are percentages. Shared subscripts a-e indicate values are not significantly different between concentrations for a single anaesthetic (within column). Shared subscripts x-z indicate values are not significantly different between anaesthetics at the same concentration (across row). Methoxyflurane was not included in the statistical analysis.

MAC	**Halothane**	**Methoxyflurane**	**Isoflurane**	**Sevoflurane**
1a	0 $_{ax}$	---	21.2 (2.3) $_{ay}$	23.0 (2.7) $_{ay}$
1.5a	0 $_{ax}$	---	68.9 (1.8) $_{by}$	81.5 (2.0) $_{bz}$
2	0.9 (0.5) $_{ax}$	0.04 (0.04)	98.9 (0.4) $_{cy}$	99.4 (0.3) $_{cy}$
1.5b	0.03 (0.03) $_{ax}$	0	64.5 (1.8) $_{by}$	84.5 (2.4) $_{dz}$
1b	0 $_{ax}$	0	11.1 (2.2) $_{dy}$	19.4 (2.5) $_{az}$
1a	0 $_{ax}$	---	21.2 (2.3) $_{ay}$	23.0 (2.7) $_{ay}$

2.5 Discussion

The objective of this study was to compare the effects of four different anaesthetics on the EEG of the chicken. Specifically this experiment compared the effects of halothane, methoxyflurane, isoflurane and sevoflurane on the EEG variable median frequency, 95% spectral edge frequency, total power and BSRs.

2.5.1 Main findings

Some of the responses of the chicken EEG were similar for halothane and methoxyflurane. Decreasing the concentrations of both halothane and methoxyflurane increased the EEG median frequency. In addition, burst suppression was absent or virtually absent with both halothane and methoxyflurane.

However, there were also some differences in EEG response to these two anaesthetics. Whereas higher concentrations of methoxyflurane caused a decrease in the spectral edge frequency this variable was lower at 1.5 MAC than at 1 or 2 MAC using halothane. In addition, increasing concentrations of halothane increased total power, while there was a tendency for higher concentrations of methoxyflurane to result in a lower total power.

The chicken EEG responded similarly to isoflurane and sevoflurane; these responses were very different from those seen with halothane and methoxyflurane. Burst suppression was present at all concentrations of isoflurane and sevoflurane, which meant that it was not possible to conduct the FFT analysis on the EEG with these two anaesthetics. Both anaesthetics caused more burst suppression than did halothane and methoxyflurane at all

concentrations, and BSR increased with increasing concentrations of both isoflurane and sevoflurane. Sevoflurane caused more burst suppression than isoflurane at the lighter concentrations. The BSR was lower at 1b MAC than at 1a MAC for isoflurane. However, the BSR was higher at 1.5b MAC than at 1.5a MAC. The reasons for these imbalances are unknown.

Generally, these results show a reduction in EEG frequency during deeper levels of anaesthesia with halothane and methoxyflurane. With isoflurane and sevoflurane there was an increase in the amount of burst suppression during deeper levels of anaesthesia.

2.5.2 Comparison of EEG responses to anaesthesia in birds and mammals

The EEG responses to anaesthesia are qualitatively similar between the chickens in the current study and mammals in previous studies. As was found in the chickens, in mammals, increasing the concentration of an anaesthetic generally causes a decrease in EEG frequencies and, with some anaesthetics, an increase in the amount of burst suppression present in the EEG (Johnson et al., 1994; Johnson and Taylor, 1998; Schwender et al., 1998; Tsushima et al., 1998; Antunes et al., 2003; Orth et al., 2006; Murrell et al., 2008; Otto, 2008). However, the magnitude of the change in the FFT variables and the onset of burst suppression sometimes appear to differ between chickens and mammals.

Below, the similarities and differences of chicken and mammalian EEG responses are discussed separately for each anaesthetic. The changes in FFT variables are difficult to compare directly with previous work because the multiples of MAC

that were used were different. In addition, some of the results from previous mammalian studies are inconsistent with each other, particularly with regard to the way specific EEG variables respond to anaesthesia.

Halothane

In mammals, an increase in halothane concentration generally results in more low frequency activity and less high frequency activity in the EEG and also causes an increase in amplitude. Therefore, as depth of anaesthesia increases there are decreases in both median frequency and 95% spectral edge frequency (Johnson et al., 1994; Tsushima et al., 1998; Antunes et al., 2003).

Consistent with this, the effect of halothane on the median frequency of the chicken's EEG was similar to that observed in mammals in terms of the trend in EEG changes, although the magnitude of the changes sometimes differed. Generally the EEG changes in the chickens were qualitatively smaller in magnitude than they were in mammals.

However, in contrast to some of these previous mammalian studies, the EEG median frequency of rats anaesthetised with halothane increased as the halothane concentration was increased between 1.25 and 1.75 MAC (Murrell et al., 2008). The median frequency of the chicken's EEG decreased as the concentration of halothane increased between 1 and 2 MAC. In rats the total power decreased with increasing anaesthetic concentration (Murrell et al., 2008), whereas in the current chickens total power increased. It is unclear what the reasons are for the differing responses to anaesthetics (i.e. increases or decreases in FFT variables); the differences could be related to differences in how avian and

mammalian nervous systems respond to anaesthesia. Alternatively it may be that the EEG responses to anaesthesia are inherently variable.

The change in the 95% spectral edge frequency also differed between previous mammalian studies and the current chickens. In horses there was a decrease in 95% spectral edge frequency as halothane concentration was increased from 0.8 to 1.2 MAC (Johnson and Taylor, 1998). In the chickens anaesthetised with halothane, the spectral edge frequency trend was not linear. As the halothane concentration increased from 1 to 1.5 MAC the mean spectral edge frequency decreased. When the concentration was increased to 2 MAC, the spectral edge frequency increased (Figure 2.8). It is unknown why the trend in 95% spectral edge frequency reversed as the halothane concentration increased.

Burst suppression has not been reported during halothane anaesthesia in mammals (Antunes et al., 2003; Orth et al., 2006; Murrell et al., 2008). In agreement with this, in the chickens, burst suppression was minimal. This indicates that the suppressive effects of halothane on the EEG are not enough to cause burst suppression.

The changes observed in the rat EEG variables during halothane anaesthesia were described as being small and potentially of little biological significance, although they were statistically significant (Murrell et al., 2008). It was suggested that the dose-response curve for these EEG variables was comparatively flat over the halothane concentrations tested (Murrell et al., 2008). In view of the relatively small changes in EEG variables during halothane anaesthesia in current experiment, there is evidence to suggest that the changes in the chicken EEG are also of little biological significance.

Methoxyflurane

Responses to methoxyflurane appear to be similar for the mammalian and chicken EEG. In horses, increasing the depth of methoxyflurane caused a decrease in median and 95% spectral edge frequencies. Burst suppression did not occur in concentrations up to 1.3 MAC (Johnson and Taylor, 1998). Similar trends in the median frequency and spectral edge frequency were seen for chickens in the current experiment and burst suppression was also absent or minimal.

Isoflurane

EEG responses to isoflurane anaesthesia in mammals are variable. In horses, there was an increase in 95% spectral edge frequency as the isoflurane concentration increased between 1.5 and 1.8 MAC, but no other significant changes in the EEG variables (Johnson and Taylor, 1998). Likewise, studies in rats reveal an increase in 95% spectral edge frequency with increased isoflurane concentrations, but no change in the EEG median frequency (Antunes et al., 2003). In contrast, studies in humans have found a decrease in the 90% spectral edge frequency with increasing isoflurane concentrations (Schwender et al., 1998). Thus, as with halothane, these EEG variables appear to show inconsistent responses to isoflurane anaesthesia. As stated for halothane, these differences may indicate a species difference in the neural response to anaesthesia, or may reflect the variable character of EEG responses to anaesthesia.

Burst suppression due to isoflurane anaesthesia has been consistently observed during mammalian studies. The BSR increased at greater isoflurane concentrations (Johnson and Taylor, 1998; Antunes et al., 2003; Murrell et al.,

2008). In chickens, isoflurane caused burst suppression to such a degree that it prevented frequency analysis of the EEG at concentrations of 1.5 and 2 MAC. It is possible that the gradual change in the EEG waveform with increasing anaesthetic concentration, of the type seen with halothane and methoxyflurane (Figures 2.7-2.12), occur in chickens at lower concentrations of isoflurane than were used in this experiment (Johnson and Taylor, 1998; Antunes et al., 2003). However, in another study of chickens, burst suppression (ratio not reported) was found at isoflurane concentrations of 0.75 MAC (Martin-Jurado et al., 2008).

In rats, the burst suppression ratio was greater than 95% at isoflurane concentrations of 1.25 MAC (Murrell et al., 2008), whereas in the chicken, BSR was only 69% at an isoflurane concentration of 1.5 MAC. The reason for the difference is unknown.

Sevoflurane

There are similarities between mammals and birds in the EEG responses to sevoflurane. Increasing sevoflurane concentration in humans caused a decrease in 90% spectral edge frequency (Schwender et al., 1998). This indicates a decrease in EEG frequency at higher concentrations of sevoflurane anaesthesia. As with isoflurane, sevoflurane caused burst suppression to such a degree that it prevented frequency analysis of the EEG. Sevoflurane seems to have a similar effect on BSRs in both bird and mammalian EEGs (Schwender et al., 1998; Tsushima et al., 1998). As in the chickens, in rats, increasing sevoflurane concentration caused increased levels of burst suppression (Murrell et al., 2008). The BSRs for sevoflurane were similar for the rats studied previously (Murrell et al., 2008) and the chickens studied here.

2.5.3 General comparison between birds and mammals

In general, it appears that anaesthetics affect the avian EEG in a similar way to the mammalian EEG. That is, greater concentrations of anaesthetics cause an increase in low frequency activity and an increase in burst suppression in the EEG (when using anaesthetic agents that cause burst suppression). This suggests that the overall mechanisms of anaesthesia in the nervous system are similar between birds and mammals. There are a few relatively minor differences in the responses to anaesthesia in terms of trends and magnitudes of EEG changes, but on closer inspection these differences are no greater than those that exist between different mammalian species. Reasons for these slight differences in EEG responses to anaesthesia between birds and mammals are not obvious, but may relate to differences in brain anatomy; this will be discussed in more detail in Chapter 4.

2.5.4 Minimum anaesthetic concentration (MAC) and the EEG

In this study, when the various anaesthetics were administered at equipotent levels of MAC the EEG measures were not the same, indicating different levels of brain activity with different agents. MAC is a measure of anaesthetic potency that is based on an agent's ability to prevent movement in response to a noxious stimulus. However, changes in brain activity are not strongly correlated with the MAC measures of anaesthetic depth (Rampil and Laster, 1992). Different anaesthetic agents cause EEG changes (e.g. BSR and frequency changes) of different magnitude and direction at the same multiples of MAC both in the current experiment and in previous studies in mammals (Tsushima et al., 1998; Antunes et al., 2003; Murrell et al., 2008).

When the EEG variables were compared at the same levels of MAC in horses, methoxyflurane had the highest median and spectral edge frequency values, halothane had intermediate values and isoflurane had the lowest values (Johnson and Taylor, 1998). In addition, burst suppression was observed with isoflurane but not halothane or methoxyflurane (Johnson and Taylor, 1998). Likewise, in rats, there was burst suppression with isoflurane and sevoflurane but not with halothane (Murrell et al., 2008).

In the present study it was not possible to statistically compare FFT variables between different anaesthetics because of the high levels of burst suppression for isoflurane and sevoflurane and because the sequence of anaesthetic concentrations differed for methoxyflurane. However, in agreement with mammalian studies, isoflurane and sevoflurane caused greater proportions of burst suppression in chickens than did halothane or methoxyflurane at equivalent multiples of MAC.

These similarities suggest that two previous conclusions applied to mammals (Murrell et al., 2008) may also apply to chickens. Firstly, different anaesthetics are likely to have different mechanisms of action within the nervous system. Secondly, MAC is a concept more related to spinal and nociceptive actions than to central or conscious brain activity. That is, although the anaesthetic MAC (the concentration needed to suppress responses to noxious stimulation) may be at the same multiple for each anaesthetic (i.e. 1, 1.5 and 2 MAC), the central nervous system activity recorded with the EEG differs between the various anaesthetics. Thus, in chickens, MAC appears to be poorly correlated with whole brain (EEG)

activity and is instead more related to mechanisms of the spinal cord (explained further below).

Two of the main effects of anaesthetics, unconsciousness and amnesia, are strongly related to cortical brain function. Cortical function during anaesthesia, which is reflected in the EEG, apparently has little relationship to the anaesthetic concentration (or partial pressure because it's a gas) required to prevent withdrawal responses to noxious stimulation, i.e. the MAC. The mechanisms of anaesthesia that cause loss of movement responses to noxious stimulation are instead likely to be linked to subcortical structures. This conclusion is supported by the observation that rats with their forebrain removed have MAC values that match those of intact control rats (Rampil et al., 1993). The lack of correlation between MAC and the EEG activity during anaesthesia may be explained by the site of action of anaesthetic agents (Antognini and Schwartz, 1993).

2.5.5 Spinal cord as site of anaesthetic action: explaining the absence of a link between MAC and the EEG

The major site of action of anaesthetics appears to be the spinal cord (Antognini, 1997). For example the partial pressure of isoflurane required to achieve MAC increased when anaesthetic was delivered solely to a mammal's brain but not to the spinal cord (Antognini and Schwartz, 1993).

If anaesthetics act primarily on the spinal cord, and the measure of anaesthetic potency, MAC, is based on a spinal mechanism, it seems less surprising that the EEG recordings differ between various anaesthetics at equipotent levels of MAC (Antognini and Schwartz, 1993). For example, in this study there was

significantly more EEG suppression (i.e. burst suppression) with isoflurane and sevoflurane than with halothane or methoxyflurane at all concentrations. MAC is related to the spinal cord, whereas the EEG is related to cortical activity.

It is not known whether there would be burst suppression with halothane or methoxyflurane at deeper concentrations.

2.5.6 Mechanisms of anaesthesia and EEG responses to noxious stimulation

EEG responses to noxious sensory input are also affected by the concentration of anaesthetic delivered to the spinal cord (Antognini et al., 2000a). Isoflurane and propofol both act on the spinal cord to indirectly suppress EEG responses to noxious stimuli (Antognini et al., 2000b; Antognini et al., 2001). Furthermore, when a stimulus is applied that bypasses the spinal cord (i.e. neural stimulation of the reticular formation, cranial to the spinal cord), neither halothane nor isoflurane prevents EEG activation (Orth et al., 2006). This suggests that the action of these anaesthetics is mediated by their action on the spinal cord.

Although anaesthesia effects such as unconsciousness and amnesia appear to be mediated primarily by indirect spinal pathways, there is also likely to be some secondary action of the anaesthetic within the brain. When anaesthesia is isolated to the brain rather than the spinal cord the anaesthetics do anaesthetise the animal, but a higher concentration is needed than if the drug was being delivered to the spinal cord (Antognini, 1997; Antognini and Wang, 1999; Antognini et al., 2000a).

2.5.7 Implications of anaesthetic effects for recording EEG responses to noxious stimuli in anaesthetised chickens

In mammals, noxious stimulation causes changes in EEG activity that can be detected when the animals are under light anaesthesia (Johnson et al., 2005b; Murrell and Johnson, 2006; Gibson et al., 2007; Murrell et al., 2007). These EEG changes are likely to be a product of the cerebral cortex processing the noxious stimulus and have been used to assess pain in mammals (Murrell and Johnson, 2006). The studies outlined in Chapters 4 and 5 of this thesis involved measurement of EEG responses to noxious stimulation in anaesthetised chickens. The information gained from the current experiment was needed for interpretation of the results from subsequent studies.

In mammals, different anaesthetic agents have different effects on baseline/spontaneous EEG as well as different effects on EEG responses to noxious stimulation. In mammals, halothane causes smaller changes in brain activity than isoflurane or sevoflurane at equipotent levels of MAC. For example there was no burst suppression in rats anaesthetised with halothane and the changes in the EEG frequencies with halothane were considered biologically insignificant (Murrell et al., 2008).

Halothane also appears to affect EEG responses to noxious stimulation to a lesser degree than do other anaesthetics. In rats, electrical stimulation of the reticular formation caused changes in EEG activity that were more obvious with halothane anaesthesia than with propofol (Antognini et al., 2006). Likewise, halothane appeared to dampen EEG activation responses of rats to a lesser

degree than did isoflurane (Orth et al., 2006). In contrast, Tsushima et al. (1998) stated that halothane suppressed neural activation in cats, and that central nervous system responses to noxious stimulation are likely to be smaller with halothane than with other agents

Halothane has been the anaesthetic of choice for several studies examining the effects of noxious stimulation on the EEG of anaesthetised mammals (Murrell and Johnson, 2006). These studies have investigated painful events in different mammalian species, including: castration in lambs (Johnson et al., 2005a) and piglets (Haga and Ranheim, 2005); velvet antler removal in deer (Johnson et al., 2005b); dehorning and slaughter in cattle (Gibson et al., 2007; Gibson et al., 2009) and a series of noxious stimuli in rats (Murrell et al., 2007).

The results from the current experiment in birds also show that halothane causes fewer changes in EEG activity when compared to isoflurane or sevoflurane, suggesting that halothane will reduce EEG responses to noxious stimulation to a lesser extent than the other anaesthetics. Therefore halothane is likely to be an appropriate anaesthetic to use in future experiments that involve EEG recording during anaesthesia. Methoxyflurane also caused minimal burst suppression. However, methoxyflurane is harder to obtain, takes longer for the anaesthesia to stabilise and there is some indication that methoxyflurane has greater analgesic properties than halothane (Johnson and Taylor, 1998). Therefore, on balance, halothane remains the preferred agent to use.

In mammals depth of anaesthesia can influence EEG responses to noxious stimuli. For example, in goats anaesthetised with isoflurane at sub-MAC concentrations, noxious stimulation activated cortical and sub-cortical structures

(midbrain reticular formation and thalamus). Once MAC was exceeded, this response was abolished (Antognini and Carstens, 1999). Therefore, for the studies recording EEG responses to noxious stimulation (Chapters 4 and 5), the chickens were lightly anaesthetised with halothane at a value close to MAC for halothane in chickens.

2.5.8 Limitations of the current experiment

The MAC values for the different anaesthetic agents were derived from different studies in the literature and the methods for determining MAC were not completely uniform.

The MAC value for methoxyflurane was also based on MAC for rats and may not have been accurate for chickens. In support of this, the chickens seemed to be at a lighter depth of anaesthesia at 1 and 1.5 MAC with methoxyflurane than with the other anaesthetics. Disturbances such as people talking in the area or doors closing would often cause eye opening or startle movements in the chickens and repetitive loud noises sometimes caused full arousal from anaesthesia. This particularly light state of anaesthesia did not occur with any of the other anaesthetic agents.

Because of this effect, methoxyflurane was administered in a different sequence (2, 1.5 and 1 MAC) to the other agents (1, 1.5, 2, 1.5 and 1 MAC). While this difference in sequence may have limited some of the statistical comparisons between the different agents, it is unlikely to have had a major effect on the results.

The recording durations were different between the four anaesthetics. This was because sevoflurane anaesthesia became stable particularly rapidly, and also the continuous burst suppression during isoflurane and sevoflurane anaesthesia made it unnecessary to record the EEG for a longer period of time. However, anaesthesia was always stable before the EEG recording was started, so this time difference is unlikely to have affected the results.

Furthermore, three anaesthetics were administered to the same group of chickens using a repeated measures design, whereas the fourth anaesthetic (halothane) was used in a different group of chickens.

2.6 Conclusions

This study investigated the effects of different anaesthetics on the EEG of chickens. Halothane caused an increase in the amount of low frequency activity, as did methoxyflurane. Halothane and methoxyflurane caused significantly less burst suppression than did isoflurane or sevoflurane. Burst suppression increased with increasing concentrations of both isoflurane and sevoflurane.

The current results were compared to mammalian studies in the literature. There were many similarities between birds and mammals in the EEG responses to anaesthesia. These similarities included lower frequency EEG activity during deeper concentrations of halothane and methoxyflurane anaesthesia, an increase in burst suppression at deeper concentrations of isoflurane and sevoflurane, and the observation that isoflurane and sevoflurane cause significantly more burst suppression than halothane or methoxyflurane. The differences in the effects of

the different anaesthetic agents on the chicken EEG were generally consistent with differences reported in mammalian studies. Based on BSR, anaesthetics did not cause the same magnitude changes in the EEG at equipotent levels of MAC. The EEG reflects cortical activity, while MAC is based on a spinal cord mechanism. The different anaesthetics having different effects on the EEG activity at the same level of MAC in chickens resembles the situation in mammals, which has been attributed to the spinal cord being the site of action of anaesthetics. Therefore, anaesthetics have the same effect on the spinal cord at the same MAC, but there can be different levels of brain activity at the same MAC. That is, effects from spinal cord to brain differ for different anaesthetics.

The EEG has been investigated as a method of monitoring depth of anaesthesia in mammals. Other measures such as loss of muscle tone, movement, eye-reflexes, or haemodynamics may be unavailable or inaccurate due to drug combinations that are used during surgery (e.g. muscle relaxants and analgesics). As the methoxyflurane and halothane concentrations were increased or decreased there were statistically significant changes in the EEG FFT variables. However, these changes were small and may not be biologically important. Furthermore, there are some contradictions in previous studies of mammals in the details of how EEG variables respond to anaesthesia. These EEG changes might also be difficult to detect in real time such as during surgery. On the other hand, the changes caused by increasing the concentration of isoflurane and sevoflurane (burst suppression) were easy to identify and monitor the changes in depth of anaesthesia. Thus the EEG may be appropriate to monitor anaesthesia in chickens with isoflurane or sevoflurane, but not halothane or methoxyflurane.

Understanding the effects of anaesthesia on the EEG is important when this information is relevant to experimental protocols, for example looking at the effects of noxious stimuli on the EEG of anaesthetised animals. The results from the current experiment are used to inform the interpretation of results from subsequent studies that use anaesthesia in chickens. Similarly to mammals, halothane appears to cause smaller changes in EEG activity than do isoflurane or sevoflurane. Halothane may therefore be a suitable agent to use in further EEG studies with chickens.

2.7 References

Alkire, M. T., Haier, R. J., and Fallon, J. H., 2000: Toward a unified theory of narcosis: brain imaging evidence for a thalamocortical switch as the neurophysiologic basis of anesthetic-induced unconsciousness. *Consciousness and Cognition,* 9: 370-386.

Antognini, J. F. and Schwartz, K., 1993: Exaggerated anesthetic requirements in the preferentially anesthetized brain. *Anesthesiology,* 79: 1244-1249.

Antognini, J. F., 1997: The relationship among brain, spinal cord and anaesthetic requirements. *Medical Hypotheses,* 48: 83-87.

Antognini, J. F. and Carstens, E., 1999: Isoflurane blunts electroencephalographic and thalamic-reticular formation responses to noxious stimulation in goats. *Anesthesiology,* 91: 1770-1779.

Antognini, J. F. and Wang, X. W., 1999: Isoflurane indirectly depresses middle latency auditory evoked potentials by action in the spinal cord in the goat. *Canadian Journal of Anaesthesia - Journal Canadien D' Anesthesie,* 46: 692-695.

Antognini, J. F., Carstens, E., Sudo, M., and Sudo, S., 2000a: Isoflurane depresses electroencephalographic and medial thalamic responses to noxious stimulation via an indirect spinal action. *Anesthesia and Analgesia,* 91: 1282-1288.

Antognini, J. F., Wang, X. W., and Carstens, E., 2000b: Isoflurane action in the spinal cord blunts electroencephalographic and thalamic-reticular formation responses to noxious stimulation in goats. *Anesthesiology,* 92: 559-566.

Antognini, J. F., Saadi, J., Wang, X. W., Carstens, E., and Piercy, M., 2001: Propofol action in both spinal cordand brain blunts electroencephalographic responses to noxious stimulation. *Sleep,* 24: 26-31.

Antognini, J. F. and Carstens, E., 2002: *In vivo* characterization of clinical anaesthesia and its components. *British Journal of Anaesthesia,* 89: 156-166.

Antognini, J. F., Bravo, E., Atherley, R., and Carstens, E., 2006: Propofol, more than halothane, depresses electroencephalographic activation resulting from electrical stimulation in reticular formation. *Acta Anaesthesiologica Scandinavica,* 50: 993-998.

Antunes, L. M., Golledge, H. D. R., Roughan, J. V., and Flecknell, P. A., 2003: Comparison of electroencephalogram activity and auditory avoked responses during isoflurane and halothane anaesthesia an the rat. *Veterinary Anaesthesia and Analgesia,* 30: 15-23.

Arhem, P., Klement, G., and Nilsson, J., 2003: Mechanisms of Anesthesia: towards integrating network, cellular, and molecular level modeling. *Neuropsychopharmacology,* 28: S40-S47.

Baars, B. J., Ramsoy, T. Z., and Laureys, S., 2003: Brain, conscious experience and the observing self. *Trends in Neurosciences,* 26: 671-675.

Dilger, J. P., 2002: The effects of general anaesthetics on ligand-gated ion channels. *British Journal of Anaesthesia,* 89: 41-51.

Dugdale, A., 2010: Chapter 8: Inhalation anaesthetic agents, *Veterinary Anaesthesia: Principles to Practice*. West Sussex, UK: Wiley-Blackwell, 64-75.

Dwyer, R., Bennett, H. L., Eger, E. I., and Heilbron, D., 1992: Effects of isoflurane and nitros oxide in subanaesthetic concentrations on memory and responsiveness in volunteers. *Anesthesiology,* 77: 888-898.

Gibson, T. J., Johnson, C. B., Stafford, K. J., Mitchinson, S. L., and Mellor, D. J., 2007: Validation of the acute electroencephalographic responses of calves

to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal,* 55: 152-157.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Hulls, C. M., Mitchinson, S. L., Stafford, K. J., Johnstone, A. C., and Mellor, D. J., 2009: Electroencephalographic responses of halothane-anaesthetised calves to slaughter by ventral-neck incision without prior stunning *New Zealand Veterinary Journal,* 57: 77-83.

Haga, H. A. and Ranheim, B., 2005: Castration of piglets: the analgesic effects of intratesticular and intrafunicular lidocaine injection. *Veterinary Anaesthesia and Analgesia,* 32: 1-9.

Johnson, C. B., Young, S. S., and Taylor, P. M., 1994: Analysis of the frequency spectrum of the equine electroencephalogram during halothane anaesthesia. *Research in Veterinary Science,* 56: 373-378.

Johnson, C. B. and Taylor, P. M., 1998: Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. *British Journal of Anaesthesia,* 81: 748-753.

Johnson, C. B., Stafford, K. J., Sylvester, S. P., Ward, R. N., Mitchinson, S. L., and Mellor, D. J., 2005a: Effects of age on the electroencephalographic response to castration in lambs anaesthetised using halothane in oxygen. *New Zealand Veterinary Journal,* 53: 433-437.

Johnson, C. B., Wilson, P. R., Woodbury, M. R., and Caulkett, N. A., 2005b: Comparison of analgesic techniques for antler removal in halothane-anaesthetised red deer (*Cervus elaphus*): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia,* 32: 61-71.

Ludders, J. W., Mitchell, G. S., and Schaefer, S. L., 1988: Minimum anaesthetic dose and cardiopulmonary dose response for halothane in chickens. *American Journal of Veterinary Research,* 49: 929-932.

Martin-Jurado, O., Vogt, R., Kutter, A. P. N., Bettschart-Wolfensberger, R., and Hatt, J. M., 2008: Effect of inhalation of isoflurane at end-tidal concentrations greater than, equal to, and less than the minimum anesthetic concentration on the bispectral index in chickens. *American Journal of Veterinary Research,* 69: 1254-1261.

Mayhew, I. G. and Washbourne, J. R., 1990: A method of assessing auditory and brainstem function in horses. *British Veterinary Journal,* 146: 509-518.

Murrell, J. C. and Johnson, C. B., 2006: Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29: 325-335.

Murrell, J. C., Mitchinson, S. L., Waters, D., and Johnson, C. B., 2007: Comparative effect of thermal, mechanical, and electrical noxious stimuli on the electroencephalogram of the rat. *British Journal of Anaesthesia,* 98: 366-371.

Murrell, J. C., Waters, D., and Johnson, C. B., 2008: Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. *Laboratory Animals,* 42: 161-170.

Naganobu, K., Fujisawa, Y., Odhe, H., Matsuda, Y., Sonoda, T., and Ogawa, H., 2000: Determination of the minimum anesthetic concentration and cardiovascular dose response for sevoflurane in chickens during controlled ventilation. *Veterinary Surgery,* 29: 102-105.

Orth, M., Bravo, E., Barter, L., Carstens, E., and Antognini, J. F., 2006: The differential effects of halothane and isoflurane on electroencephalographic responses to electrical microstimulation of the reticular formation. *Anesthesia and Analgesia,* 102: 1709-1714.

Otto, K. A., 2008: EEG power spectrum analysis for monitoring depth of anaesthesia during experimental surgery. *Laboratory Animals,* 42: 45-61.

Raj, A. B. M. and O'Callaghan, M., 2004: Effect of amount and frequency of head-only stunning currents on the electroencephalogram and somatosensory evoked potentials in broilers. *Animal Welfare,* 13: 159-170.

Rampil, I. J. and Laster, M. J., 1992: No correlation between quantitative electroencephalographic measurements and movement response to noxious stimuli during isoflurane anaesthesia in rats. *Anesthesiology,* 77: 920-925.

Rampil, I. J., Mason, P., and Singh, B. S., 1993: Anesthetic potency (MAC) is independent of forebrain structures in the rat. *Anesthesiology,* 78: 707-712.

Schwender, D., Daunderer, M., Klasing, S., Finsterer, U., and Peter, K., 1998: Power spectral analysis of the electroencephalogram during increasing end-expiratory concentrations of isoflurane, desflurane and sevoflurane. *Anaesthesia,* 53: 335-342.

Simons, A. J. R., Boezeman, E. H. J. F., and Pronk, R. A. F., 1989: Automatic EEG monitoring of anaesthesia. *In* Jones, J. G. (ed.), *Bailliere's Clinical Anaesthesiology: International Practice and Research, Vol.3 number 3*. London: Bailliere Tindall, 623-646.

Steffey, E. P., 1996: Inhalation anesthetics. *In* Thurmon, J. C., Tranquilli, W. J., and Benson, G. J. (eds.), *Lumb and Jones' Veterinary Anesthesia 3rd edition*. Illinois: Williams and Wilkins, 297-329.

Steriade, M., Amzica, F., and Contreras, D., 1994: Cortical and thalamic cellular correlates of electroencephalographic burst-suppression. *Electroencephalography and clinical neurophysiology,* 90: 1-16.

Tsushima, K., Shingu, K., Ikeda, S., Kimura, H., Yamada, K., and Murao, K., 1998: Suppressive actions of volatile anaesthetics on the response capacity in cats. *Canadian Journal of Anaesthesia - Journal Canadien D' Anesthesie,* 45: 240-245.

Velarde, A., Ruiz-de-la-Torre, J. L., Rosello, C., Fabrega, E., Diestre, A., and Manteca, X., 2002: Assessment of return to consciousness after electrical stunning in lambs. *Animal Welfare,* 11: 333-341.

Waizer, P. R., Baez, S., and Orkin, L. R., 1973: A method for determining minimum alveolar concentration of anesthetic in the rat. *Anesthesiology,* 39: 394-397.

Chapter 3. Development of the electroencephalogram in chickens before and after hatching

Chick hatching on day 21

Some of the findings from this chapter were presented at the 43rd Congress of the International Society for Applied Ethology in Cairns, Australia 2009.

McIlhone, A. E., Beausoleil, N. J., Mellor, D. J., Mitchinson, S. L., and Johnson, C. B., 2009: Development of electrical activity and responses to noxious stimuli in the chicken brain. *43rd Congress of the International Society for Applied Ethology*: p106 (Abstract).

3.1 Abstract

This study aimed to investigate at what point, before or after hatching, the chick might develop the capacity for consciousness. This is important because the capacity for consciousness determines whether or not the animal can suffer.

Brain electrical activity can indicate the presence or absence of consciousness. The brain activity of chickens before and after hatching was monitored using an electroencephalogram (EEG). The EEG was recorded from three chicks at each day of age between days 12 and 20 of a 21-day incubation. The EEG was also recorded from 11 hatched chicks of different ages up to four days after hatching.

Brain activity was apparent from incubation day 13 onwards. Initially the EEG appeared intermittently, but it became continuous by around day 16. Spectral analysis of the EEGs showed an increasing amount of high frequency activity up to day 17. This activity diminished around the time of hatching but re-emerged in the newly-hatched chick. The spectral analysis variables median frequency, 95% spectral edge frequency and total power all fitted cubic regressions ($P<0.05$).

The decrease in high frequency EEG activity before hatching may be related to the neuroinhibitory effects of an oxygen shortage in the chick towards the end of incubation. The results were not able to provide a definitive answer on when the chick might develop the capacity for conscious. However, with the available evidence, it seems unlikely that the undisturbed chick would be conscious before hatching, and consciousness is likely to emerge gradually after the chick has hatched.

3.2 Introduction

3.2.1 The importance of consciousness

An important question in assessing animal welfare is: "is the animal conscious?" Without consciousness an animal cannot perceive the affective (emotional) components of any positive or negative experiences and so there would be no impact on its wellbeing (Mellor and Diesch, 2006). For example, pain is both a sensory and an emotional experience, so that without the emotional component there would be no unpleasantness associated with nociception (Price et al., 2002).

Chicken embryos are used in medical research (Stern, 2005) and may be subjected to procedures that could be painful or distressing if they were performed on a conscious animal (e.g. exposing the underdeveloped chick during incubation). In addition, large numbers of chicks are incubated and reared after hatching in the poultry industry, and it is important to consider the potential for welfare compromise both before and after hatching. This requires an assessment on the potential for consciousness in those chicks. The objective of this study was to investigate the onset of consciousness in domestic chickens (*Gallus domesticus*). That is, we wanted to determine at what time, between the egg being laid and adulthood, the chick might develop the capacity for consciousness.

3.2.2 Definition of consciousness

It is important to have a clear definition of consciousness for this study. Consciousness is often used interchangeably with the term awareness. In this context to be conscious means that sensory input is perceived and processed by

higher levels of the brain. Responses to sensory input are therefore not simply reflexes (Sommerville and Broom, 1998).

Some authors describe multiple levels of consciousness (Edelman et al., 2005). For example primary consciousness, during which the animal demonstrates perception and motor events; and higher order consciousness, which includes a greater sense of self, reflection on primary consciousness and advanced abilities to construct past and future events (Edelman et al., 2005; Seth et al., 2005). All mammals are thought to at least possess primary consciousness, which includes a sense of perception that may be important when considering the animal's welfare, but it is more difficult to judge the conscious capacity of non-mammalian species (Edelman et al., 2005).

Here, consciousness is defined as a state in which an animal is sensitive to both its internal and external environment in ways that can influence its affective (emotional) experience and, in turn, its welfare. This definition is similar to that of Broom (1988) where the welfare of an animal is its state as regards its attempts to cope with its environment. Therefore, by saying an animal is conscious it is acknowledged that the animal could feel pain, fear, anxiety and other emotions which would be unpleasant for the animal, so the animal's welfare could be compromised by those experiences.

3.2.3 Measures of and prerequisites for consciousness

Mature mammals are generally accepted as having the capacity for consciousness (Seth et al., 2005). However, the subjective experience of consciousness makes it difficult to measure. So, on what basis could it be agreed

that an animal is or is not conscious? In practice an individual can only know their own experience of consciousness; the experience credited to animals is based on assumptions and examining their behaviour, anatomy and physiology (Weiskrantz, 1995; Edelman et al., 2005).

People have studied non-human mammals and non-mammalian species to assess whether those animals have: a) neural structures that could generate consciousness, b) similar neural dynamics (circuits and activity) to those found in animals that are accepted as having the capacity for consciousness and c) behaviours that suggest capacity for memory and perceptive states. Birds of various species have been found to fulfil all these criteria and there is a strong likelihood of them being capable of consciousness as it is defined here (Edelman et al., 2005). The evidence for this is explained below.

a) Neural prerequisites for consciousness

Consciousness is a product of the complex workings of the animal's central nervous system. The brain integrates sensory inputs from multiple areas and other information, including memory, and uses this to create current experience (Butler et al., 2005). Consciousness cannot be located in any one brain area. Instead it appears to be generated by the network of information transfer between multiple regions (Seth et al., 2005).

In mammals, the cerebral cortex and thalamus play a crucial role in the experience of consciousness (Seth et al., 2005). Evidence from stroke and brain injury patients supports the importance of the thalamo-cortical circuit and the mesencephalic reticular formation in consciousness. For example, small disruptions to the cortex can affect the experience of consciousness, yet damage

to the lower brainstem or the thalamus can abolish consciousness. Damage to regions such as the hippocampus or cerebellum does not seem to affect consciousness. It appears that the lower brainstem maintains consciousness, whereas the cortex and thalamus modulate the experience of consciousness (Seth et al., 2005).

If a certain neural structure present in one animal species is missing in another this does not always imply that its function is also absent, as there may be analogous systems operating to fulfil that function. Different species may have evolved their own experience of consciousness, appropriate to their habitat and way of life and within the bounds of their body structure and nervous system (Butler et al., 2005).

From a basic anatomical view, the avian brain appears very different from that of the mammal. The mammalian brain has a cerebral cortex which has a folded appearance and forms a mantle over much of the rest of brain. This cortex also has a layered neural structure (the neurons are lined up and layered in sheets within the cortex) (Medina and Reiner, 2000). In contrast, the neurons in the avian cerebral hemispheres are functionally arranged into nuclei (groups of neurons). The avian hemispheres lack the distinctive folded-mantle appearance of the mammalian cortex, and do not posses the same layered structure (Medina and Reiner, 2000). However, the avian brain has circuitry that resembles that in the mammalian brain in terms of the connections between regions that have particular functions. The neural circuit within the bird's dorsal pallium is similar to the mammalian ganglia-cortico-thalamic loop, which is involved in generating consciousness (Medina and Reiner, 2000).

If anatomically different structures generate consciousness in different species, there may be a range of different experiences of consciousness, each attuned for the animal in question (Butler et al., 2005). This seems appropriate when one considers the number of different niches occupied by animals and their different sensory capabilities, for example an earthworm compared to a rodent. Animals that are able to undertake more complex behavioural tasks are likely to have more complex nervous systems and a different experience of consciousness from animals that have more simplistic lifestyles (Weiskrantz, 1995).

b) Neural activity

Previous authors report that in many respects the electrical activity of the wakeful adult avian brain is very similar to that of mammals in terms of the shape and frequencies of the EEG waveform (Ookawa, 2004). However, other studies report differences between avian and mammalian brain electrical activity during sleep, for example the absence of sleep spindles and the high prevalence of unihemispheric sleep in birds (Ayala-Guerrero and Vasconcelos-Duenas, 1988; Ayala-Guerrero, 1989; Ookawa, 2004; Edelman et al., 2005). It is difficult to conclude what this may mean in terms of consciousness, except that the wakeful EEG of mammals and bird appear to be similar.

c) Behavioural assessments

Some studies have attempted to find behavioural tests that could be used to assess animals' capacity for consciousness. However, animals can perform a range of behaviours without the experience of consciousness. In mammals, movements mediated at a sub-cortical level do not need conscious involvement to be performed (Lee et al., 2005). For example, a noxious stimulus can cause an animal to withdraw a limb from the stimulus. However, this withdrawal is a

reflex movement, initiated by peripheral nociceptors activating a motor response via the spinal cord, and does not necessarily involve the higher brain function or consciousness (Lee et al., 2005). If an animal has the necessary neural structures, a signal is relayed to its brain as the reflex is occurring, and if the animal is conscious and awake, it will perceive the noxious stimulus as pain. Yet the reflex itself can occur independently of conscious awareness of the event (Lee et al., 2005; Mellor et al., 2005).

Sleep arousal responses involve an afferent signal spreading through the reticular activating system and the thalamus potentially to the cortex causing the animal to wake up. However, these responses can initiate changes in posture or breathing, either with or without cortical/conscious involvement, so conscious wakefulness does not always occur (McNamara et al., 1998; McNamara et al., 2002).

Therefore, if behaviour is to be used as an indicator of consciousness, then the tests need to avoid reflex behaviours and instead must use more complex brain functions and interactive behaviours. For example, such behavioural tests often require the use of anticipation and adaptation (Weiskrantz, 1995) for example, transitive inference and problem solving (Butler and Cotterill, 2006). This way the test is more likely to be linked to a measure of consciousness.

Animals that demonstrate advanced cognitive abilities, such as the ability to problem solve, are also likely to possess consciousness (Butler and Cotterill, 2006). However, consciousness cannot necessarily be ruled out in animals that do not display such cognitive abilities (Butler and Cotterill, 2006). A limiting factor in behavioural tests is that the range of animals that can be tested is

restricted by whether or not they can be trained to respond appropriately in the test conditions (Seth et al., 2005).

In many cognitive tests, adult birds of a particular species perform complex behaviours that require thought processes linked with consciousness (Butler et al., 2005; Butler and Cotterill, 2006). These behaviours include the use of tools, transitive inference, sophisticated song learning, use of spatial memory and, in some cases, word comprehension (Edelman et al., 2005; Butler and Cotterill, 2006).

Despite differences in neural anatomy, the similarities in the neural capacity and behaviour between birds and mammals provide strong evidence for the presence of consciousness in the adults of at least some bird species (Edelman et al., 2005; Butler and Cotterill, 2006).

3.2.4 Onset of consciousness in young animals

Accepting that the adult chicken is capable of consciousness, this study aims to determine the point during development at which consciousness first becomes apparent. It seems highly unlikely that consciousness could be present straight after conception, as there are only a few cells present and no neural structures (Romanoff, 1960; Rogers, 1995). Yet the domestic chicken is a precocial species, that is, it hatches in a well developed state, and is active and interacts with its environment within hours of hatching (Cusick and Peters, 1973). This suggests that the neural structures necessary for consciousness are likely to be present before the chick hatches out of its egg.

3.2.5 Brain needs to be functional for consciousness to be present

In addition to the required brain structures being present, these also need to be functional for the animal to support consciousness. Animals can have all the neural structures required for consciousness and still be unconscious, with states of sleep, anaesthesia and coma being three examples of this (Boveroux et al., 2008). Therefore, rather than looking for the presence of certain brain structures in chicks at different ages, it may be more appropriate to use measures of brain function to see when the chick might first develop the capacity for consciousness.

The neural structures necessary for consciousness in the lamb are present before birth. Yet throughout gestation there are several mechanisms acting on the foetus to inhibit electrical activity of the cerebral cortex until after birth (Mellor and Gregory, 2003; Mellor et al., 2005; Mellor and Diesch, 2006). The foetus is exposed to various placental chemicals including adenosine, allopregnanolone, pregnanolone, prostaglandin D_2, and a placental peptide, as well as warmth, cushioned tactile stimulation and buoyancy, all of which have potential to inhibit cerebral cortical electrical activity (Mellor and Diesch, 2006, 2007).

After birth the lamb begins pulmonary respiration, and arterial oxygen tensions increase, which reduces adenosine inhibition of cerebral cortical activity. The exposure to allopregnanolone and pregnanolone and their anaesthetic and sedative actions is reduced, facilitating the onset of awareness. Furthermore, increased oestrogen secretion from the dam before and during birth may have an arousing effect on the lamb. Other environmental factors may also have potential to arouse the lamb immediately after birth. These include first exposure to colder temperatures than those *in utero*, hard surfaces, licking by the mother and gravity (Mellor and Gregory, 2003; Mellor and Diesch, 2006, 2007).

It is possible that, like the foetal-lamb, the pre-hatched chick could be under the influence of environmental conditions that maintain unconscious states until after hatching. For example, oxygen supply to the chick is limited by diffusion across the egg shell (Romanoff, 1960) which may limit brain function (Kraaier et al., 1988; Ozaki et al., 1995). The chick may be exposed to neuroinhibitory hormones present in the yolk (Lipar et al., 1999; Mostl et al., 2001). The chick also becomes increasingly cramped with its neck folded within the egg before hatching which seems to affect brain activity and arousal behaviour (Corner et al., 1973; Bekoff and Sabichi, 1987). These factors may have inhibitory effects on the chick's brain activity, which could influence the onset of consciousness (Mellor and Diesch, 2007).

3.2.6 The electroencephalogram (EEG)

The EEG records the electrical activity of the brain, and because the EEG is strongly linked to cortical activity, it has been used to assess the potential for consciousness in animals (Velarde et al., 2002; Baars et al., 2003; Raj and O'Callaghan, 2004).

3.2.7 Onset of consciousness in mammals and chicks

Forty to fifty years ago the EEG was used to explore some aspects of the development of brain activity in young mammals and birds (Tuge et al., 1960; Ellingson and Rose, 1970). The domestic chick exhibits behavioural and EEG evidence of consciousness within hours after hatching (Cusick and Peters, 1973), suggesting that its brain is likely to have developed sufficient functionality to support consciousness before hatching. Measurement of pre and post-hatching

EEG waveforms may therefore provide an indication of when the onset of consciousness occurs.

There are a few limitations in the previous EEG studies in chicks. Due to limitations in the technology available at the time, most analyses of the EEG were done visually and described simply as counts of "waves per second" (w.p.s), equivalent to the unit Hertz or Hz. More information may now be gained from up-to-date digital technology that permits quantitative spectral analysis of the EEG waveforms (Murrell and Johnson, 2006). There are also periods during incubation for which there are few reports of brain electrical activity, particularly from days 18 to 20. Finally, conclusions regarding the state of the chick around the time of hatching are conflicting, with some reports stating that the chick is conscious during hatching (Bakhuis and Van de Nes, 1979; Bakhuis and Bour, 1980), while others stating that it is not (Peters et al., 1965).

More recently the EEG has been used, together with behaviour, to draw inferences about the onset of consciousness in mammalian species (Diesch et al., 2009b). The literature has also been reviewed regarding the onset of consciousness in chickens (Mellor and Diesch, 2007).

3.2.8 Study objective

The overall goal of this study was to estimate the time of the onset of consciousness in chicks. As a starting point, the specific aim of this study was to use quantitative EEG analysis to re-examine the onset and development of brain electrical activity in chicks during incubation and after hatching.

3.3 Materials and Methods

The objective of this study was to collect a series of electroencephalogram (EEG) recordings to evaluate how brain activity develops in the domestic chicken (*Gallus domesticus*) before and after hatching. All procedures were approved by the Massey University Animal Ethics Committee (protocol number 07/61).

3.3.1 Animals

Forty six fertile chicken eggs and three one-day-old male chicks, all of the Hyline Brown layer strain, were obtained from a commercial hatchery (Golden Coast Commercial, New Plymouth, New Zealand). The eggs were delivered to Massey University within one day of being laid and were then incubated at 38.5°C (± 0.5°C) in a still air, Brinsea Octagon 10 incubator (Brinsea® Products Ltd, North Somerset, United Kingdom). Most of the eggs were placed in the incubator on the day of arrival. However, to manage the numbers of eggs due to hatch each day, six eggs were stored at 7°C for between one and 14 days before being transferred to the incubator. The eggs were turned daily while refrigerated; this involved slowly tilting the eggs from side to side five to six times.

The eggs were incubated with their rounded end (base) facing upwards and were turned automatically by the incubator, which gradually tilted them by 180° every hour, for the first 19 days of the 21-day incubation. On day 19 the turning mechanism was inactivated and the eggs were left in the base-upwards position for the remainder of the incubation period. The humidity during incubation was regulated according to the manufacturer's instructions, but the level of humidity was not measured. The recommended relative humidity levels for this model of incubator were 40-50%, with an increase to 70% or more after the first chick starts

to pip the eggshell (Brinsea Octagon 10 User Instructions). These humidity levels are similar to those recommended by others for chicken eggs (Hamburger and Oppenheim, 1967; Speciale Jr et al., 1975). To achieve the recommended humidity levels, one of the incubator trays was filled with water for the first 19 days of incubation and both trays were used on day 20. The incubator temperature and the water level in the trays were checked daily.

The eggs were candled on days 10, 15, 18 and 20 to follow the chicks' growth. Candling involved shining a 10W light against the shell of the egg and thereby revealing a silhouette of its contents. The chick and yolk components were visible as dark shadows, whereas the air cell, located at the base of the egg, looked clear and empty.

The hatched chicks were reared under a 24-hour light regime in groups of two to four. They were provided with a heat lamp, wood-shavings substrate, a perch and food and water *ad-libitum* (Harvey's chick starter crumbles, Ingham Feeds and Nutrition, Hamilton, NZ).

3.3.2 Experimental protocol

EEG recordings were collected from at least two chicks on each day of incubation from day 12 through to day 20. A recording was collected from one chick at day eight, but because no EEG was seen, recording at this age was not repeated. EEGs were also recorded from 11 hatched chicks, which ranged in age from immediately after to four days after hatching.

The methodology differed slightly between the three developmental groups. For clarity these groups were defined as follows:

a) *Pre-hatched chicks* were those that were still incubating inside their eggs.
b) *Newly-hatched chicks* were those that had completed their incubation and had just broken out of their egg. EEG recording electrodes were applied within 0.5 to 2 minutes of the chick breaking its head, neck and thorax out of the egg.
c) *Hatched chicks* were those that were two hours to four days old (post hatch).

3.3.3 Accessing Pre-hatched chicks

To record the EEG of a pre-hatched chick, an egg at the required stage of incubation was randomly selected from the incubator. The method for accessing the pre-hatched chicks was based on that of Hamburger and Oppenheim (1967). Each egg was candled to identify the size and shape of the air cell. The air cell orientation and position of any visible blood vessels were outlined on the eggshell with pencil. The base of the egg was pierced using a pair of micro-dissecting forceps, which were then used to remove the shell from over the air cell area to gain access to the underlying chorioallantoic membrane. The opened egg was placed in a purpose-built heated water coil made of copper piping that was connected to a heat-pad water pump. This setup kept the egg warm by circulating warm (~39˚C) water around it.

The chorioallantoic membrane of the egg is highly vascular (Burton and Tullett, 1985). To minimise disruption of the chick's circulation, a small amount of warm

saline was applied to the membrane to make it partially translucent and thereby reveal the major blood vessels running across its surface. The membrane was then tented upwards with a pair of blunt forceps and opened in an area that caused minimal disruption to the large blood vessels. Thermal cautery was used to reduce blood loss from smaller vessels that had been severed or damaged as the membrane was opened.

For chicks used on days 12-16 of incubation, the dark pigmentation of their eyes provided a visual aid for locating the head within the amnion. The orientation and greater size of chicks from day 16 of incubation onwards allowed relatively easy access to their heads. The chick's head is usually closer to the air cell from day 16 onwards. The curved tip of a pair of haemostats (artery forceps) was used to lift the head out of the egg and rest it on the edge of the eggshell opening. An exception was made with chicks from days 19-20 of incubation, which were disturbed as little as possible. Care was taken to avoid damaging the delicate tissues of the chick or the fragile membranes and blood vessels.

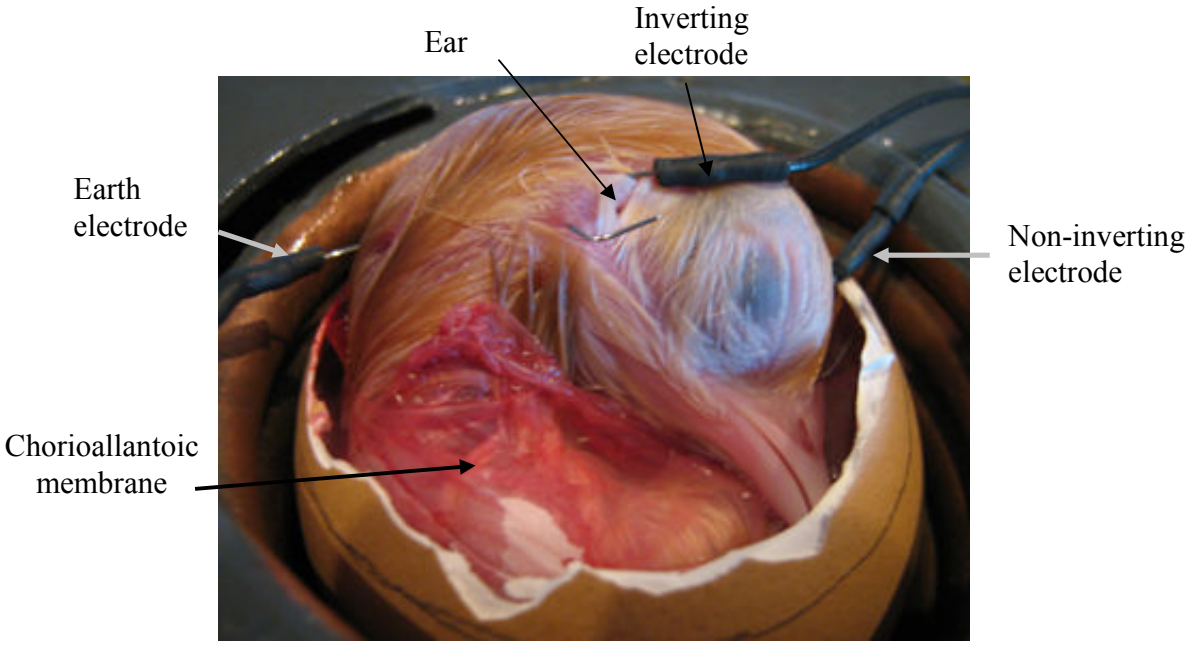


Figure 3.1 Experimental setup for recording EEG from a pre-hatched chick. This chick was used on day 19 of incubation. The pencil mark on the egg shell shows the outline of the air cell detected during candling before the egg was opened. The egg is inside the heated-water copper coil used to keep the chick warm.

3.3.4 EEG recording setup for pre-hatched chicks

Three 27-gauge subcutaneous, stainless-steel needle electrodes (Viasys Healthcare, Surrey, England) were used to record a single channel of EEG from the right side of the chick's head (Figure 3.1). The electrode positions were based on the montage described for horses by Mayhew and Washbourne (1990). The electrode sites were: lateral to the comb (non-inverting electrode); caudal to the external auditory meatus (inverting electrode); and dorso-medially on the back of the neck, caudal to the base of the skull (earth or ground electrode). The electrode cables were made of a flexible 1mm diameter insulated copper wire.

The three EEG recording electrodes were connected to a breakout box, plugged into a physiological signal amplifier (Iso-Dam isolated physiological signal amplifier, World Precision Instruments, Sarasota FL, USA). The amplifiers provided a signal gain of 1000 times and recorded frequencies between 0.1 Hz and 0.1 kHz, i.e. high-pass filters were set at 0.1 Hz and low-pass filters were set at 0.1 kHz. The amplifier fed into an analogue-to-digital converter (Powerlab, ADInstruments Ltd, Sydney, Australia), which digitised the EEG signal at 1000 points/second. All the EEG recordings were stored on an Apple personal computer using Chart Version 5.5 digital recording software (AD Instruments Ltd).

After the electrodes were in place, when possible, 30 minutes of EEG were recorded with the chick's head out of the egg. The chick's head was then returned, as closely as possible, to its original position inside the egg. For chicks on days 19 and 20 of incubation the head and neck were left folded within the egg and their position was only disturbed slightly during electrode placement. The head position inside the egg sometimes had to be altered to keep either the inverting or non-inverting electrode above the albumen to reduce the chances of the EEG circuit short-circuiting across the albumen fluid.

The chick was disturbed as little as possible during the recording. However, overhead lighting was required for visual observations and the sound of people in the vicinity may also have disturbed the chicks. The recording period for each chick generally lasted for two to four hours, although in some cases, longer recordings were collected (see below).

3.3.5 Age and sex verification

At the end of recording, each chick was euthanised with an intrathoracic injection of sodium pentobarbitone. The chick was then removed from the egg and its age was verified by comparing its appearance and body dimensions to the descriptions of chick development published by Hamburger and Hamilton (1992). The features used to determine age from days 12-18 were: length of beak from anterior angle of nostril to tip of bill; and length of the third (longest) toe (Hamburger and Hamilton, 1992). These measurements were taken with a pair of Vernier callipers.

From day 19-20 the measurements are less diagnostic because the toe length is virtually unchanged and the bill may have shortened due to sloughing of the peridermal covering (Hamburger and Hamilton, 1992). Aging at these stages was instead based on the number of days the egg had been in the incubator. The age was further evaluated by examining features such as the amount of blood in the chorioallantoic membrane and the extent of absorption of the yolk sack, which is usually half enclosed in the body by day 20 (Hamburger and Hamilton, 1992).

The Hyline Brown chicken has been selectively bred so that the female chicks have tan/brown down feathers and the males have pale yellow down feathers (Hyline.International, 2009). The gender of chicks was therefore recorded from day 15 of incubation, after which the colouration of the down was sufficiently distinct to allow their sex to be determined.

3.3.6 Longer-term recordings from pre-hatched chicks

Attempts were made to collect EEG recordings over several days from pre-hatched chicks. A multiple-day EEG recording from a single chick would remove some of the inter-chick variability and provide a more continuous indication of the development of the chick's EEG. EEG was recorded continuously from day 16 until day 21 of incubation for one chick. In addition, EEG was recorded from two chicks overnight from day 19 until day 20.

Specific environmental conditions were needed to maintain an incubating chick long-term while its EEG was being recorded. For example:

Temperature

Temperature fluctuations affect the development of the chick (Hamburger and Hamilton, 1992) and can also affect the EEG (Simons et al., 1989). The heated-water coil, described above, provided warmth to maintain a constant temperature, and the egg and water-coil were both kept inside a polystyrene box to reduce heat loss.

Humidity

Low humidity can cause the albumen and membranes to dry out, which would compromise the chick's survival and/or cause them to stick to the egg shell during hatching. If the humidity is too high the egg will not lose the necessary quantities of water during incubation and/or the chick will be sticky after it hatches, so its down will not "fluff up" properly (Brinsea Octagon 10 User Instructions). A piece of plastic film was placed loosely over the open end of the

egg to reduce evaporative water loss, and a few drops of warm physiological saline were dripped on the chick or the membranes if they appeared to be drying out.

Sterility

An incubating egg provides a warm and nutrient rich environment for growth of microorganisms. It was therefore important to reduce the opportunity for microbial contamination of the egg after it was opened. The electrodes were sterilised before use and were placed on the chick using sterile tweezers and forceps. When the chick's head was out of the egg it rested on a sterile surgical drape. Once the chick's head was back inside the egg, a piece of clean, non-sterile plastic film was placed loosely over the opening to reduce contamination of the egg with airborne microorganisms.

3.3.7 EEG recordings from newly-hatched chicks

The two newly-hatched chicks had their EEG recorded for two to three hours from the time their head was free from the egg. The electrode setup was the same for these chicks as it was for the pre-hatched chicks. The needle electrodes were attached as soon as the head had broken free from the egg. During the recording period, the chicks were kept on a polypropylene cloth resting on a heat-pad. An observer was always present to assist the chicks if they became tangled in the electrode cables. The first chick was alone during the recording period, whereas the second chick had the first as a companion.

3.3.8 EEG recording from hatched chicks

EEGs were recorded from nine hatched chicks, which ranged in age from two hours to four days old. Electrode positions were the same as they were for the newly-hatched chicks. However, these hatched chicks were physically more active than the newly-hatched and pre-hatched chicks, and it was necessary to anaesthetise them to place the electrodes.

The chicks were anaesthetised by placing them in a Perspex induction chamber. Halothane in oxygen was then delivered into the chamber (Rhodia Halothane, Merial Australia Pty, Parramatta, NSW). The initial delivery of anaesthetic was 0.5% halothane with an oxygen flow rate of 2 $Lmin^{-1}$. The halothane percentage was then gradually increased until the chick lost its righting reflex and was behaviourally unresponsive to moderate tactile stimulation. The electrodes were inserted and EEG was recorded as the chicks were recovering from anaesthesia.

During recovery from anaesthesia the chicks began to move, and this disturbed the electrodes and therefore disrupted the EEG recording. Silver/silver-chloride electrodes establish an electrical circuit more quickly than stainless steel electrodes can, allowing the EEG signal to be re-established more promptly after the chick moved (Kamp and Lopes Da Silva, 1999). Therefore, after using the stainless steel electrodes with the first four hatched chicks, silver/silver-chloride electrodes were used for the remaining seven.

The silver/silver-chloride EEG electrode sites were the same as for the stainless steel electrodes. The hatched chicks were kept on a warm surface during the recording periods, with supervision and another chick for company, if one was

available. The chicks were euthanised with an intrathoracic injection of sodium pentobarbitone at the end of the experiment.

3.3.9 EEG Analysis (a) Analysis of EEG states

There were two methods used to assess EEG activity in the chicks before and after hatching. The first analysis examined the states of the EEG during the recording and how much time was spent in each of these states. The second analysis used the Fast Fourier Transformation to determine how the EEG changed at the different ages of development.

Analysis of EEG states

During development the EEG recordings were identified as being movement artefact or one of three waveforms: active EEG, intermediate EEG or isoelectric EEG defined as follows:

> *Active EEG*: spontaneous activity with an amplitude of >5-10 μV for minimum 1 second
> *Intermediate EEG*: Amplitude (voltage) generally <5 μV but with sporadic, short duration spikes with an amplitude of around 10 μV
> *Isoelectric EEG*: amplitude continuously <5 μV
> *Artefacts*: isolated angular spikes with an amplitude of >50 μV, of low frequency usually <0.5 Hz

The amount of the recording occupied by each of these three states was calculated for each chick and expressed as a percentage. These percentages were calculated by examining a series of 10-minute blocks for each chick and noting

the number of seconds in which the recording was active, intermediate or isoelectric EEG or movement artefact.

Each 10-minute block was examined 1 minute at a time for ease of counting. Each minute was examined from start to finish two or three times and counts were made of the number of seconds when the EEG was active, intermediate, or isoelectric.

Sometimes movement artefact prevented identification of the recording as representative of any of the three activity states. These time periods were excluded from the analysis. The percentage values therefore represented the "*percent of the recording that could be identified as one of the three states*". This prevented the results from being biased by the amount of artefact in the recording. For each chick, the following values were calculated:

For each block of 10 minutes (=600 seconds):
Seconds of active EEG + intermediate EEG + isoelectric EEG = total count
Seconds of active EEG/total count * 100 = percent active EEG
Seconds of intermediate EEG/total count * 100 = percent intermediate EEG
Seconds of isoelectric EEG/total count * 100 = percent isoelectric EEG

Defining the 10-minute blocks:

The 10-minute time blocks are outline in Table 3.1. The 10-minute blocks were examined at: 0-10 and 20-30 minutes of recording with the chick's head out of the egg. After its head had been returned to the egg, blocks were examined at 0-10, 20-30, 60-70 and 180-190 minutes. If the recording continued, 10 minute blocks

were examined at 24 hour intervals. In the text these 10-minute blocks will be referred to based on the time, and whether the head of the chick was in or outside the shell, e.g. 0-10HO = time block 0-10 minutes into the recording with the chick's head still outside the egg, 60-70HI = 60-70 minutes in to the recording and the chick's head is back inside the egg.

Table 3.1. Description of the time blocks used for the analysis of EEG states

Block	Chick status	Time
0-10 HO	Head out	0-10 minutes
20-30 HO	Head out	20-30 minutes
0-10 HI	Head in	0-10 minutes
20-30 HI	Head in	20-30 minutes
60-70 HI	Head in	60-70 minutes
180-190 HI	Head in	180-190 minutes
24 Hr HI	Head in	24 Hours
2, 3, 4 & 5 days HI	Head in	2, 3, 4 & 5 days

Statistics for the analysis of EEG states

After calculating the percent values for each 10-minute block the percent values across the time blocks were averaged to give a single mean value for each chick.

These mean values were linearly regressed against the day of age, excluding the results from day 20 using Minitab version 15.1 (2006).

A linear regression was also performed on a log transformed odds ratio of the results, but the outcome was similar to the first regression, so the simplest analysis will be presented in the results.

3.3.10 EEG Analysis (b) Spectral analysis of the EEG

Analysis of the EEG also involved examining the different frequencies of brain electrical activity and calculating the contribution that each of these made to the overall waveform. Four-second segments (epochs) of EEG that were free of movement artefacts were manually selected from within the recordings. A total of 34 chicks were used in this analysis. The number of segments collected varied between chicks because movement artefacts often made sections of the EEG unusable. Between 2 and 44 segments were collected for each chick from various locations throughout the recording.

A video camera synchronised with the EEG recorded the behaviour of both the pre-hatched and hatched chicks during the EEG recording. This synchronised video was played back to clarify the presence or absence of movement artefacts in sections of EEG. Artefacts due to respiratory movements were low frequency, smooth and regular and therefore identifiable against the faster, random EEG signal.

Each 4-second epoch of EEG was processed using the Fast Fourier Transformation (FFT) by means of a purpose written software program (Spectral Analyser, C.B. Johnson, 2002). The FFT programme also applied a 30 Hz low-pass filter, which excluded signals over 30 Hz from the analysis. The FFT generates a frequency spectrum, which is a graphical indication of the contribution that each frequency makes to the EEG waveform within the epoch. Analysis of the EEG was based on the area under this frequency spectrum curve. Parameters derived from the frequency spectrum included: the *total power* of the

EEG, measured as the total area under the frequency spectrum; the *median frequency*, the frequency below which half the total power is located; and the *95% spectral edge frequency*, the frequency below which 95% of the total power is located (Murrell and Johnson, 2006).

3.3.11 Statistical analysis of FFT variables

These FFT results were analysed in two ways. Firstly all the raw multiple samples collected from each chick were included. Thus, there were multiple samples from each chick (between 2 and 44 samples) and multiple chicks at most of the ages (between 1 and 5 at each age). The second analysis took an average over the values for each chick, so that each chick contributed only one value to the FFT variable regression analysis (Figure 3.2).

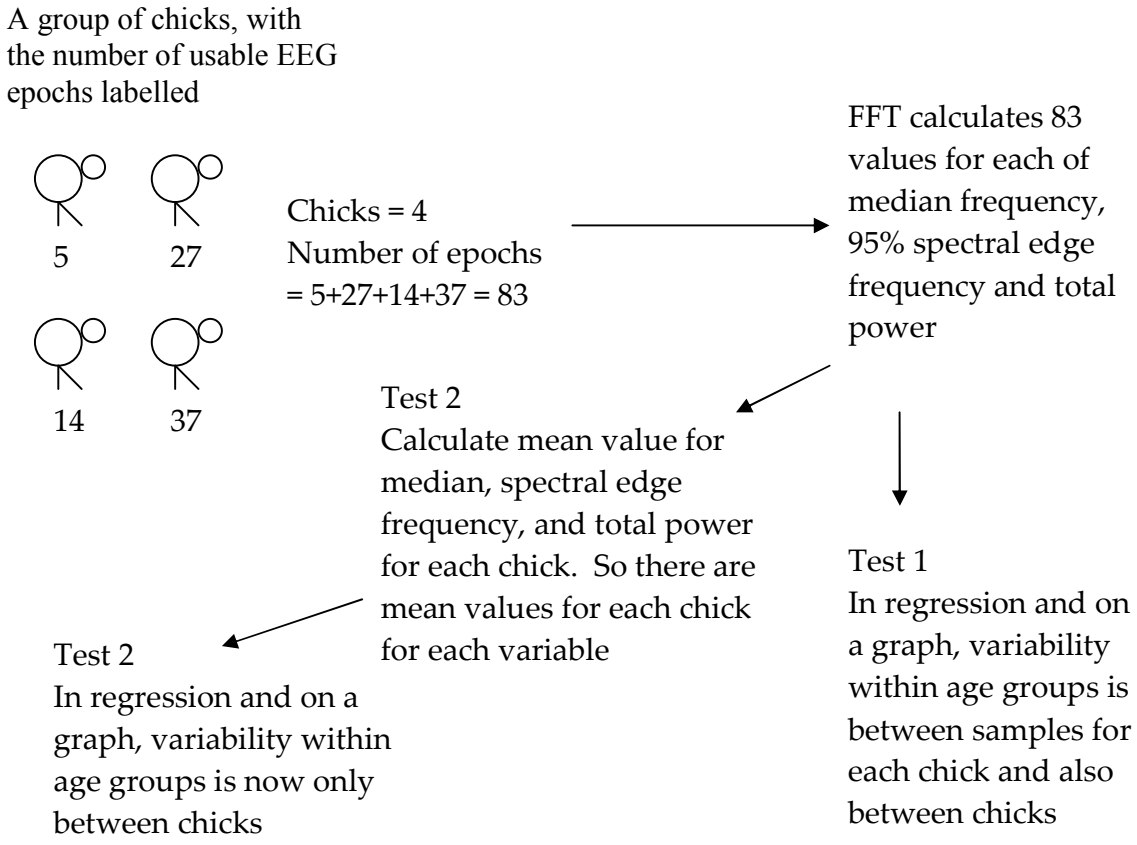


Figure 3.2 A theoretical example of the two methods used for regression

The statistical analyses were conducted using Minitab version 15.1 (2006). A cubic regression was used to detect a developmental trend in the median frequency, 95% spectral edge frequency and total power of the EEGs. The regression tested for linear, quadratic and cubic relationships between each of the EEG variables and the age of the chick. The cubic relationship was the best fit for each variable.

The equations used were:

Median frequency = age x age^2 x age^3

95% Spectral edge frequency = age x age^2 x age^3

Total power = age x age^2 x age^3

3.4 Results

3.4.1 General details

Incubation outcome

Forty-two of the 46 fertile eggs provided pre-hatched or hatched chicks that could be used in the study. Four chicks died prior to any direct experimental intervention, one of them at about day 12 and three at days 18-20 of incubation.

Age and gender of the chicks

The number of days that the egg had been in the incubator usually aligned with the chick's age based on morphological measurements described by Hamburger and Hamilton (1992). If there was any discrepancy between the two values then the developmental stage based on measurements was used to designate the chick's age. Of the chicks whose gender could be identified, three were female and 24 were males.

Candling and accessing pre-hatched chicks

Candling was an effective method of determining the position of both the chick and the major blood vessels within the egg. Thermal cautery was effective for haemostasis of small blood vessels, but not large vessels. For large vessels, it was best to apply pressure if the vessel was sited against the egg shell, or to clamp the vessel with forceps if its end was free in the albumen. For chicks of 12-15 days of incubation in particular, survival was quickly compromised if large vessels were damaged.

3.4.2 Numbers of chicks used and difficulties with EEG recordings

The youngest chick used in the study was eight days through incubation; the oldest was four days post hatching. The number of chicks at each developmental stage varied and is shown in Table 3.2. In pre-hatched chicks the EEG electrodes were easily displaced because the chicks' skin was very fragile until days 15-16 of incubation. Movement artefacts were common in chicks both pre- and post-hatching, and led to recordings from seven chicks being excluded from FFT analysis. Recordings from the four youngest chicks were excluded because no EEG was detected.

Table 3.2 Numbers of chicks studied at each day of age before and after hatching, and the number used in the FFT analysis.

Chick age (days of incubation)	Number studied	Number in FFT analysis
8	1	0 (no active EEG present)
12	3	0 (no active EEG present)
13	5	3
14	3	3
15	5	5
16	3	3
17	5	5
18	4	4
19	3	2
20	2	2
21/newly hatched*	2	2
21 (day of hatching**)	3	3
1 day post hatch	2	0
2 days post hatch	1	1
4 days post hatch	3	1
Total	45	34

* *Newly-hatched chicks* were those that had just broken out of their egg. EEG electrodes were applied within 0.5 to 2 minutes of the chick exiting the egg.

** *Hatched chicks* were those from which the EEG was collected two hours to four days after hatching

3.4.3 Behaviour of newly-hatched chicks

Newly-hatched chicks mostly slept. While awake, they were calmest when they had a companion. Chicks were mobile for short intervals within minutes of hatching. If a chick hatched alone it vocalised in a shrill tone and made efforts to move around the holding box. When a chick had company it would vocalise and the other chick would respond. The vocalisations would then change to a trilling twitter of a lower pitch and volume than the first sounds. The chicks would then settle down close together and continue to cheep back and forth to each other until they fell asleep.

3.4.4 Behaviour of hatched chicks

Older hatched chicks that had been anaesthetised for electrode placement spent most of their time asleep even after the time the anaesthetic had worn off. Usable EEG was only collected during these sleep intervals.

Therefore, between the newly-hatched and hatched chicks, all EEG records were taken when the chicks were either sleeping naturally or recovering from anaesthesia. Hatched chicks were also calmest when they had a companion.

3.4.5 EEG presence and duration during incubation

The number of chicks used in the analysis of EEG states is shown in Table 3.3. The total time spent in active EEG, intermediate EEG or isoelectric EEG, expressed as a percentage of the total recording time at each age is shown in Figure 3.3. The remaining percentage represents the proportion of the total

recording that was contaminated by movement artefacts or other disruptions to the recording which prevented classification of the trace into any of the three EEG states.

Table 3.3 Number of pre-hatched chicks used in the analysis of EEG state at several time points before hatching. HO = head outside the egg, HI = head has been returned to inside the egg.

	Number of chicks in analysis							
Day of incubation	0-10 HO	20-30 HO	0-10 HI	20-30 HI	1hr HI	3hr HI	24hr HI	2,3,4 & 5 days HI
8	1	1	-	-	-	-	-	-
12	1	-	1	-	-	-	-	-
13	5	4	3	3	1	-	-	-
14	3	2	3	3	3	2	-	-
15	5	4	5	5	5	1	-	-
16	3	3	2	2	2	1	1*	1*
17	5	5	4	4	3	-	-	-
18	4	3	4	4	4	2	-	-
19	-	-	3	3	3	3	2**	-
20	-	-	2	2	2	2	-	-

* Long-term recording lasting from day 16 to day 21 of incubation

** Long-term recordings lasting from day 19 to day 20 of incubation

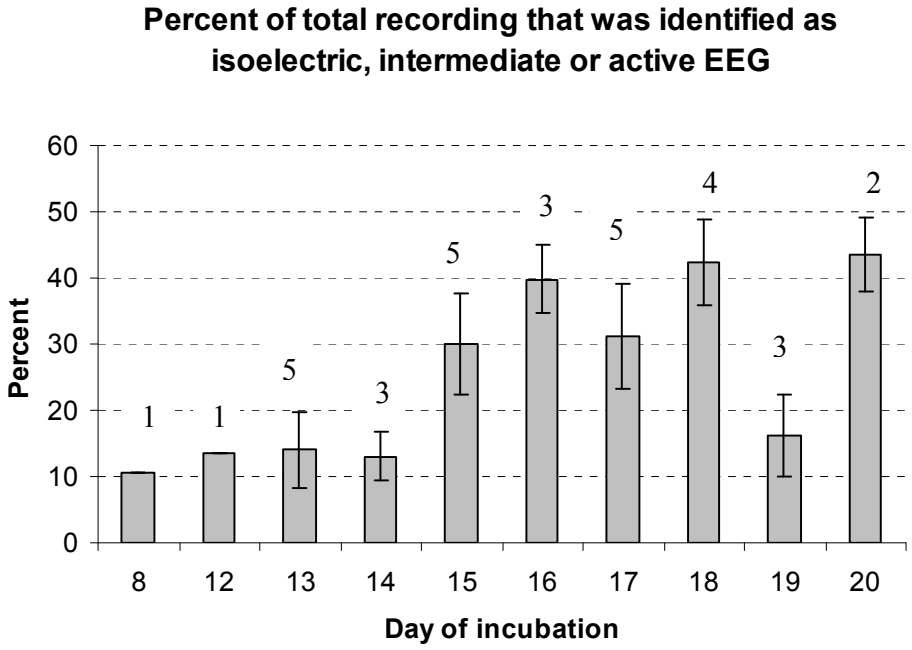


Figure 3.3 Mean (SEM) percent of time that the waveform could be identified as active, intermediate or isoelectric EEG. During the remainder of the time, the recording was contaminated by movement artefact and could therefore not be classified into any of the three states. The numbers above each bar indicate the number of chicks that contributed to the results at that age.

Active EEG

The amount of active EEG present at each age was very similar between the different 10 minute blocks. Therefore, a mean was calculated over all the time points at each age for active EEG, intermediate EEG and isoelectric EEG. More detailed graphs showing proportions of active, intermediate and isoelectric EEG at the different time-points are included in Appendix B.

The EEG waveform emerged gradually during development. It was first seen in pre-hatched chicks at 13 days of incubation. Initially active EEG appeared in epochs separated by periods of isoelectric activity. The percentage of active EEG increased significantly until incubation day 19, and decreased on day 20 (Figure 3.4). The results matched a linear regression between days 8 and 19 ($F(1,28) = 61.08$, $P<0.05$, $R^2=67.4\%$). The regression equation was: % Active EEG = -119 + 10.5 Age. Active EEG was present 100% of the time in the readable traces from all hatched chicks (not shown in Figure 3.4).

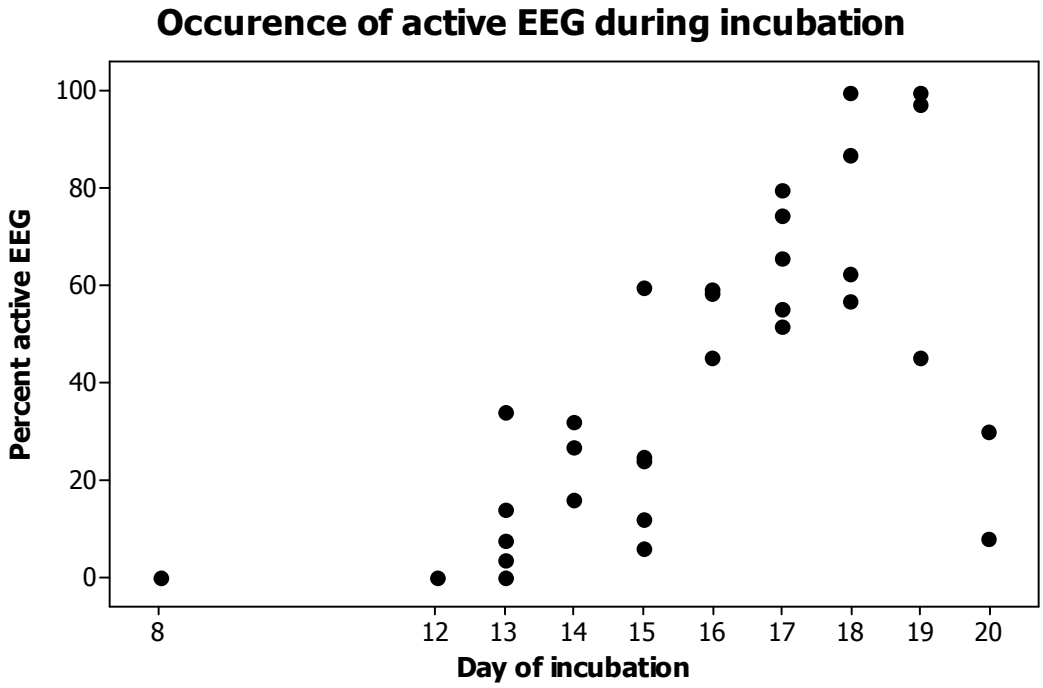


Figure 3.4 Mean percent of EEG that was active EEG in recordings from chicks at different stages of incubation. Each point represents the mean for an individual chick.

Intermediate activity

The amount of intermediate activity appeared to decrease between days 14-19 and was high on day 20 of incubation (Figure 3.5). However, these results did not fit a linear regression between days 8 to 19 ($F(1,28)=1.33$, $P>0.05$, $R^2=1.1\%$). The regression equation was: % intermediate EEG = 76 – 2.35 Age.

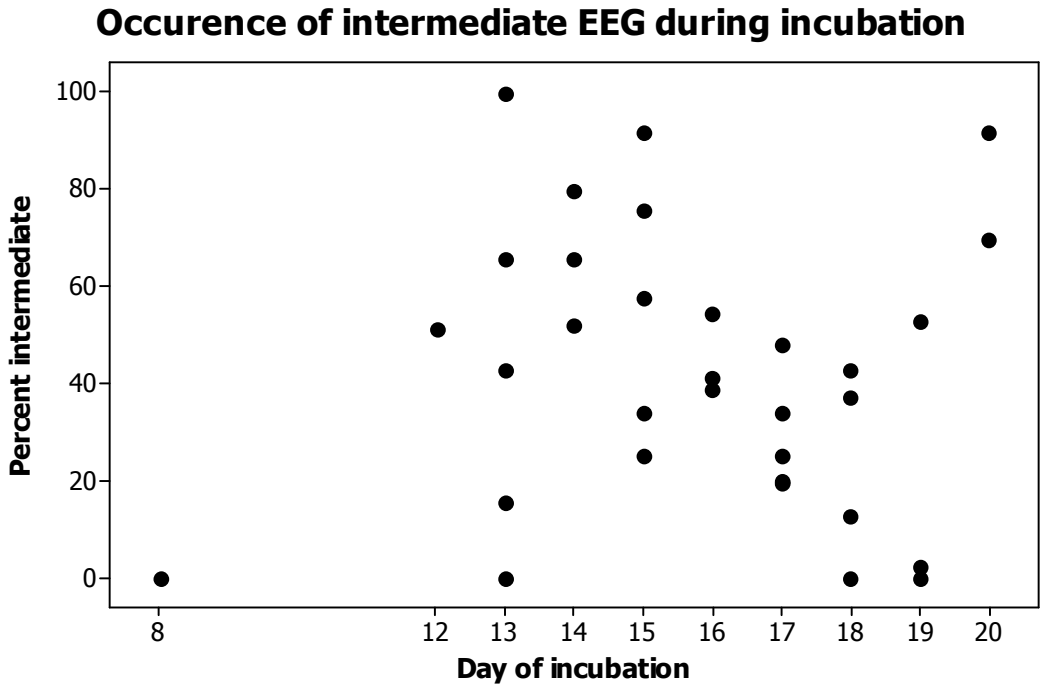


Figure 3.5 Mean percent of the EEG that was intermediate EEG in chicks at different stages of incubation. Each point represents the mean for an individual chick.

Isoelectric activity

The percentage of isoelectric activity decreased during incubation, approaching zero by day 16 of incubation (Figure 3.6). The results from days 8 to 19 matched a linear regression ($F(1,28)=27.34$, $P<0.05$, $R^2=47.6\%$). In most chicks, no

isoelectric activity was found after day 16 (see Table 3 for the number of chicks examined at each age).

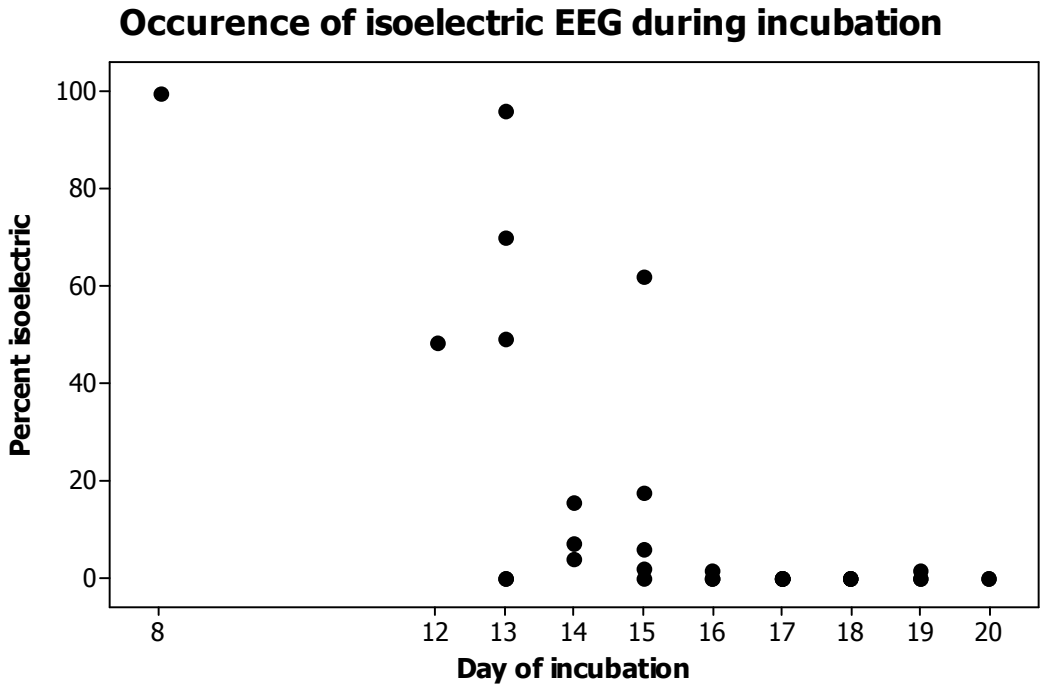


Figure 3.6 Mean percent of EEG that was isoelectric EEG in chicks at different stages of incubation. Each point represents the mean for an individual chick.

3.4.6 Relative proportions of active EEG, intermediate EEG and isoelectric EEG

At day 8 the trace was isoelectric and at day 12 only isoelectric and intermediate EEG was found. All three patterns were present from day 13 until about day 16, when isoelectric activity virtually disappeared. After day 16, only active EEG and intermediate activity were present. The marked decline in active EEG at day 20 was matched by a marked increase in intermediate activity.

3.4.7 Long-term EEG recordings from individual chicks over consecutive days

Success rates for long-term recordings were low, with only 5 of the 13 chicks providing recordings that lasted longer than 8 hours (Table 2). However, short-term recordings from all of these chicks were included in the analysis of results.

Two long-term EEG recordings began in chicks at day 19 of incubation and lasted for about 32 hours. Both chicks hatched on what would have been day 20 of incubation. Both chicks started breathing movements late in day 19, four to five hours after electrode placement. The chicks performed hatching behaviour, but their appearance after hatching was somewhat different from normally hatched chicks. They shuffled on their hocks more frequently, and the yolk of the second chick had not been fully absorbed. Apart from this the chicks appeared healthy and interacted with their surroundings and other chicks in ways similar to those of chicks from this study that hatched at day 21 of incubation.

3.4.8 Comparison of results from short-term and long-term recordings

The percentages of active EEG, intermediate EEG or isoelectric EEG in readable traces (Figures 3.7a, 3.7b and 3.7c) differed depending on whether the recordings were made over hours (short term) or days (long term). However, this comparison was restricted because, for the long term observations, only one chick provided EEGs from day 16 to day 21, and only two chicks provided overnight EEGs from day 19 to day 20. The difference between values for short- and long-term recordings on day 16 for active EEG (Figure 3.7a) and intermediate EEG (Figure 3.7b) occurred despite the fact that the chicks had been treated similarly at the time point for recording. There was however, only one

chick in each group at day 16. The results for chicks on day 17 and 18 of incubation were similar in that proportions for the long-term recording were close to the standard error range for the short term recordings. Numerical differences between values from short- and long-term recordings were evident on days 19 and 20.

The readable parts of the EEG recording from one chick between days 16 and 21 consisted entirely of active EEG and intermediate EEG, as there was no isoelectric EEG (Figures 3.7a, 3.7b, 3.7c). The readable traces recorded from the two chicks from day 19 of incubation until after hatching consisted entirely of active EEG (Figures 3.7a, 3.7b, 3.7c).

a)

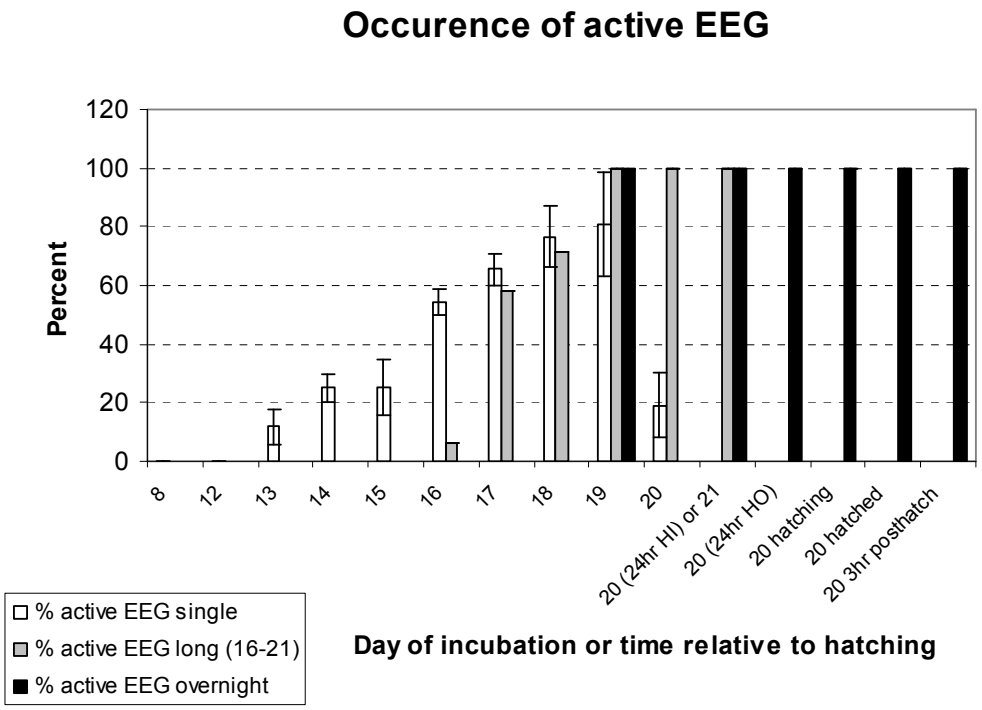


Figure 3.7a Mean % of total EEG that was active EEG at different ages of development. Key: single = 1 value (mean) for pre-hatched chicks at each age of incubation; long (16-21) = long term values from one chick that were obtained between days 16 and 21 of incubation; overnight = the mean values for two chicks that had their EEGs recorded overnight from day 19 of incubation through to hatching on day 20. Error bars = SEM. Note: on the x-axis, day "20 (24hr HI) or 21" was day 20 of incubation for the overnight chick, but day 21 for the *Single day* chicks

b)

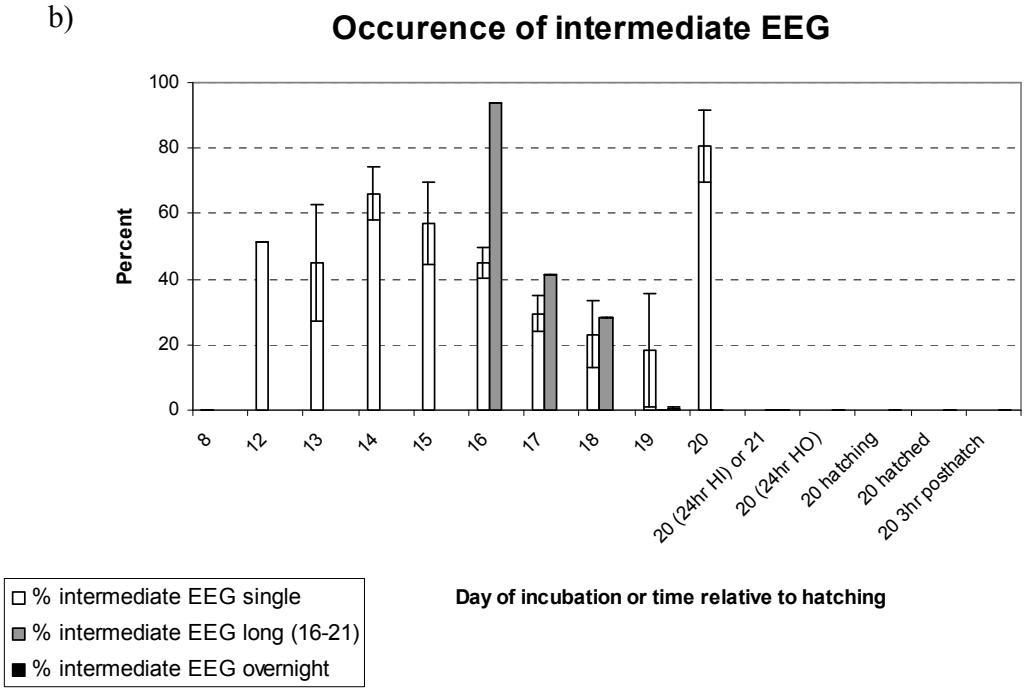


Figure 3.7b Mean % of total EEG that was intermediate EEG at different ages of development. Key: single = 1 value (mean) for pre-hatched chicks at each age of incubation; long (16-21) = long term values from one chick that were obtained between days 16 and 21 of incubation; overnight = the mean values for two chicks that had their EEGs recorded overnight from day 19 of incubation through to hatching on day 20. Error bars = SEM. Note: on the x-axis, day "20 (24hr HI) or 21" was day 20 of incubation for the overnight chick, but day 21 for the *Single day* chicks

c)

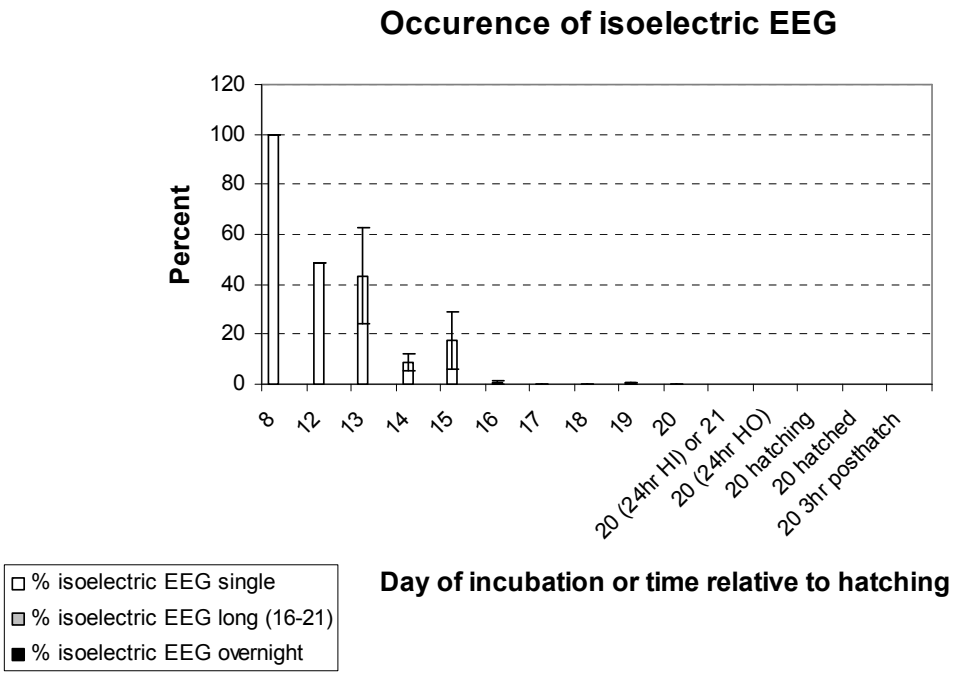


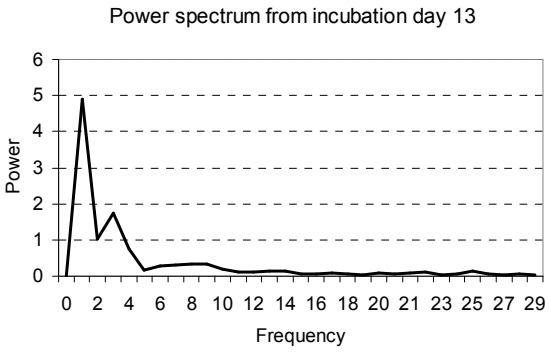
Figure 3.7c Mean % of total EEG that was isoelectric EEG at different ages of development. Key: single = 1 value (mean) for pre-hatched chicks at each age of incubation; long (16-21) = long term values from one chick that were obtained between days 16 and 21 of incubation; overnight = the mean values for two chicks that had their EEGs recorded overnight from day 19 of incubation through to hatching on day 20. Error bars = SEM. Note: on the x-axis, day "20 (24hr HI) or 21" was day 20 of incubation for the overnight chick, but day 21 for the *Single day* chicks

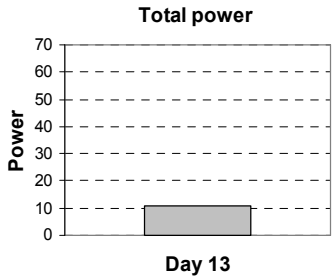
3.4.9 Development of raw EEG traces

The following figures illustrate the changes in the EEG recordings at different ages of development (Figures 3.8a-f). The duration of the EEG and the amount of high frequency activity increased from day 13 to day 18. On day 20 there was less high frequency activity than in previous days (Figure 3.8e). There was an increase in high frequency activity seen in the EEG of the newly-hatched chick (Figure 3.8f). The EEG amplitude increased from day 13 to day 18; there was

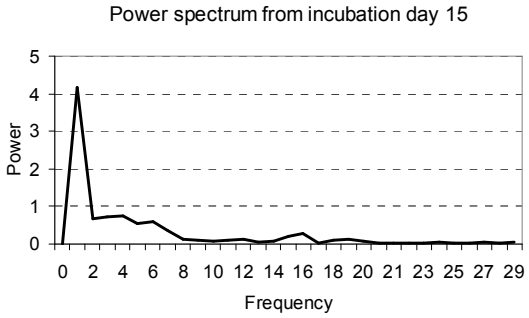
then a decrease in the amplitude of the EEGs from days 19 to 20. The amplitude increased again after the chick hatched. These EEGs were also analysed using the FFT spectral analysis which enabled quantitative statistical analyses, these will be discussed shortly.

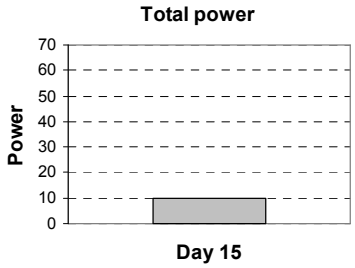
a) Day 13



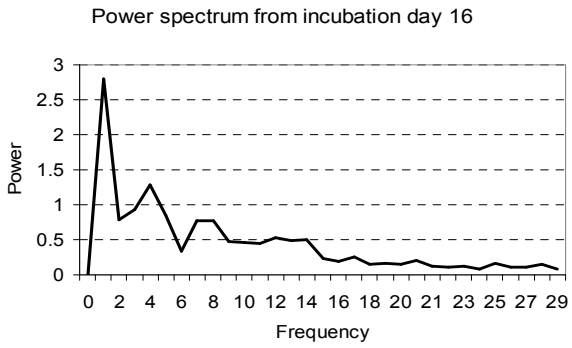


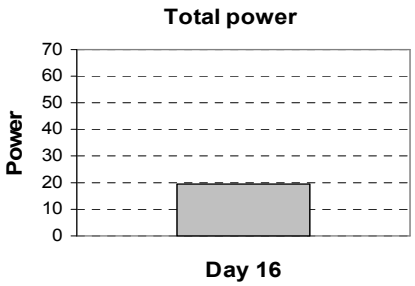
b) Day 15



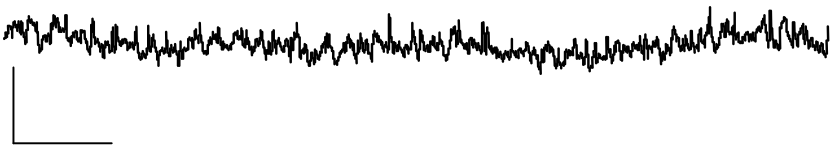


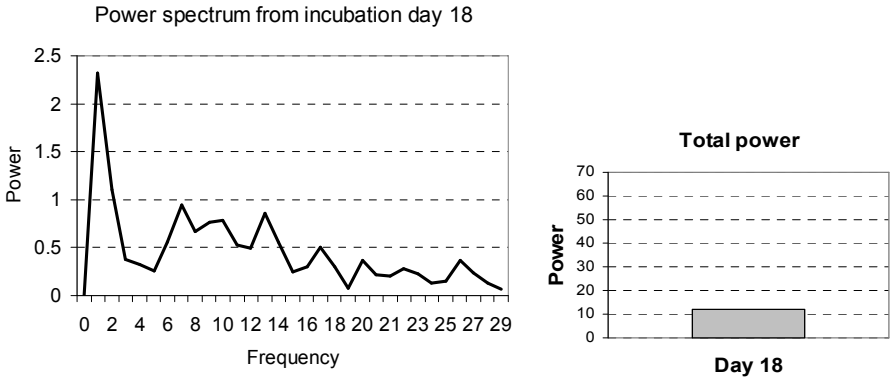
c) Day 16



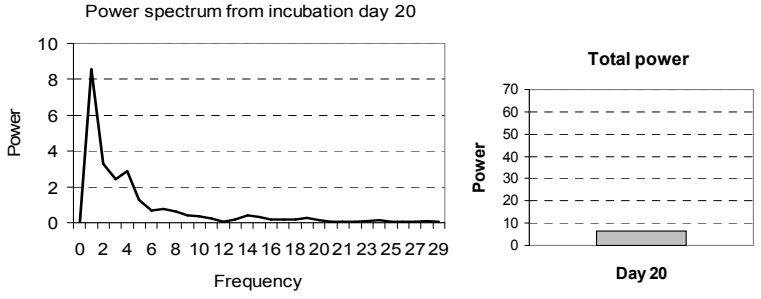


d) Day 18





e) Day 20



f) Newly-hatched chick

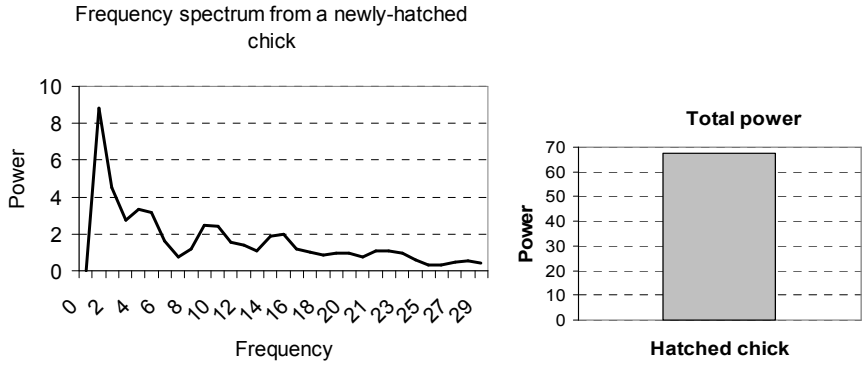


Figure 3.8a-f Examples of segments of EEG taken from six different chicks between days 13 and 20 of incubation, and in a newly hatched chick. Each EEG segment is accompanied by its power spectrum and an indication of the total power of the EEG. The units on the power scale (y-axis) are arbitrary (μV^2); also note the difference in scale on each power axis. The L-shaped scale markers are 40 μV vertical and 0.5s horizontal. The EEG segments are 4 seconds in duration.

3.4.10 FFT analysis

Sample sizes

Ideally, the analysis would include an equal number of chicks at each age of incubation, and each of these chicks would provide an equal number of EEG epochs for inclusion in the FFT analysis. However, the number of chicks at each age varied, and, because of between-chick variation in movement artefacts, so did the number of analysable EEG epochs from each chick.

Cubic regression tests were used to test for the presence of a trend in the three EEG variables: median frequency, 95% spectral edge frequency and total power. Because of the two types of variation in the sample sizes, these regression tests were conducted in two ways. The first test used all of the raw values, so all of the chicks at each age were included in the analysis, with each chick contributing a different number of EEG epochs. The second set of tests used mean values. These means were taken across the EEG variables for each chick, so that each chick had 3 averages, one for each of median frequency, spectral edge frequency and total power. Variability was still present between chicks of the same age, except for the hatched chicks, where there was only one of each age.

Regression outcome

There were 34 chicks used in the regression analysis, with each chick contributing between 2 and 44 samples to the analysis.

When multiple samples from each chick were included in the analysis there was a significant cubic relationship between median frequency and age ($F(3,598) = 41.73$, $P<0.05$). The median frequency of the EEGs increased between days 13 and 18 of incubation and then decreased during days 19 to 20. After hatching, the median frequency increased for the first 8 hours but then decreased again in the 2 and 4 day old chicks (Figure 3.9a). This trend was broadly similar for the 95% spectral edge frequency. There was an increase in 95% spectral edge between days 13 and 15, there was only a slight decrease before hatching and values increased again after hatching. There was a significant cubic relationship between 95% spectral edge frequency and age ($F(3,598) = 57.52$, $P<0.05$) (Figure

3.9b). Total power was higher in the hatched chicks than in the pre-hatched chicks and during the incubation period there seemed to be a tendency for total power to be highest around day 16 to 17. Total power also showed a significant cubic relationship with age ($F(3,598) = 262.53$, $P<0.05$) (Figure 3.9c).

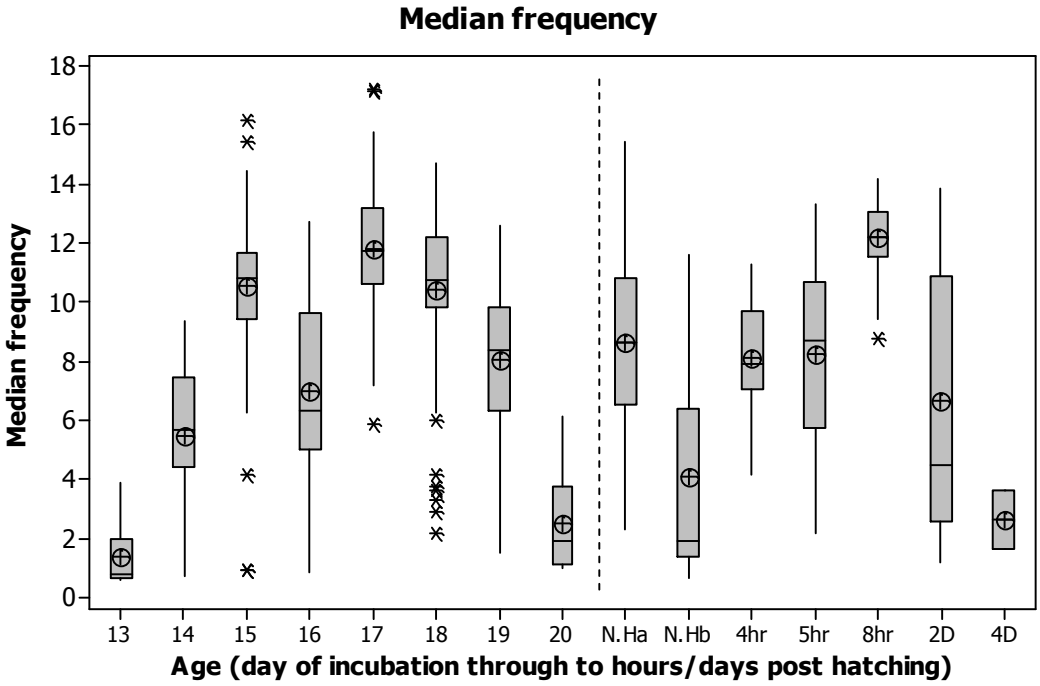


Figure 3.9a Box plot displaying the median frequency of chick EEGs before and after hatching. + symbol = mean for the box. Each box and whisker before hatching covers the median and inter-quartile ranges for that age, which includes multiple samples from each chick in the age group (see Table 2 for numbers of chicks in each age). After hatching, each box and whisker represents multiple samples from only one chick for each age. X-axis scale is days of incubation with hatching occurring on day 21 at the dotted line (N.H = newly hatched on day 21). N.Ha was a newly-hatched chick that provided an EEG recording from 4min to 3hrs post hatch, N.Hb was a chick that provided a recording from 2hr to 4hr post hatch. The hatched chick's age is given in hours or days post-hatching (D = days). * = outliers.

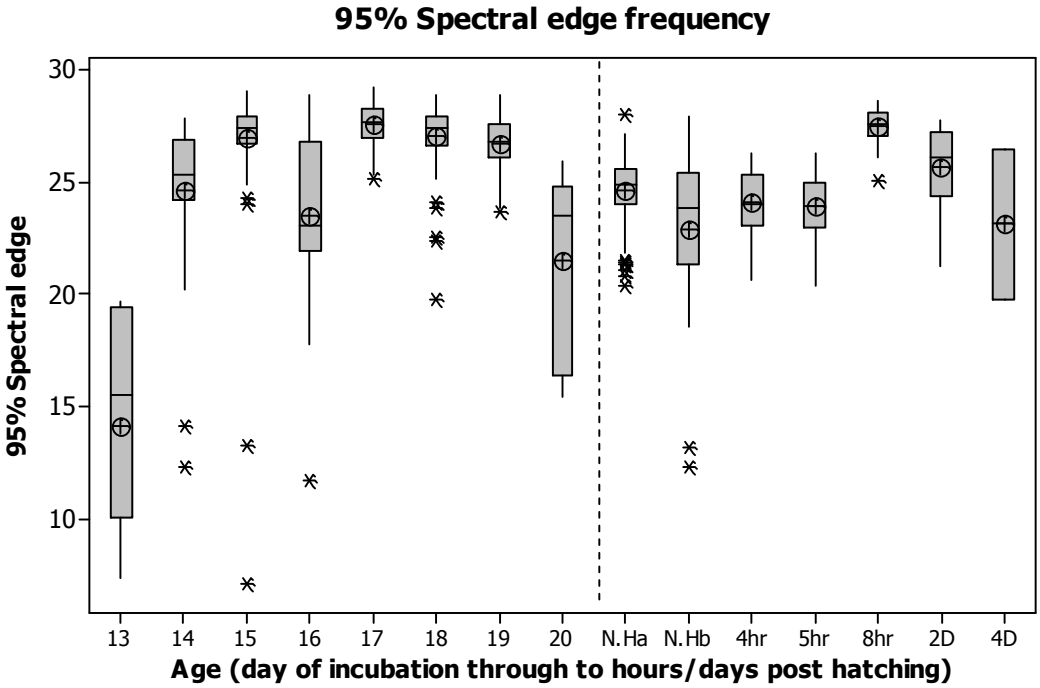


Figure 3.9b Box plot displaying the 95% spectral edge frequency of chick EEGs before and after hatching. + symbol = mean for the box. Each box and whisker before hatching covers the median and inter-quartile ranges for that age, which includes multiple samples from each chick in the age group (see Table 2 for numbers of chicks in each age). After hatching, each box and whisker represents multiple samples from only one chick for each age. X-axis scale is days of incubation with hatching occurring on day 21 at the dotted line (N.H = newly hatched on day 21). N.Ha was a newly-hatched chick that provided an EEG recording from 4min to 3hrs post hatch, N.Hb was a chick that provided a recording from 2hr to 4hr post hatch. The hatched chick's age is given in hours or days post-hatching (D = days). * = outliers.

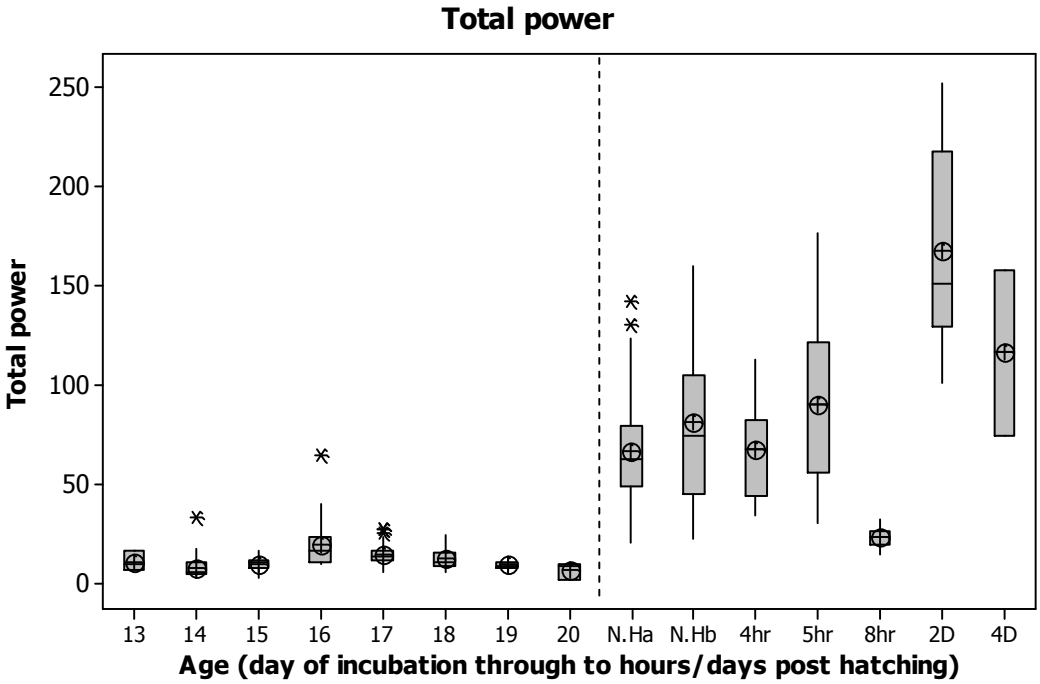


Figure 3.9c Box plot displaying the total power of chick EEGs before and after hatching. + symbol = mean for the box. Each box and whisker before hatching covers the median and inter-quartile ranges for that age, which includes multiple samples from each chick in the age group (see Table 2 for numbers of chicks in each age). After hatching, each box and whisker represents multiple samples from only one chick for each age. X-axis scale is days of incubation with hatching occurring on day 21 at the dotted line (N.H = newly hatched on day 21). N.Ha was a newly-hatched chick that provided an EEG recording from 4min to 3hrs post hatch, N.Hb was a chick that provided a recording from 2hr to 4hr post hatch. The hatched chick's age is given in hours or days post-hatching (D = days). * = outliers.

When each chick contributed only one value (the mean of all samples) to the analysis, similar results were found (Figures 3.10a, b and c). The cubic regression trend was still present for the averaged results of median frequency (F (3,30) =

4.43, P<0.05), 95% spectral edge frequency (F (3,30) = 5.29, P<0.05) and total power (F (3,30) = 19.83, P<0.05).

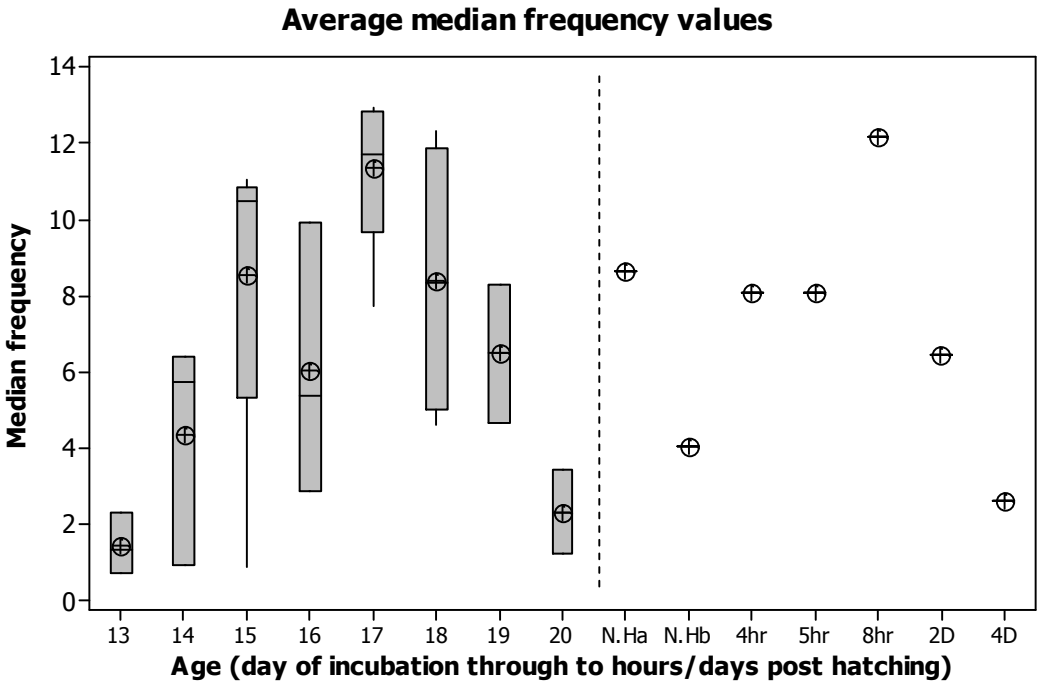


Figure 3.10a Box plot displaying the average median frequency of chick EEGs before and after hatching. + symbol = mean for the box. Each box and whisker before hatching covers the median and inter-quartile ranges for that age, to which each chick contributed one value (mean of the multiple samples from that chick) at that age. After hatching, each symbol represents the value from only one chick for each age. X-axis scale is days of incubation with hatching occurring on day 21 at the dotted line (N.H = newly hatched on day 21). N.Ha was a newly-hatched chick that provided an EEG recording from 4min to 3hrs post hatch, N.Hb was a chick that provided a recording from 2hr to 4hr post hatch. The hatched chick's age is given in hours or days post-hatching (D = days).

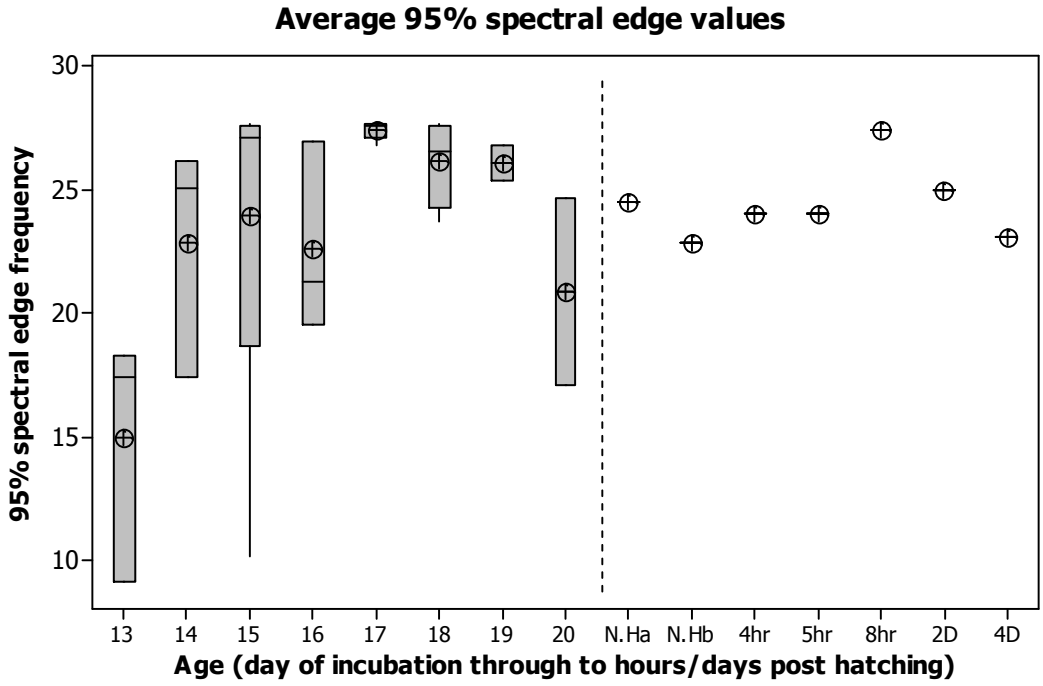


Figure 3.10b Box plot displaying the average 95% spectral edge frequency of chick EEGs before and after hatching. + symbol = mean for the box. Each box and whisker before hatching covers the median and inter-quartile ranges for that age, to which each chick contributed one value (mean of the multiple samples from that chick) at that age. After hatching, each symbol represents the value from only one chick for each age. X-axis scale is days of incubation with hatching occurring on day 21 at the dotted line (N.H = newly hatched on day 21). N.Ha was a newly-hatched chick that provided an EEG recording from 4min to 3hrs post hatch, N.Hb was a chick that provided a recording from 2hr to 4hr post hatch. The hatched chick's age is given in hours or days post-hatching (D = days).

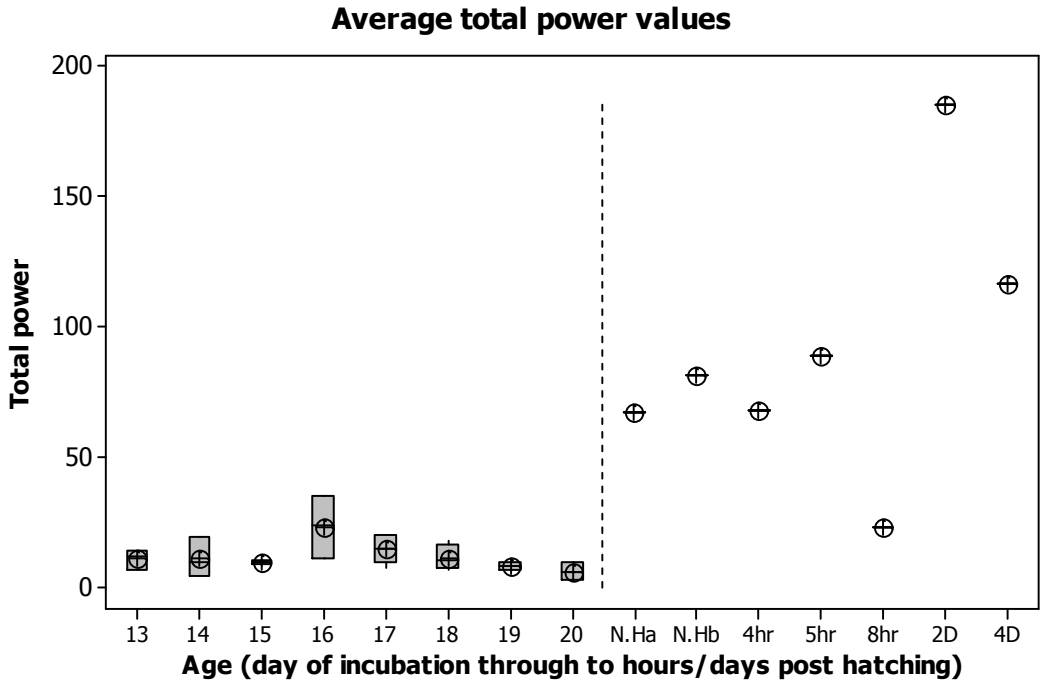


Figure 3.10c Box plot displaying the average total power of chick EEGs before and after hatching. + symbol = mean for the box. Each box and whisker before hatching covers the median and inter-quartile ranges for that age, to which each chick contributed one value (mean of the multiple samples from that chick) at that age. After hatching, each symbol represents the value from only one chick for each age. X-axis scale is days of incubation with hatching occurring on day 21 at the dotted line (N.H = newly hatched on day 21). N.Ha was a newly-hatched chick that provided an EEG recording from 4min to 3hrs post hatch, N.Hb was a chick that provided a recording from 2hr to 4hr post hatch. The hatched chick's age is given in hours or days post-hatching (D = days).

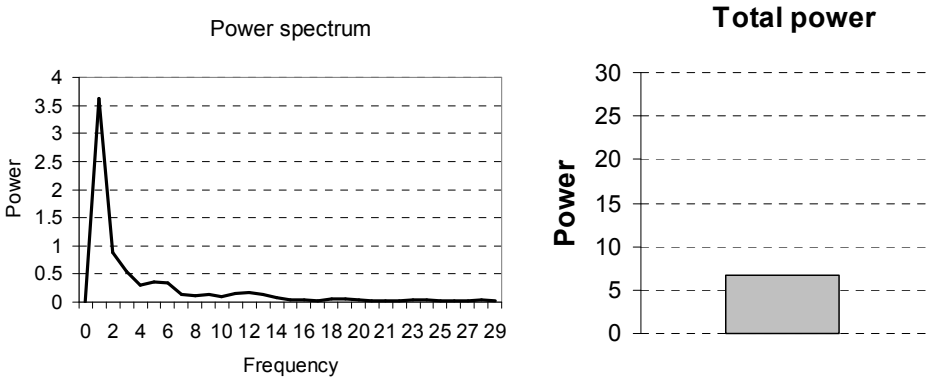
3.4.11 Effect of head position on FFT variables

There did not appear to be any consistent differences in the EEG variables between the "head out" and "head in" recordings (results not shown). However, the sample sizes were too small to make statistical comparisons.

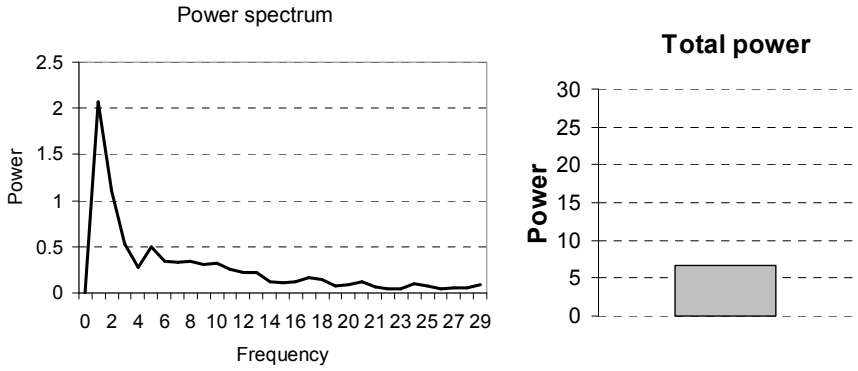
3.4.12 Later stages of incubation and hatching

The following is a description of the changes in the EEG around the time of hatching (Figures 3.11a-e). Only one chick could be followed in detail throughout this time, but its results have been compared with those of other chicks when possible. This chick had the electrodes placed on day 19 of incubation, the recording then continued for a further 33 hours. The chick hatched on what would have been day 20 of incubation.

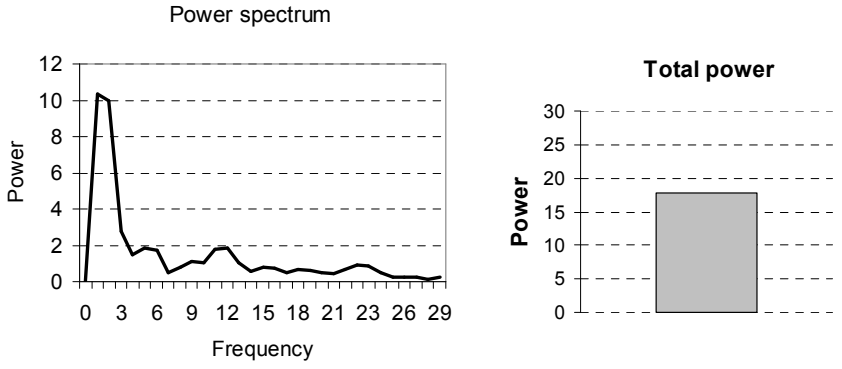
a) Day 19, after 27 minutes of recording, about 27.5 hours before hatching



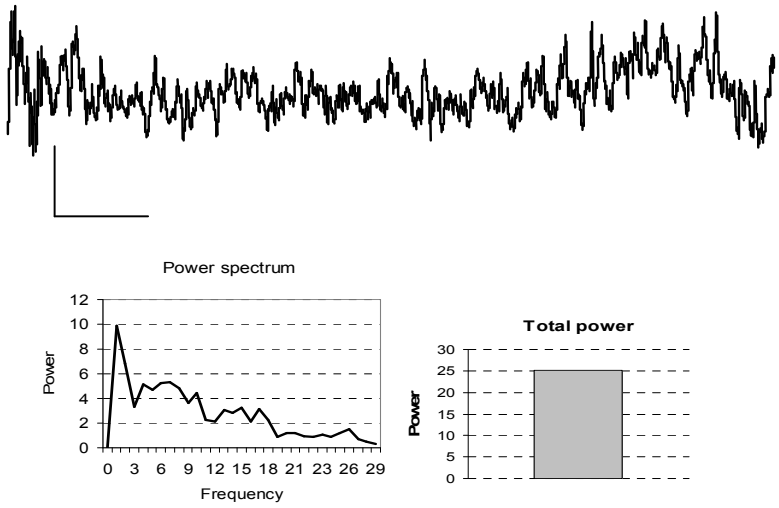
b) Day 19, after 10 hours of recording, about 18 hours before hatching



c) Day 20, after 23.25 hours of recording, about 4.5 hours before hatching



d) Day 20, after 29 hours of recording, and about 1 hour and 7 minutes after hatching



e) Day 20, after 30 hours of recording, and about 2 hours after hatching

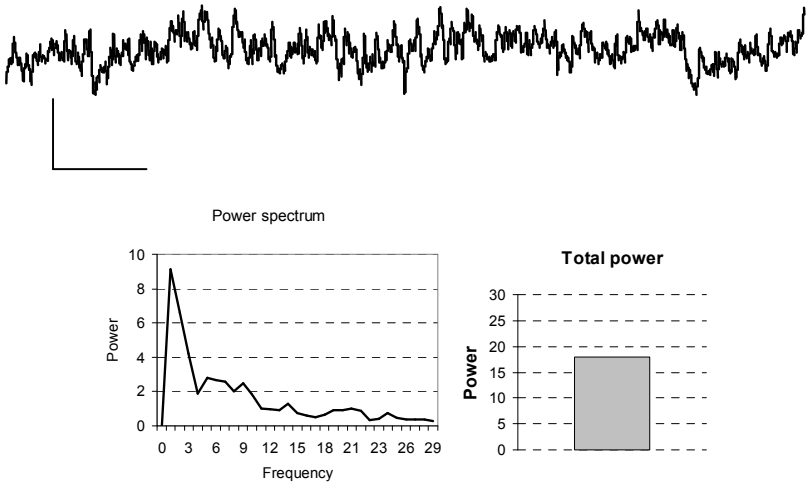


Figure 3.11a-e Segments of EEG taken from the recording that went from day 19 to day 20 of incubation. The L-shaped scale markers are 40 μV vertical and 0.5s horizontal. Each EEG segment is accompanied by its power spectrum. The units on the power scale (y-axis) are arbitrary (μV^2); also note the difference in scale on each power axis. The EEG segments are 4 seconds duration.

This chick's EEG was very low amplitude and low frequency at the start of day 19 when the recording began (Figure 3.11a). These EEG characteristics were observed on day 19 in both of the chicks that had their EEGs recorded overnight from day 19 to day 20, and were similar to those EEGs from single-day recording chicks on days 19 and 20. There appeared to be increases in the amount of high frequency activity approximately 18 and 10 hours before hatching (Figures 3.11b and 11c respectively); these are reflected as increases in the median frequency (Figure 3.12a). There was an increase in EEG amplitude approximately 4.5 hours before hatching (Figure 3.11c) and again after hatching (Figure 3.11d and e). The 95% spectral edge frequency had an increasing trend between 22 and 10 hours before hatching, and then levelled off (Figure 3.12b). The total power had an increasing trend between 22 hours before hatching and the time of hatching, it then decreased slightly (Figure 3.12c). The EEG from this hatched chick appeared similar to those recorded from previous hatched chicks.

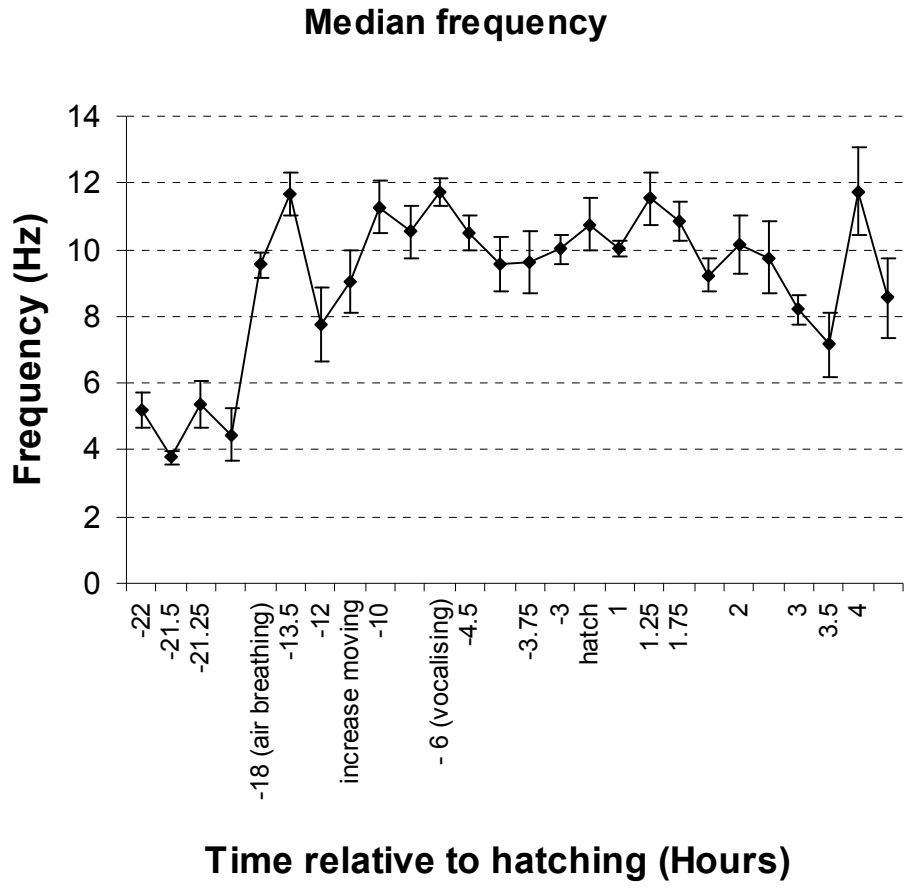


Figure 3.12a Line graph showing median frequency of the chick's EEG over time between days 19 and 20. The recording period stretched from day 19 of incubation until 4 hours after hatching, on day 20. The first point on the graph is 6 hours into the recording, 22hrs before hatching (hence time relative to hatching = -22 hours); the subsequent time-points are taken at unequal intervals beyond this. Each point is the mean (SEM) across 4s epochs at that time point.

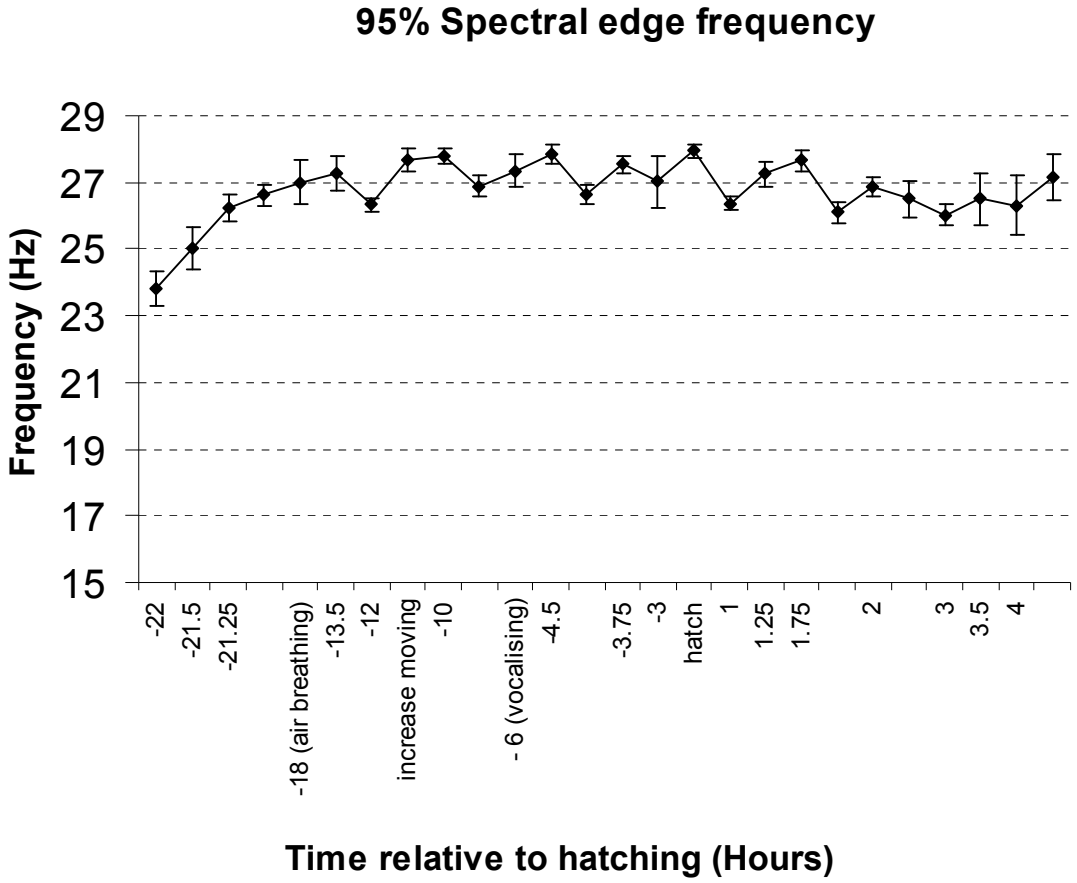


Figure 3.12b Line graph showing 95% spectral edge frequency of the chick's EEG over time between days 19 and 20. The recording period stretched from day 19 of incubation until 4 hours after hatching, on day 20. The first point on the graph is 6 hours into the recording, 22hrs before hatching (hence time relative to hatching = -22 hours); the subsequent time-points are taken at unequal intervals beyond this. Each point is the mean (SEM) across 4s epochs at that time point.

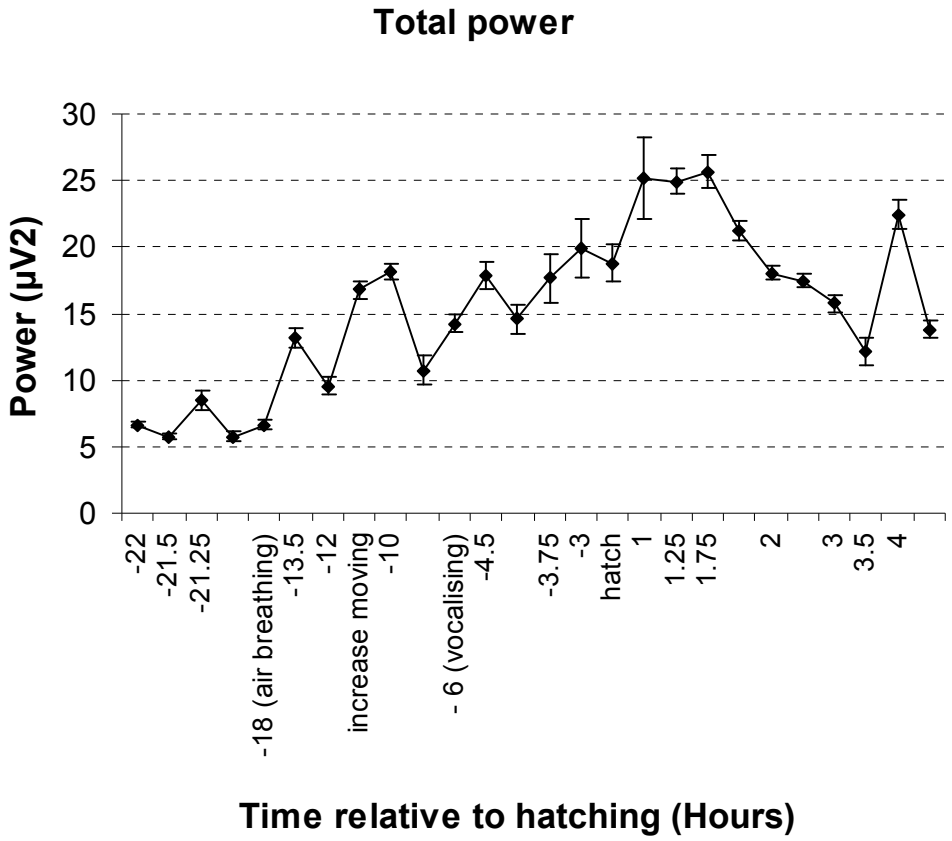


Figure 3.12c Line graph showing total power of the chick's EEG over time between days 19 and 20. The recording period stretched from day 19 of incubation until 4 hours after hatching, on day 20. The first point on the graph is 6 hours into the recording, 22hrs before hatching (hence time relative to hatching = -22 hours); the subsequent time-points are taken at unequal intervals beyond this. Each point is the mean (SEM) across 4s epochs at that time point.

3.5 Discussion

This Discussion will outline the main findings from the study. These will then be discussed with reference to relevant literature. The limitations of the study will be outlined. The conclusion will discuss the implications for the onset of consciousness in chickens.

The main findings from this experiment were:

1. The chick's brain activity was first detected using EEG on day 13 of incubation, comprising both active EEG and intermediate EEG
2. From days 13 to 15, the brain activity was interspersed with isoelectric EEG, the prevalence of which diminished towards zero by day 16 or 17
3. The high-frequency components of the EEG increased until day 17, reflected by increases in median frequency and 95% spectral edge frequency
4. The EEG amplitude decreased on days 19-20 and the frequencies were lower than at days 17-18
5. The high frequency EEG activity increased after hatching reflected as slight increases in median frequency and 95% spectral edge frequency. The frequencies were higher than on day 20 and there was also a large increase in total power
6. Only active EEG was observed after hatching

Based on these findings, the onset of those features of brain activity represented by the EEG was therefore gradual in the pre-hatched chicks, and these continued to develop after hatching. The EEG activity decreased in power and lost some

high frequency activity during the day before hatching, but increased again after hatching.

3.5.1 Use of the EEG and its relationship to consciousness

In order for an animal to be conscious it needs to have a sufficiently complex nervous system and the nervous system needs to be functional - the animal needs to be awake. Animals can have the neural anatomy necessary for consciousness and yet be unconscious for example during sleep, anaesthesia or coma or due to trauma, hypoxia or hypothermia (Simons et al., 1989; Ozaki et al., 1995; Boveroux et al., 2008). Therefore, a measure of brain functioning is useful to determine whether or not they are conscious. The EEG has been used to assess brain activity and function. In mammals the EEG records activity from the cortical cells, which are linked to consciousness (Velarde et al., 2002; Raj and O'Callaghan, 2004; Murrell and Johnson, 2006). The EEG recorded from chickens is similar to that seen in mammals and is believed to be generated by its cerebral hemispheres (Corner et al., 1973).

3.5.2 Onset of the EEG in incubating chicks

In terms of the timing of the onset of EEG and interpretation of different states, the current results were similar to previous reports. In contrast to early chick studies, the present results provide a quantitative analysis of EEG frequency and power characteristics. These results were also interpreted with reference to modern ideas, i.e. the onset of consciousness.

Previous experiments examining the development of brain activity in chicks generally note the first appearance of EEG on day 13 of incubation, with the

epochs of electrical activity being brief and interspersed with periods of isoelectric recording (Garcia-Austt Jr, 1954; Ellingson and Rose, 1970). The electrical activity at this age was reported to be of low voltage and low frequency, although the reported frequencies differ between studies (Table 3.4) (Ellingson and Rose, 1970).

The cerebral hemispheres in the chicken appear to be physiologically immature before day 14 of incubation (Peters et al., 1956). EEG spike responses to strychnine, an epileptic activity generator, first appear on day 13 of incubation. The spikes are similar to those observed in mammals, in which they originate in the cortex (Garcia-Austt Jr, 1954). The EEG signals emerging from the chicken brain are therefore probably based on the avian equivalent of cortical activity. The limited detection range of the electrodes would prevent them from picking up significant activity from deeper structures (Garcia-Austt Jr, 1954). The brain activity of chicks is also unresponsive to metrazol, another seizure-inducing agent, until day 14 of incubation (Peters et al., 1956).

Table 3.4. Qualitative and quantitative descriptions of developmental changes in the EEG of chicks as reported in previous studies. Columns 3 and 6 contain results from complementary or parallel studies. Units of frequency reflect those reported in each paper: w.p.s., waves per second and c.p.s., cycles per second, which were usually counted manually. These units are equivalent to Hertz (Hz), as reported in the present results in column 1. Empty spaces indicate the study did not report results for that day of development.

Age and median frequency range from present study	**Garcia-Austt Jr, 1954**	**Combined Peters et al., 1956 and Peters et al., 1965**	**Tuge et al., 1960**	**Sharma et al., 1963**	**Combined Speciale Jr et al., 1975, Cusick and Peters, 1973 and Corner and Bakhuis, 1969**
< 12 days No EEG		No EEG, just electrical activity associated with muscle movement artefact	No EEG activity	Day 4: 2-4 c.p.s 5-10 μV	
13-14 days 3-4 Hz	2.5-3 w.p.s, 5-10 μV intermittent	Isoelectric	No EEG activity	Day 13-16: 10-30 c.p.s, 10-20 μV	
15-16 days 6-8 Hz	Continuous by day 15. Day 16: big change in EEG: 3-4 w.p.s, 20-100μV Superimposed on previous waves	Day 15: isoelectric interrupted by slow waves 4-7 w.p.s. Day 16: sustained activity, 3-4, 8-12 and up to 30 w.p.s hemispheres not synchronised	Day 15: doubtful activity Day 16: irregular sporadic activity		
17-18 days 8-11 Hz			Day 17: rhythmical, 12-18 c.p.s, 10-30 μV; 10 c.p.s, 5 μV		
19-20 days 2-6 Hz	Day 20: 'dysrhythmic', voltage decrease	Irregular, moderate amplitude 5-30μV, 2-10 w.p.s			6 hrs before hatching: large amplitude slow waves with periods of activation
Hatching No EEG captured		Irregular waves of 5-75μV and 2-20 w.p.s			High amplitude slow potentials with periodic decreases in amplitude
Newly hatched 4-8 Hz (asleep)		16-22 up to 30 w.p.s superimposed on 1-4 w.p.s. 30-40, 60-70 w.p.s during movement			Drowsy, with periods of waking, slow wave sleep and paradoxical sleep. Sleep: 3-25 Hz, with reductions in amplitude during arousal
Post hatch 2-12 Hz (asleep)		2-3 weeks old. Alert: 20-40 μV 20-30 w.p.s. Asleep: 50-250μV, 3-8 w.p.s interrupted every 10-15s with low voltage fast waves lasting 2-4 s	3-day old, 15-30 c.p.s, 10-60 μV Adult – 20-120 μV, 30 c.p.s		

One other EEG study did not detect electrical activity until day 15 of incubation (Tuge et al., 1960). In contrast, another study reported the presence of spontaneous brain activity as early as day four of incubation (Sharma et al., 1963). The reasons for these different outcomes are unknown. In the present study a single chick was tested on day eight of incubation, but no EEG activity was found. Likewise, no active EEG was seen in any of the chicks on day 12 of incubation, therefore it was decided that it was not worthwhile testing more chicks at earlier ages. The current results agree with previous conclusions that there is little brain activity before day 13 of incubation (Garcia-Austt Jr, 1954; Peters et al., 1956; Ellingson and Rose, 1970).

3.5.3 Changes in EEG during incubation

During the course of incubation the chick EEG increased in amplitude and also frequency (seen as increases in the median frequency and 95% spectral edge frequency) until day 19 to 20. In the later stages of incubation there was also a reduction in the amount of isoelectric activity, as has been found in previous studies (Ellingson and Rose, 1970). A possible reason for the increase in voltage may be an increase in the capacity of the neurons to depolarise in synchrony and increased numbers of neurons being connected and depolarising (Sharma et al., 1963). The first appearance of EEG activity on day 12-13 also coincides with the development and increased activity of several biochemical processes in the chick's brain (Garcia-Austt Jr, 1954).

The EEG was continuous, either as active or intermediate EEG, by day 16-17. Previous work reports that low voltage fast waves appeared on day 16, superimposed on the slower frequency waves (Peters et al., 1956; Ellingson and

Rose, 1970) (Table 6) and that the EEG was virtually continuous at this time (Peters et al., 1956). Other EEG studies showed more high voltage and high frequency activity on day 16. This activity continued to increase until day 19 (Garcia-Austt Jr, 1954; Peters et al., 1956). These EEG changes were seen in the current study as an increase in the high frequency activity up to day 18. The increase in high frequency activity may represent maturation of brain activity and therefore an increased potential for consciousness in the chick.

3.5.4 Anatomical development

The chick's brain begins to differentiate at around day two of incubation. Development and growth continues throughout incubation and after hatching. The peak period of neural formation in the forebrain is around incubation day 8. Synapse formation between neurons progresses at a maximal rate at around day 15 of incubation and continues throughout incubation and until about two weeks post hatching. Maturation of the synapses continues for a longer period, with changes occurring from three to ten weeks post hatch (Rogers, 1995). Thus, there is a high rate of synapse formation at around the time EEG is becoming established, and the development of neural networks and synapses continues after hatching as does the development of the EEG (Peters et al., 1956; Peters et al., 1965).

3.5.5 EEG development in other species

Studies on EEG development in other bird species have revealed similar patterns to those found in the current study, although the timing of the patterns relative to hatching varies. Pigeon chicks differ from domestic chicks in that they are altricial, that is they hatch in a less developed state and cannot fend for

themselves for several days after hatching (Tuge et al., 1960). The EEG in the pigeon chick does not emerge until four to five days after hatching. The activity then increases, exhibiting similar characteristics to those seen in during development in the domestic chick, and resembles adult patterns 14 to 17 days after hatching. The difference in timing is likely to be due to differences in the level of anatomical and physiological development between species (Tuge et al., 1960). The pattern of EEG development is very similar across species. However the extent of EEG development relative to birth or hatching varies between altricial and precocial species for both mammals and birds.

The guinea pig is a precocial mammalian species. EEG is present in the foetal guinea pig, and is similar to that of the adult, by 46 days of the 66 day gestation (Flexner et al., 1950). The onset of the EEG in the guinea pig follows structural and biochemical developments in cortical cells of the brain (Flexner et al., 1950). In contrast, the albino rat, an altricial mammalian species, first shows spontaneous EEG activity four to 12 days after birth (Tuge et al., 1960; Diesch et al., 2009b). Initially EEG epochs are interspersed with isoelectric periods; but the EEG becomes continuous around postnatal day 8, and resembles the adult EEG around 18 days after birth (Tuge et al., 1960). It seems likely that the precocial species would have the capacity for consciousness earlier in life than the altricial species.

3.5.6 Environmental factors that may influence the EEG

In precocial mammals, there is a collection of neuroinhibitory factors unique to foetal life that may delay the onset of consciousness until after birth (Mellor and Gregory, 2003; Mellor et al., 2005; Mellor and Diesch, 2006). It is possible that a

similar group of neuroinhibitors within the egg may also act on the chick to affect its brain activity and thus the timing of the onset of consciousness in relation to hatching. These factors could include hormones within the yolk, which is absorbed by the chick during incubation, the chick's cramped posture within the egg and the oxygen status of the chick (Mellor and Diesch, 2007). In the discussion that follows, many of the concepts and references are discussed by and referenced from Mellor and Diesch (2007).

a) Yolk hormones

Yolk structure and absorption

Inside the egg, embryonic membranes support the chick as it grows. The chorioallantoic membrane facilitates gas exchange with the environment (as will be discussed later), and the yolk sac absorbs the yolk. The yolk sac contains a vascular network that transports nutrients from the yolk to the chick. The yolk contains the nutrients, growth factors and hormones the chick needs for development during incubation (Romanoff, 1960).

The yolk is not a uniform structure; instead it is made up of several concentric layers (Falen et al., 1991; Hutchison et al., 1992). The composition of the yolk and the concentration of nutrients and hormones can therefore change throughout the yolk. Differences in yolk hormones concentration in different layers of the yolk reflect changes in maternal hormones during the days prior to ovulation as the yolk layers were deposited (Lipar et al., 1999).

The yolk sac absorbs the yolk gradually throughout incubation. The yolk's layered structure persists for at least the first 11 days of incubation, after which time the borders between layers appear less distinct (Falen et al., 1991). The

outer layers of yolk are absorbed first, progressing to the inner layers. Variation in the yolk composition across the layers will therefore result in different quantities of factors being absorbed by the chick at different stages of development, although some mixing may occur. Variations in yolk hormone concentration are likely to have a role in development of the chick (Lipar et al., 1999). At the end of incubation the remnant yolk is absorbed into the abdomen and continues to supply the chick with nutrients for a few days after hatching (Romanoff, 1960).

Hormones in the yolk and the potential for neuroinhibition

As well as nutrients and growth factors, also present in the yolk are hormones that can influence the growth and function of the nervous system. Some factors may act directly on the nervous system. Alternatively, some compounds, such as cholesterol, may be metabolised to form biologically active factors e.g. pregnenolone and progesterone (Pignataro et al., 1998; Tsutsui et al., 2003).

The concentration of progesterone is greatest in the outermost layers of the yolk, and decreases in the inner layers (Lipar et al., 1999; Mostl et al., 2001). Therefore, the developing chick would initially be exposed to high levels of progesterone, and the levels would decrease during incubation as the yolk is absorbed.

Concentrations of 17β-oestradiol are lowest in the external layers of the yolk and increase towards the centre (Lipar et al., 1999). The amount of oestrogen delivered to the chick may initially decrease through to day 14. However, around day 20 there is a large increase in oestrogen, which raises the levels well above the initial values (Elf and Fivizzani, 2002). Therefore, the developing chick would receive increasing quantities of oestradiol towards the end of incubation.

Neuroinhibition

The gamma-aminobutyric acid (GABA) system is a neuroinhibitory mechanism that regulates activity in the central nervous system. Compounds including drugs and neurosteriods, such as progesterone derivatives, can have a depressive effect on the central nervous system by influencing the GABA inhibitory system i.e. modulating GABA receptor binding (Viapiano and Fiszer de Plazas, 1998; Viapiano et al., 1998).

Progesterone from the yolk is used in the synthesis of neuroactive steroids in the brain of the pre-hatched chick from day 11 onwards (Pignataro et al., 1998). Due to the concentration gradient through the yolk, the chick would be exposed to high levels of progesterone earlier in incubation and lower concentrations as incubation progressed (Lipar et al., 1999; Mostl et al., 2001). If yolk progesterone was to have an effect on the chick it is likely that there would be a greater inhibitory effect on neural activity early on, when progesterone levels are higher. However, there may be even more of an effect after day 11 of incubation, when the brain is able to synthesise additional neuroactive metabolites (Pignataro et al., 1998). The neuroinhibitory effects are likely to be less strong as the chick approaches hatching as the concentrations of progesterone in the yolk decrease. This reduction would allow the hatched chick to be aroused and responsive to its environment after hatching.

The GABA system is particularly sensitive to binding of steroids around the time of hatching (Viapiano et al., 1998). Other progesterone metabolites that enhance GABA binding (e.g. 5β-pregnan-3β-ol-20-one) are also more active around hatching (Pignataro et al., 1998). The effects of these factors may promote

neuroinhibition in the chick and suppress the potential for consciousness until after hatching has occurred.

In mammals, oestrogen has an activating effect on the foetal, newborn and adult behaviour (Mellor and Gregory, 2003). The concentrations of oestrogen in the yolk are low until the time immediately prior to hatching (Elf and Fivizzani, 2002). Therefore, if chickens respond to oestrogen in a similar way to mammalian young, any arousing effect is not likely to occur until the time of hatching.

b) Oxygen status and onset of breathing

Another factor which may influence the potential for consciousness is the oxygen status of the chick before and after hatching. Hypoxia can have a limiting effect on brain activity and consciousness (Gurvitch and Ginsburg, 1977; Kraaier et al., 1988; Ozaki et al., 1995). The chick may be in a slightly hypoxic environment prior to hatching (Freeman and Misson, 1970), which could affect the potential for consciousness.

While the chick is incubating inside the egg it acquires oxygen and eliminates carbon dioxide via diffusion across the porous shell (Burton and Tullett, 1985). The chorioallantoic membrane is a highly vascular structure that grows with the chick and lines the entire inside of the shell by around day 14 of incubation (Freeman and Misson, 1970). Capillaries in the chorioallantoic membrane acquire oxygen and release carbon dioxide via diffusion at the shell surface. These compounds are transported to and from the chick via the chorioallantoic vessels (Tazawa, 1980; Burton and Tullett, 1985). Both the chorioallantoic vessels

and the yolk sac vessels enter and exit the chick through the abdomen (Romanoff, 1960).

Also lining the shell are the inner and outer shell membranes. After the egg has been laid an air space appears between the inner and outer shell membranes at the base (rounded pole) of the egg. As incubation progresses this air space, also referred to as the air-cell, enlarges as water is lost from the egg contents (Freeman, 1974a). Diffusion occurs across all areas of the egg, including above the air-cell (Burton and Tullett, 1985).

Throughout incubation the chick grows to take up most of the space inside the egg. As the chick increases in size, its metabolic rate increases, as do its oxygen demands. Haemoglobin concentration, haematocrit levels and erythrocyte counts all increase later in incubation and therefore increase the oxygen carrying capacity of the blood (Tazawa, 1980). The concentration of gasses in the air-cell change during incubation, with the oxygen levels falling and carbon dioxide levels rising (Tazawa, 1980). Due to the increase in oxygen demand and decrease in oxygen availability, the chick gradually becomes hypoxic and hypercapnic before the onset of pulmonary respiration (Burton and Tullett, 1985). However, the exact trigger for the onset of lung breathing is unknown (Vince et al., 1975) Between days 8-18 of incubation the oxygen partial pressure (PO_2) in the blood (arterial and venous) falls and carbon dioxide partial pressure (PCO_2) rises (Tazawa et al., 1971). Arterial and venous PO_2 remain relatively stable from day 17 until the start of respiratory movements (Freeman and Misson, 1970). On day 18 there is a fall in arterial PCO_2 ($PaCO_2$). Yet, as the chick approaches the onset of air breathing on day 19-20 there is a slight increase in $PaCO_2$, although this is not considered to be of great enough magnitude to be the sole stimulus for

initiating lung respiration (Vince et al., 1975). At around day 19 the chick begins respiratory movements. This activity does not initially contribute to gas exchange, but aerates the respiratory system and stimulates its further development (Burton and Tullett, 1985).

On day 20 the chick begins the first stages of hatching – internal pipping, that is, breaking through the chorioallantoic membrane. The beak becomes angled upwards and pushes up against the chorioallantoic membrane so that it pushes the membrane into the air-cell. The beak scrapes back and forth against the membrane, and makes clapping movements (opening and closing of the beak). The membrane is gradually worn away and the beak pierces through (Kuo and Shen, 1937; Vince et al., 1975). Once air breathing begins, after internal pipping, there is a slight decrease in $PaCO_2$, followed by rises in both $PaCO_2$ and PaO_2 (Vince et al., 1975).

True breathing can occur either before or after the chorioallantoic membrane is pierced (Kuo and Shen, 1937; Vince et al., 1975), although the lungs may not be fully functional until after internal pipping (Freeman, 1974b). There is also very little air beneath the membrane, so the chick needs to pierce through it in order to obtain more oxygen (Kuo and Shen, 1937). Once the beak has pierced the membrane the chick can breathe air from the air-cell (Burton and Tullett, 1985).

Pulmonary respiration causes increases in both arterial and venous PO_2 (Freeman and Misson, 1970). Approximately 8-9 hours after breathing has started the chick breaks a hole in the egg shell over the air cell (external pipping). The chick then has access to atmospheric air and there is an increase in oxygen in the air cell (Freeman, 1974b). Arterial oxygen levels increase slightly and there is a large

increase in venous oxygen. The PCO_2 levels in the blood decrease as the egg shell is broken (Freeman and Misson, 1970).

Before external pipping, there is a period of time when respiration is shared between the chorioallantoic membrane and the lungs and both are important. If diffusion across the egg is prevented prior to external pipping, there are significant increases in chick mortality before hatching. After the chick has pipped the egg shell it can survive solely on pulmonary respiration (Visschedijk, 1968). It takes approximately 24 hours for the chick to transition from diffusional gas exchange across the eggshell to true pulmonary respiration (Burton and Tullett, 1985). Once the inner-membrane of the air cell has been pierced and pulmonary respiration has started the chorioallantoic membrane begins to dry out. In time the circulation through this membrane ceases and it is no longer functional (Visschedijk, 1968).

Oxygen consumption by the chick increases over time: firstly when it begins respiratory movements on day 19-20 of incubation, secondly at the time of internal pipping and thirdly at the time of hatching (Freeman, 1974b; Burton and Tullett, 1985). This increase in oxygen supply would be necessary to meet the oxygen demands in the physical activity of hatching (Burton and Tullett, 1985).

During incubation and prior to pipping and the onset of pulmonary respiration arterial oxygen levels are as low as about 20 mmHg, but they rise to about 37 mmHg after internal pipping and to about 40 mmHg after pipping the shell. Similar values have been recorded in mammalian foetuses (rabbits and sheep) (Freeman and Misson, 1970). In mammals the EEG is noticeably reduced by low oxygen levels, and recovers after birth when lung respiration increases

oxygenation (Mellor and Diesch, 2007). After hatching there is a dramatic increase in PaO_2 and a decrease in $PaCO_2$. The 1-day-old hatched chick has arterial oxygen levels of 109 mmHg (Freeman and Misson, 1970).

The hypoxic environment prior to internal pipping and hatching may affect the chick's brain activity. On days 19 and 20 there was a reduction in EEG activity (Figures 3.4 and 3.9a); this may have been caused by the hypoxia. In humans, hypobaric hypoxia, from simulated changes in altitude, causes slowing of the EEG (Kraaier et al., 1988; Ozaki et al., 1995). Other forms of hypoxia, such as disruption of cerebral blood supply, cause similar EEG changes (Gurvitch and Ginsburg, 1977).

c) Posture of the chick

While the chick is inside the egg it is possible that arousal is suppressed by the restraint of the shell (Corner et al., 1973). The head and neck position appear to be particularly important. From days 18-20 the chick is not able to stretch or straighten its neck. Towards the end of incubation the chick is confined with its neck curled around so that the head is tucked under the right wing. The head is not freed until the shell has been broken (Bekoff and Sabichi, 1987). The incubating chick is most aroused during periods of hatching behaviour when the neck and head are free from the shell. Freeing the limbs or wings does not elicit arousal (Corner et al., 1973).

A hatched chick, up to 4 days of age, will fall asleep when it is restrained in a position resembling that of a chick inside an egg. In addition, when the chick is restrained and asleep it is often not aroused by stimuli that would wake a chick that is sleeping unrestrained (Corner et al., 1973). During this type of restraint

the EEG of the chick changes from an active/wakeful waveform to one that represents sleep (Corner et al., 1973). The restraint will also elicit hatching behaviour in young hatched chicks. This hatching behaviour can be prevented if a local anaesthetic is used to block sensory input from the folded neck (Bekoff and Sabichi, 1987). Therefore the restraint of the chick inside the egg may contribute to the decreased EEG activity before hatching, and the release of the chick may contribute to the increase in EEG activity seen after hatching.

3.5.7 Hatching

By day 19 the chick has adopted the hatching position. The head is tucked under the right wing and the beak is horizontal below the chorioallantoic membrane, beneath the air-cell (Vince et al., 1975). In the days before hatching, little fluid remains in the amniotic cavity that surrounds the chick (Burton and Tullett, 1985). After the chick has broken through the egg shell and has stated breathing atmospheric oxygen there is usually a quiet phase which can last for variable lengths of time, but often several hours (in this study between the time ranged between 1 and 11 hours). The next phase of hatching happens quickly. The chick uses its beak to break the shell around the inside circumference of the egg. The chick then pushes the base of the egg open and can exit the egg through the opening it has created (Freeman, 1974b).

The timing of hatching must be carefully controlled so that the chick is both physiologically and behaviourally able to cope with the external environment. The chick needs to have absorbed its yolk into its abdomen, although not depleted its supply of nutrients (Bekoff and Sabichi, 1987).

The initiators of hatching behaviour are not well understood, and may actually be a combination of several factors. For example, the chick adopts the hatching posture with a bent neck a few days before hatching, so the sensory information alone cannot be sufficient to elicit hatching behaviour. Additional causes may be that the sensory pathways do not mature until before hatching, or that the pathways may be inhibited by other factors until immediately prior to hatching, as discussed above (Bekoff and Sabichi, 1987).

3.5.8 EEG in the days before hatching

The pre-hatched chicks in the present study had EEG with lower voltage and frequency on the days immediately prior to hatching (days 19-20 of incubation) than in previous days. The observed depression in EEG activity may be related to an experimentally induced oxygen shortage in the chick at this time, and may therefore differ from the normal EEG of a non-instrumented chick.

The chick's oxygen status may have been affected by the experimental setup in two ways. First, the chorioallantoic membrane was disrupted when it was opened to gain access to the chick, and this may have put the chick in an artificially hypoxic/hypercapnic state by limiting the potential for diffusion and affecting blood flow. Second, both the shell and the chorioallantoic membrane were open, so once the chick started breathing, it had access to greater levels of oxygen than it would have had under normal conditions.

To explore these effects, it is worthwhile to examine in more detail the chicks that had their EEGs recorded on day 20. There were two sets of chicks. Two chicks had their EEGs recorded solely on day 20 of incubation, and two chicks had their

EEGs recorded overnight starting on day 19 and extending to 20 when hatching occurred. In the multi-day recording group, active EEG was present throughout day 20. In contrast, the traces from the single-day recording chicks were mostly occupied by intermediate activity. These findings could be interpreted with reference to the chick's oxygenation status.

When the EEG electrodes were placed on the single day chicks (for the day 20 recording) neither of them had engaged in internal pipping, nor had they started pulmonary respiration. The EEG on day 20 had a low median frequency and low total power (Figures 3.9a and 3.9c). Prior to internal pipping on day 20 these chicks on were likely to have been in a hypoxic and/or hypercapnic state (Tazawa et al., 1971; Vince et al., 1975), which may have depressed their brain activity.

Respiratory movements started during the recording, but it was not clear at what point they started functional lung respiration. The onset of breathing is a gradual process in the chick. It usually takes at least two hours before the respiratory tract is aerated enough to begin functioning (Vince and Tolhurst, 1975; Burton and Tullett, 1985). When breathing started these chicks would then have had access to the higher oxygen content of atmospheric air, as opposed to that of the air-cell air, which would have been available under normal circumstances.

The chicks that had their EEGs recorded overnight were exposed to different conditions to the single day chicks on day 20. These chicks were instrumented on day 19. The manipulations may have stimulated the chick to start breathing earlier than usual, an idea supported by the chicks hatching the following day

(on day 20). The chicks that had their EEG recorded solely on day 20 (before pipping) had depressed brain activity. The chicks that were instrumented the previous day, and that had potentially been breathing air for several hours, had a more active day 20 EEG. There were increases in median frequency, 95% spectral edge frequency, and total power over the recording period for this group (Figures 3.12a,b and c), which may be related to increased oxygenation prior to hatching. These results could support the concept that the decrease in active EEG and predominance of intermediate EEG observed on day 20 in the single day recordings was a result of low oxygen and its effects on the brain activity.

Another study reported a decrease in EEG voltage on day 20 at the time when the chick starts pulmonary respiration (Garcia-Austt Jr, 1954). Those chicks had been given curare to prevent muscular movements. Respiration was therefore inhibited and the chicks died of anoxia within two hours (Garcia-Austt Jr, 1954) so any later increases in EEG associated with air breathing, such as those seen in the current study, were not observed.

3.5.9 EEG at the time of hatching

Evidence from previous studies suggests that consciousness is not needed for hatching. Pigeons hatch just as effectively as domestic chicks, yet they do not show signs of EEG activity until four days after hatching (Tuge et al., 1960). Chicks can also hatch, using normal hatching behaviour, after their cerebral hemispheres have been removed (Corner et al., 1973).

The state of the chick, in terms of consciousness, around the time of hatching has previously been debated. Some studies state that the chick may be conscious

during the hatching process (Bakhuis and Van de Nes, 1979; Bakhuis and Bour, 1980). Others state that the chick is not conscious at the time (Peters et al., 1965).

Unfortunately, the current study was unable to record a usable EEG during the hatching process due to high levels of movement artefact. However, the EEG before hatching exhibited lower frequency activity than that recorded from many of the pre-hatched chicks and the hatched chicks. This would imply that the chick is unlikely to have been conscious immediately before or during hatching.

Some previous reports state that the EEG during hatching resembles a sleep state, except that the struggling movements do not always coincide with changes in the EEG as they would in normal sleep (Corner et al., 1973). Other studies report the presence of high amplitude slow waves throughout hatching, with periodic reductions in amplitude, again similar to sleep (Corner and Bakhuis, 1969; Speciale Jr et al., 1975). Based on behaviour and EEG, it has been suggested that, physiologically, the pre-hatched chick is neither asleep nor awake, but is in a coma-like stupor without awareness (Peters et al., 1965).

Around the time of hatching the EEG does not seem to respond to sensory input. Pre-hatched chicks may show behavioural responses to stimuli such as sound. However, there is apparently no arousal in cerebral activity, as is seen in hatched chicks. Therefore, it is possible that these behaviours are a result of lower level reflex arcs without conscious involvement (Peters et al., 1965).

On day 20 chicks may open their eyes, but this is not necessarily accompanied by a change in the EEG pattern (Peters et al., 1965). Similarly, the EEG can change to lower voltage high frequency activity, usually associated with arousal, without

any muscular activity (Speciale Jr et al., 1975). As hatching approaches, the chick has more frequent bursts of behavioural activity, which are better correlated with changes in EEG activity, but there are still occasions where the two occur separately (Speciale Jr et al., 1975). The evidence therefore seems to indicate that, as mentioned above, the chick is not, and does not need to be, conscious during hatching.

3.5.10 Onset of alert behaviour after hatching

The first signs of alertness in the chick are seen after the egg shell is broken open and the chick released (Corner et al., 1973). The waking behaviours have been described as wide open eyes, erect posture, orienting the head and body (Corner and Bakhuis, 1969). Releasing and stretching of the head and neck in particular is necessary for the chick to display these behaviours. As mentioned earlier, releasing only the wings and/or legs of the chick does not stimulate signs of wakefulness (Corner and Bakhuis, 1969; Corner et al., 1973).

The chick's posture is quite limp immediately after hatching. Over time, the chick raises its head and body, rearing onto its hocks (tarsometatarses) (Cusick and Peters, 1973). During some of the chick's early rearing behaviours their eyes may remain closed and there is little difference in the cerebral activity from sleeping (Cusick and Peters, 1973). The chick initially shuffles in this hock-rearing position, and then eventually it rights itself onto its feet and begins upright walking and running. Immediately after hatching the chick is only active for very short periods (Cusick and Peters, 1973). Much of the time after hatching is taken up by sleeping. Chicks have more sustained periods of arousal around 1.5 – 2 hours after hatching. The behaviour in these arousal states

involves high muscle tone, well directed responses to sensory stimuli and brief performances of more complex behaviour and longer periods of eye opening (Peters et al., 1965). After 1-2 hours behaviour and EEG become more correlated, alert behaviour is more frequently accompanied by a low voltage high frequency EEG while sleep is seen as low frequency high voltage, as occurs in older chicks (Peters et al., 1965; Corner et al., 1973; Cusick and Peters, 1973). After 4-6 hours the chick spends more time upright, and when it is awake the chick's EEG resembles that of a 10-day-old chick (Cusick and Peters, 1973).

The forebrain is apparently not required for the initial brief startle behaviours. Those can occur in decerebrate chicks i.e. chicks that have had their cerebral hemispheres removed (Corner and Bakhuis, 1969). However, the cerebral hemispheres are required for sustained arousal and more coordinated behaviours, otherwise the chick becomes subdued over time (Corner and Bakhuis, 1969). So, while the chick may not need to be conscious immediately after hatching, the neural structures that facilitate consciousness are required for the chick to sustain normal behaviour subsequently.

The sequence for the onset of alert behaviour has been reported to take from 6-12 hours (Cusick and Peters, 1973). Yet the chicks in the current study were mobile on their feet, running, drinking, manipulating objects with their beaks and interacting with other chicks within 2 hours of hatching. These behavioural observations were not quantified or recorded consistently. One interaction illustrated the complex behaviours demonstrated by the chicks soon after hatching. About 2 hours after hatching, one chick was manipulating the wood-shaving substrate with his beak. A second chick ran over and took the shaving from the first chick and ran away with it. The first chick then chased the other

one around the box. Similar interactions were seen between several of the other chicks within the same time frame.

The onset of alert behaviour may have been quicker in this experiment because of the environment in which the chicks were housed. The observation area for alert behaviour reported in previous studies was an infant incubator, which is a fairly barren environment, and the chicks were observed in isolation (Corner et al., 1973). In the current study the chicks were in a box with wood-shaving substrate, a heat lamp, food, water, a perch and another chick for company. There was greater opportunity for chicks to perform normal behaviours and the presence of another chick may have been a stimulating factor for the onset of arousal.

While there are small bursts of arousal behaviour within moments of hatching, the onset of a state of sustained conscious probably occurs more gradually. Based on behaviour consciousness first seems to appear as the chick is waking up after hatching. The chick may therefore be aware of its surroundings and be able to perceive pain shortly after hatching, yet awareness of these experiences and of sensory inputs is likely to increase as time passes. Likewise, among precocial mammals, it is likely that the newborn lamb becomes more aware as time passes (Mellor and Gregory, 2003).

3.5.11 Experimental limitations

Long term recordings

In most cases, the experimental setup was not sufficient to meet the requirements of the incubating chick long term. The heated-water coil maintained the egg at

an appropriate temperature for incubation, but the evaporative water-loss was high, which disrupted the humidity levels inside the egg. The albumen dried on the chicks, making them sticky, and the membranes also became dry. Despite using sterile equipment, there was evidence of some microbial contamination of the eggs. One egg had a white culture, and another developed a brown film, growths that were not identified.

The long-term survival of the chicks was also compromised by consequences of incising the chorioallantoic membrane to gain access to the chicks. Opening the membrane would have affected the blood flow and also the capacity of the membrane to pick up oxygen. In some cases the main chorioallantoic blood vessels darkened after three to four hours, which may have indicated a lower oxygen content of the blood. Disruption to blood supply and/or blood loss was considered to be the most likely causes of death in the pre-hatched chicks that died. Disruption of blood supply may also have affected the condition of the pre-hatched chicks during the shorter term recordings, which may have influenced the EEG recordings for example, a reduction in blood flow and oxygen delivery to the brain can affect the EEG (Gurvitch and Ginsburg, 1977; Kraaier et al., 1988; Ozaki et al., 1995).

Both of the chicks that had their EEGs recorded continuously from day 19 to day 20 hatched on day 20. Although it is not uncommon for chicks to hatch on day 20 rather than 21 (Hamburger and Hamilton, 1992), the manipulation of the chick may have stimulated early hatching. The egg shell had been broken, so once the chicks started breathing they had access to atmospheric oxygen, as opposed to the oxygen concentration that is usually limited by diffusion through the egg shell before the chick breaks itself through the egg.

Effects of anaesthesia on the results

It is difficult to say whether or not the chick is conscious before hatching, but the current evidence suggests that consciousness first occurs after hatching. The EEG median frequencies appeared similar between the day 15 to 19 pre-hatched and hatched chicks, and the 95% spectral edge frequencies sometimes appeared slightly lower in the hatched chicks compared to the day 15 to 19 pre-hatched chicks. Higher frequency activity is generally associated with a greater level of arousal or wakefulness (Murrell and Johnson, 2006). However, the hatched chicks in these experiments were asleep and/or recovering from anaesthesia while their EEG was being recorded. The "hatched chick" EEGs were therefore not an indication of the EEG of a conscious chick, but a sleeping or anaesthetised chick. Anaesthesia generally causes a lower frequency EEG waveform (Simons et al., 1989). Similar EEG responses have been reported in chicks with increasing anaesthetic doses of sodium pentobarbitone (Ookawa, 1972) and in the chickens in the experiments discussed in Chapter 2. These changes in EEG with sleep/anaesthesia are likely to have lowered the median and spectral edge frequency values for the hatched chicks used in the current study. If the chicks had not been anaesthetised and were awake during the recordings then the EEG activity might have included more high frequency activity and therefore higher values for the median and 95% spectral edge frequency.

Electrode circuit and total power

Although the EEG frequencies may have been similar before and after hatching, the total power was much higher in the hatched chicks than in any of the pre-hatched ages. The increase in total power, when accompanied by the same

frequency waveform as in pre-hatched chicks, may indicate higher voltage EEG and an overall increase in the EEG activity. This change may be a consequence of a biological event caused by removal of the inhibitory factors acting inside the egg, increased neuron firing or greater recruitment of groups of neurons (Simons et al., 1989). However, the total power might also have been affected by changes in the electrode circuit conditions, as follows.

When the chicks were in the egg they were either bathed in albumen fluid (early in incubation) or at least moist (in later stages of incubation) from the humidity inside the shell. This moisture provides a path for an electrical circuit. The overall voltage of the EEG recording circuit is then split between the EEG electrodes and the moisture bridge. The EEG activity is still detected, but, if a large component of the circuit is passing across the moisture bridge, then the voltage of the EEG waveform will be lowered. When the chick has hatched and dried off there is less chance of the circuit splitting, so the overall EEG voltage will be larger. This situation could have resulted in the apparent increase in total power between the pre-hatched and hatched chicks. The frequencies of the EEG would not be affected by this circuit. The different frequencies of the EEG waveform would still be present and any reduction in voltage would occur equally across all of the frequencies.

Electrode position

In this study the electrodes were placed in the same position for each chick, and on the same (right) side of the head, so the results would not have been affected by any variation in activity at different electrode sites. However, another study reported greater activity in the left compared to the right hemisphere of chicks

and that the frontal electrodes contributed more to the EEG power than did the posterior electrodes (Hunter et al., 1999). Overall, the study reported the same developmental pattern in the EEG at each of the electrode sites (Hunter et al., 1999).

In addition, changes in anatomy of the bird cranium may have influenced the EEG with age. In particular, analysis of changes in the EEG amplitude is influenced by changes in skull size and thickness as the bird grows and develops. However, analysis of the frequency parameters, as was undertaken in the present study, may be less affected by this growth and can be used to examine changes in brain electrical activity with time (Hunter et al., 1999).

3.6 Conclusions and implications for consciousness

The present results are not definitive, but when considered with reference to previous literature they suggest that, in relatively undisturbed conditions, the chick is not likely to be conscious until after hatching.

Based on the present results and previously published reports (Table 6), the development of consciousness in the chick could be described in a set of merging phases. In the very early stages of incubation the chick is unlikely to be conscious because the nervous system is not sufficiently developed. The EEG did not pick up activity from the chick's brain until around the 13th day of incubation. After day 13 the EEG may be present, yet, neither the EEG nor the chick's posture are definite indicators of sleep or wakefulness as they exist in a hatched chick (Peters et al., 1965).

The development of the nervous system and the increases in high frequency activity indicate that the potential for consciousness increases throughout incubation. However, the chemical neuro-inhibitors and other factors mentioned above could suppress consciousness in the pre-hatched chick. From days 13 to 16 the potential for conscious is still low. The EEG activity was sporadic, the neural circuits are still developing and conditions inside the egg may suppress cerebral activity.

From day 16-18 the EEG activity was continuous, there was an increase in high frequency activity and a greater amplitude as the EEG began to resemble that of the hatched chick. Yet there are still likely to be a number of environmental factors, such as low oxygen, chick posture and neuroinhibitory hormones acting on the chick's brain. Unfortunately there were no EEGs recorded from conscious hatched chicks that could be used for comparison in this experiment.

Even on day 19, the pre-hatched chick may show a behavioural response to sensory stimuli, but there was no change in the EEG towards the low-voltage fast waves seen in the hatched chick (Peters et al., 1965). There also seemed to be a reduction in the EEG activity before hatching, so the potential for consciousness at this time seems low, provided that the chick is left undisturbed.

When an animal goes from a state of sleep or rest to a state of arousal or alertness there is a change in the EEG from low frequency high voltage to high frequency low voltage. This EEG change can be induced by any form of sensory input that arouses the animal to a state of alertness (Moruzzi and Magoun, 1949). In mammals, the system that induces this change in brain activity is thought to comprise reticular relays located in the brain stem, mediated partly by the

thalamus, and acting on the cortex (Moruzzi and Magoun, 1949). Similar systems may exist in birds.

It seems likely that the neural mechanisms for consciousness in the chick are in place and close to being functional by day 20 of incubation. One theory is that these physiological systems may need some form of stimulus to initiate functional activity (Peters et al., 1965). This stimulus may be delivered during the hatching process. As the chick hatches its nervous system receives a large influx of sensory inputs which may influence the maturation of neural circuits and activate pathways important in the arousal of the chick (Peters et al., 1965).

Although the current study suggests that the chick is unlikely to be conscious before hatching, these experiments were conducted in chicks that had been disturbed as little as possible. In the later stages of incubation it is possible that disturbance of the chick may have an arousing effect, particularly if the chick is freed from its egg or has access to atmospheric oxygen.

In order to draw some clearer conclusions on the onset of consciousness, more information is needed. Other studies evaluating the onset of consciousness have looked at the brain's capacity to process sensory inputs, such as noxious stimulation (Diesch et al., 2009b, 2009a); a similar experiment may be useful here. By investigating how the brain responds to sensory inputs, it may be possible to determine the level of processing that occurs at different stages of development. Evaluating brain responses to noxious stimuli would be useful and relevant for animal welfare because higher brain processing (consciousness) has to occur for an animal to perceive pain (Mellor and Diesch, 2006).

Previous work has found that noxious stimuli can cause identifiable changes in the EEG of anaesthetised mammals (Murrell and Johnson, 2006). Those animals are anaesthetised and therefore not perceiving pain, yet their EEG was still sufficiently responsive that it would change in response to a noxious stimulus. If an animal showed an EEG response to a noxious stimulus, this would indicate that the animal is likely to have the neural capacity for consciousness, and the potential to perceive pain at higher levels. It would therefore be useful to examine the neural processing of noxious sensory input in the brain of chicks at different ages of development.

3.7 References

Ayala-Guerrero, F. and Vasconcelos-Duenas, I., 1988: Sleep in the dove *Zenaida asiatica*. *Behavioral and Neural Biology,* 49: 133-138.

Ayala-Guerrero, F., 1989: Sleep patterns in the parakeet *Melopsittacus undulatus*. *Physiology and Behavior,* 46: 787-791.

Baars, B. J., Ramsoy, T. Z., and Laureys, S., 2003: Brain, conscious experience and the observing self. *Trends in Neurosciences,* 26: 671-675.

Bakhuis, W. L. and Van de Nes, J. C. M., 1979: The causal organization of climax behaviour in the domestic fowl (*Gallus domesticus*). *Behaviour,* 70: 185-230.

Bakhuis, W. L. and Bour, H. L. M. G., 1980: The behavioural state during climax (hatching) in the domestic fowl (*Gallus domesticus*). *Behaviour,* 73: 77-105.

Bekoff, A. and Sabichi, A. L., 1987: Sensory control of the initiation of hatching in chicks: effects of a local anaesthetic injected into the neck. *Developmental Psychobiology,* 20: 489-495.

Boveroux, P., Bonhomme, V., Boly, M., Vanhaudenhuyse, A., Maquet, P., and Laureys, S., 2008: Brain function in physiologically, pharmacologically and pathologically altered states of consciousness. *International Anesthesiology Clinics,* 46: 131-146.

Broom, D. M., 1988: The scientific assessment of animal welfare. *Applied Animal Behaviour Science,* 20: 5-19.

Burton, F. G. and Tullett, S. G., 1985: Respiration of avian embryos. *Comparative Biochemistry and Physiology,* 82A: 735-744.

Butler, A. B., Manger, P. R., Lindahl, B. I. B., and Arhem, P., 2005: Evolution of the neural basis of consciousness: a bird-mammal comparison. *BioEssays,* 27: 923-936.

Butler, A. B. and Cotterill, R. M. J., 2006: Mammalian and avian neuroanatomy and the question of consciousness in birds. *Biological Bulletin,* 211: 106-127.

Corner, M. A. and Bakhuis, W. L., 1969: Developmental patterns in the central nervous system of birds. V. cerebral electrical activity, forebrain function and behavior in the chick at the time of hatching. *Brain Research,* 13: 541-555.

Corner, M. A., Bakhuis, W. L., and van Wingerden, C., 1973: Sleep and wakefulness during early life in the domestic chicken, and their relationship to hatching and embryonic motility. *In* Gottlieb, G. (ed.), *Behavioural Embryology*. New York: Academic Press, 245-266.

Cusick, C. J. and Peters, J. J., 1973: Electromyographic, electroencephalographic, and behavioural changes during the onset of erect posture in newly hatched chicks. *Poultry Science,* 53: 1456-1462.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009a: Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Laboratory Animals,* 00: 1-9.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009b: Electroencephalographic responses to tail clamping in anaesthetized rat pups. *Laboratory Animals,* 43: 224-231.

Edelman, D. B., Baars, B. J., and Seth, A. K., 2005: Identifying hallmarks of consciousness in non-mammalian species. *Consciousness and Cognition,* 14: 169-187.

Elf, P. K. and Fivizzani, A. J., 2002: Changes in sex steroid levels in yolks of the leghorn chicken, *Gallus domesticus*, during embryonic development. *Journal of Experimental Zoology,* 293: 594-600.

Ellingson, R. J. and Rose, G. H., 1970: Ontogenesis of the electroencephalogram. *In* Himwich, W. A. (ed.), *Developmental Neurobiology*. Illinois: Charles C Thomas Publisher, 441-474.

Falen, S. W., Szeverenyi, N. M., Packard Jr, D. S., and Ruocco, M. J., 1991: Magnetic resonance imaging study of the structure of the yolk in the developing avian egg. *Journal of Morphology,* 209: 331-342.

Flexner, L. B., Tyler, D. B., and Gallant, L. J., 1950: Biochemical and physiological differentiation during morphogenesis. X. onset of electrical activity in developing cerebral cortex of fetal guinea pig. *Journal of Neurophysiology,* 13: 427-430.

Freeman, B. M. and Misson, B. H., 1970: pH, pO_2 ad pCO_2 of blood from the foetus and neonate of *Gallus Domesticus*. *Comparative Biochemistry and Physiology,* 33: 763-772.

Freeman, B. M., 1974a: Gaseous exchange and oxygenation of the embryo. *In* Freeman, B. M. and Vince, M. A. (eds.), *Development of the Avian Embryo*. London: Chapman and Hall Ltd, 119-159.

Freeman, B. M., 1974b: Physiology of hatching. *In* Freeman, B. M. and Vince, M. A. (eds.), *Development of the Avian Embryo*. London: Chapman and Hall Ltd, 249-260.

Garcia-Austt Jr, E., 1954: Development of electrical activity in cerebral hemispheres of the chick embryo. *Proceedings of the Society for Experimental Biology and Medicine,* 86: 348-352.

Gurvitch, A. M. and Ginsburg, D. A., 1977: Types of hypoxic and posthypoxic delta activity in animals and man. *Electroencephalography and clinical neurophysiology,* 42: 297-308.

Hamburger, V. and Oppenheim, R., 1967: Prehatching motility and hatching behavior in the chick. *Journal of Experimental Zoology,* 166: 171-204.

Hamburger, V. and Hamilton, H. L., 1992: A series of normal stages in the development of the chick embryo. *Developmental Dynamics,* 195: 231-272.

Hunter, M., Battilana, M., Bragg, T., and Rostas, J. A. P., 1999: EEG as a measure of developmental changes in the chicken brain. *Developmental Psychobiology,* 36: 23-28.

Hutchison, M. J., Lirette, A., Etches, R. J., Towner, R. A., and Janzen, E. G., 1992: Research note: an assessment of egg yolk structure using magnetic resonance imaging. *Poultry Science,* 71: 2117-2121.

Hyline.International, 2009: Technical Bulletin: Identification of Hy-Line parent chicks. URL http://www.hyline.com/userdocs/library/Parent_identification.pdf

Kamp, A. and Lopes Da Silva, F., 1999: Technological basis of EEG recording. *In* Niedermeyer, E. and Lopes Da Silva, F. (eds.), *Electroencephalography: basic principles, clinical applications, and related fields, 4th edition*. Baltimore: Lippincott Williams & Wilkins, 110-121.

Kraaier, V., Van Huffelen, A. C., and Wieneke, G. H., 1988: Quantitative EEG changes due to hypobaric hypoxia in normal subjects. *Electroencephalography and clinical neurophysiology,* 69: 303-312.

Kuo, Z. Y. and Shen, T. C. R., 1937: Ontogeny of embryonic behaviour in aves. XI. Respiration in the chick embryo. *Journal of Comparative Psychology,* 24: 49-58.

Lee, S. J., Ralston, H. J. P., Drey, E. A., Partridge, J. C., and Rosen, M. A., 2005: Fetal pain: a systematic multidisciplinary review of the evidence. *Journal of the American Medical Association,* 294: 947-954.

Lipar, J. L., Ketterson, E. D., Nolan Jr, V., and Casto, J. M., 1999: Egg yolk layers vary in the concentration of steroid hormones in two avian species. *General and Comparative Endocrinology,* 115: 220-227.

Mayhew, I. G. and Washbourne, J. R., 1990: A method of assessing auditory and brainstem function in horses. *British Veterinary Journal,* 146: 509-518.

McNamara, F., Wulbrand, H., and Thach, B. T., 1998: Characteristics of the infant arousal response. *Journal of Applied Physiology,* 85: 2314-2321.

McNamara, F., Lijowska, A. S., and Thach, B. T., 2002: Spontaneous arousal activity in infants during NREM and REM sleep. *Journal of Physiology,* 538: 263-269.

Medina, L. and Reiner, A., 2000: Do birds possess homologues of mammalian primary visual, somatosensory and motor cortices. *Trends in Neurosciences,* 23: 1-12.

Mellor, D. J. and Gregory, N. G., 2003: Responsiveness, behavioural arousal and awareness in fetal and newborn lambs: experimental, practical and theraputic implications. *New Zealand Veterinary Journal,* 51: 2-13.

Mellor, D. J., Diesch, T. J., Gunn, A. J., and Bennet, L., 2005: The importance of 'awareness' for understading fetal pain. *Brain Research Reviews,* 49: 455-471.

Mellor, D. J. and Diesch, T. J., 2006: Onset of sentience: the potential for suffering in fetal and newborn farm animals. *Applied Animal Behaviour Science, 100*: 48-57.

Mellor, D. J. and Diesch, T. J., 2007: Birth and hatching: key events in the onset of awareness in the lamb and chick. *New Zealand Veterinary Journal,* 55: 51-60.

Moruzzi, G. and Magoun, H. W., 1949: Brain stem reticular formation and activation of the EEG. *Electroencephalography and clinical neurophysiology,* 1: 455-473.

Mostl, E., Spendier, H., and Kotrschal, K., 2001: Concentration of immunoreactive progesterone and androgens in the yolk of hens' eggs (*Gallus domesticus*). *Wiener Tierarztliche Monatsschrift,* 88: 62-65.

Murrell, J. C. and Johnson, C. B., 2006: Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29: 325-335.

Ookawa, T., 1972: Avian wakefulness and sleep on the basis of recent electroencephalographic observations. *Poultry Science,* 51: 1565-1574.

Ookawa, T., 2004: The electroencephalogram and sleep in the domestic chicken. *Avian and Poultry Biology Reviews,* 15: 1-8.

Ozaki, H., Watanabe, S., and Suzuki, H., 1995: Topographic EEG changes due to hypobaric hypoxia at simulated high altitude. *Electroencephalography and clinical neurophysiology,* 94: 349-356.

Peters, J., Vonderahe, A., and Schmid, D., 1965: Onset of cerebral electrical activity associated with behavioural sleep and attention in the developing chick. *Journal of Experimental Zoology,* 160: 255-262.

Peters, J. J., Vonderahe, A. R., and Powers, T. H., 1956: The functional chronology in developing chick nervous system. *Journal of Experimental Zoology,* 133: 505-518.

Pignataro, L., Colman Lerner, A. A., Baranao, J. L., and Fiszer de Plazas, S., 1998: Biosynthesis of progesterone derived neurosteroids by developing avian CNS: *in vitro* effects on the $GABA_A$ receptor complex. *International Journal of Developmental Neuroscience,* 16: 433-441.

Price, D. D., Barrell, J. J., and Rainville, P., 2002: Integrating experiental-phenomenological methods and neuroscience to study neural mechanisms of pain and consciousness. *Consciousness and Cognition,* 11: 593-608.

Raj, A. B. M. and O'Callaghan, M., 2004: Effect of amount and frequency of head-only stunning currents on the electroencephalogram and somatosensory evoked potentials in broilers. *Animal Welfare,* 13: 159-170.

Rogers, L. J., 1995: *The Development of Brain and Behaviour in the Chicken*. Cambridge: Cab International, 273 pp.

Romanoff, A. L., 1960: *The Avian Embryo*. New York: The Macmillan Company, 1305 pp.

Seth, A. K., Baars, B. J., and Edelman, D. B., 2005: Criteria for consciousness in humans and other mammals. *Consciousness and Cognition,* 14: 119-139.

Sharma, K. N., Dua, S., Singh, B., and Anand, B. K., 1963: Electro-ontogenesis of cerebral and cardiac activities in the chick embryo. *Electroencephalography and clinical neurophysiology,* 16: 503-509.

Simons, A. J. R., Boezeman, E. H. J. F., and Pronk, R. A. F., 1989: Automatic EEG monitoring of anaesthesia. *In* Jones, J. G. (ed.), *Bailliere's Clinical Anaesthesiology: International Practice and Research, Vol.3 number 3*. London: Bailliere Tindall, 623-646.

Sommerville, B. A. and Broom, D., M, 1998: Olfactory awareness. *Applied Animal Behaviour Science,* 57: 269-286.

Speciale Jr, S. G., Nowaczyk, T., and Jouvet, M., 1975: A longitudinal study of bioelectric activity in the pre- and post-hatch chick. *Developmental Psychobiology,* 9: 539-549.

Stern, C. D., 2005: The chick: a great model system becomes even greater. *Developmental Cell,* 8: 9-17.

Tazawa, H., Mikami, T., and Yoshimoto, C., 1971: Respiratory properties of chicken embryonic blood during development. *Respiration Physiology,* 13: 160-170.

Tazawa, H., 1980: Oxygen and CO_2 exchange and acid-base regulation in the avian embryo. *American Zoologist,* 20: 395-404.

Tsutsui, K., Matsunaga, M., and Ukena, K., 2003: Biosynthesis and biological actions of neurosteroids in the avian brain. *Avian and Poultry Biology Reviews,* 14: 63-78.

Tuge, H., Kanayama, Y., and Yueh, C. H., 1960: Comparative studies on the development of EEG. *Japanese Journal of Physiology,* 10: 211-220.

Velarde, A., Ruiz-de-la-Torre, J. L., Rosello, C., Fabrega, E., Diestre, A., and Manteca, X., 2002: Assessment of return to consciousness after electrical stunning in lambs. *Animal Welfare,* 11: 333-341.

Viapiano, M. S. and Fiszer de Plazas, S., 1998: Comparative modulation by 3α,5α and 3β,5β neuro steroids of GABA binding sites during avian central nervous system development. *Neurochemical Research,* 23: 155-161.

Viapiano, M. S., Mitridate de Novara, A. M., and Fiszer de Plazas, S., 1998: Neurosteroid modulation of GABA binding sites in developing avian central nervous system. *Neurochemistry International,* 32: 291-298.

Vince, M., Misson, B. H., and Freeman, B. M., 1975: Blood gas partial pressures and the onset of lung ventilation in the chick embryo. *Comparative Biochemistry and Physiology,* 51A: 457-463.

Vince, M. A. and Tolhurst, B. E., 1975: The establishment of lung ventilation in the avian embryo: the rate at which lungs become aerated. *Comparative Biochemistry and Physiology,* 52A: 331-337.

Visschedijk, A. H. J., 1968: The air space and embryonic respiration: 1. the pattern of gaseous exchange in the fertile egg during the closing stages of incubation. *British Poultry Science,* 9: 173-184.

Weiskrantz, L., 1995: The problem of animal consciousness in relation to neurophysiology. *Behavioural Brain Research,* 71: 171-175.

Chapter 4. Effects of noxious stimulation on the electroencephalogram of the chicken

"Kestral"

Some of the findings from this chapter were presented at the 43rd Congress of the International Society for Applied Ethology in Cairns, Australia in 2009.

McIlhone, A. E., Beausoleil, N. J., Mellor, D. J., Mitchinson, S. L., and Johnson, C. B., 2009: Development of electrical activity and responses to noxious stimuli in the chicken brain. *43rd Congress of the International Society for Applied Ethology*: p106 (Abstract).

Also, at the 10th World Congress of Veterinary Anaesthesia in Glasgow, Scotland in 2009.

McIlhone, A. E., Beausoleil, N. J., Mellor, D. J., Mitchinson, S. L., and Johnson, C. B., 2009: Effect of noxious stimulation on the electroencephalogram of anaesthetised chickens. *10th World Congress of Veterinary Anaesthesia*: p161 (Abstract).

4.1 Abstract

The electroencephalogram (EEG) has been used to record brain electrical responses to noxious stimulation in anaesthetised mammals. This study tested whether a similar protocol could be used in chickens.

Two groups of 10 chickens were used in this study. All chickens were anaesthetised using halothane in oxygen. Anaesthesia was maintained with 0.9-1% end-tidal halothane and the EEG was recorded continuously. The EEG and anaesthesia were stable for 15 minutes before treatments started. The first 10 chickens were given a 5 second toe-clamp stimulus using a pair of haemostats. The other 10 chickens each received four noxious stimuli, delivered 15 minutes apart: 1) 55°C rod on the skin under the wing for five seconds; 2) 50 Hz 50 V electrical stimulation of the lateral plantar nerve for two seconds; 3) five second skin pinch using haemostats; and 4) feather removals from the leg and chest. Spectral analysis of the EEG generated the variables: median frequency, 95% spectral edge frequency and total power.

Electrical stimulation caused an increase in median frequency five to ten seconds after stimulation ($P<0.05$), 95% spectral edge frequency and total power were unchanged. The EEG variables following the other stimuli did not differ from baseline ($P>0.05$).

The chicken's EEG appeared to be less responsive to noxious stimulation than the mammalian EEG. This discrepancy between birds and mammals may relate to differences in brain anatomy. Another method may be needed to investigate the processing of noxious sensory inputs in birds.

4.2 Introduction

Previous studies in various mammalian species have used the electroencephalogram (EEG) to record responses to noxious stimuli during anaesthesia (Gibson et al., 2007; Murrell et al., 2007). When the mammal is lightly anaesthetised with halothane, the brain activity is still responsive to noxious sensory inputs (Murrell and Johnson, 2006). This model has been used in welfare studies to record responses to potentially painful husbandry procedures (Murrell et al., 2003; Johnson et al., 2005a; Johnson et al., 2005b; Gibson et al., 2007; Gibson et al., 2009). The current study aimed to investigate whether a similar model could be used in birds.

4.2.1 Pain in animals

Pain in animals has been defined as "an aversive sensory and emotional experience representing awareness by the animal of damage or threat to the integrity of its tissues" (Molony and Kent, 1997). Noxious stimuli activate nociceptive nerve fibres in animal tissues which is a process referred to as nociception. These nerve fibres send signals to the spinal cord which relays signals towards the brainstem, thalamus, cortex and limbic system where the signal can be interpreted as pain (Bromm and Lorenz, 1998). If the sensory input is not processed by the higher brain areas to be perceived consciously then the process is still called nociception. Pain involves an emotional response, therefore an animal needs to be conscious in order for it to experience pain (Lee et al., 2005).

Pain provides a vital survival function to the animal, alerting it to potential tissue damage and providing it with the opportunity to subsequently identify and

avoid pain-inducing situations. However, the emotional component of pain is, by definition, unpleasant, and prolonged or intense pain can therefore have strong negative effects on the animal's welfare (Bromm and Lorenz, 1998).

For this reason, being able to assess pain in animals is important, but it can be difficult because of the language barrier between animals and humans. Pain assessments therefore rely on behavioural and physiological variables that change in response to pain or nociception in that species (Gentle, 1992).

The behavioural indicators of pain are not always obvious and can be diverse. Different species, ages or individuals among animals may exhibit different pain behaviours (Morton and Griffiths, 1985; Mellor et al., 2000). Some animals, particularly prey species, may also hide their pain behaviours (Hawkins, 2006). In experimental trials it can be difficult to measure the efficacy of analgesic drugs when trying to distinguish between pain, fear, stress and any sedative effects of the drugs i.e. determining whether the animal is subdued because of pain, or because the analgesic has a sedative effect (Hawkins, 2006). In order to assess pain in any situation the observer needs to be familiar with the animal's normal and abnormal behaviours (Morton and Griffiths, 1985).

It is also helpful to follow physiological changes that occur when an animal experiences pain or noxious sensory input. Once the behavioural and physiological changes have been identified suitable measures can be selected to assess pain in experimental, clinical and other general conditions (Molony and Kent, 1997; Mellor et al., 2000).

4.2.2 Cerebral cortex is involved in the conscious perception of pain

Multiple cortical and subcortical regions in the mammalian brain are involved in pain processing. Several experiments have used imaging techniques such as functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) to examine the activity of various brain regions during pain in mammals (Apkarian et al., 2005).

4.2.3 EEG responses to noxious stimuli

The EEG reflects cortical processing. Changes in the EEG due to nociception indicate changes in cortical activity including the processing of neural signals relating to pain (Murrell and Johnson, 2006). Event related potentials (ERPs) and the EEG may be useful tools for examining acute (short term) pain in animals. Studies with sheep identified increases in brain activity as the intensity of a noxious electrical stimulus increased. The amplitude of ERPs and the EEG frequency were both greater with increased stimulus intensity (Morris et al., 1997).

In the horse, noxious sensory input in the form of castration caused an increase in the high-frequency component and a decrease in the low frequency component of the EEG (Murrell et al., 2003). Similar changes are seen in response to various noxious stimuli in other mammals (Johnson et al., 2005a; Johnson et al., 2005b; Murrell and Johnson, 2006; Gibson et al., 2007; Murrell et al., 2007; Gibson et al., 2009). The changes that occur in the EEG during noxious stimulation seem to represent the acute phase of pain. The EEG variables that change following dehorning in cattle, return to pre-treatment values within two

minutes of the event, despite other evidence that the pain may continue for a greater length of time (Gibson et al., 2007).

4.2.4 Minimal anaesthesia model

In mammals, halothane anaesthesia causes less depression of cortical activity than many other anaesthetic agents (Johnson and Taylor, 1998; Murrell et al., 2008). This discovery has lead to the development of a model for recording the EEG of anaesthetised mammals and investigating the effects of noxious stimulation on the EEG (Johnson et al., 2005b). In this minimal-anaesthesia model the animal is unconscious and therefore not perceiving pain. However, under halothane anaesthesia noxious sensory inputs still cause identifiable changes in the EEG (Murrell et al., 2003). It is therefore possible to humanely record a response to a stimulus that would be painful if the animal were conscious. This methodology enables the use of negative controls without the concerns of causing pain in an animal (Murrell et al., 2007). For example, in a study on dehorning cattle, two groups of calves were dehorned. One group received prior treatment with a local anaesthetic, while the other group (negative control) did not receive the local anaesthetic. EEG responses to dehorning were recorded in both groups and the results could be compared between the two groups. However, because the calves were under general anaesthetic, neither group was conscious to experience the pain associated with the dehorning (Gibson et al., 2007).

EEG responses to noxious stimulation can be variable. In rats, there was an increase in median frequency following electrical and thermal stimuli, but not after a mechanical stimulus. There was also a decrease in total power after

electrical and mechanical stimulation, but not after the thermal stimulation. There were no changes in 95% spectral edge frequency following any of these stimuli (Murrell et al., 2007). Therefore when recording EEG responses to noxious stimuli, the experimental setup needs to be carefully controlled (e.g. controlling anaesthetic depth, consistent delivery of the stimulus) to try and limit other sources of variability (Murrell et al., 2007)

An advantage of using the EEG for pain assessment is that the EEG reflects cortical processing which is correlated with consciousness. Accordingly, when the EEG responds to noxious stimulation, the changes in brain activity are likely to indicate the cortical processing of pain and are therefore linked to the cognitive experience and perception of pain (Craig et al., 1996). Therefore, the EEG indices are apparently linked to what would be the cognitive perception of pain, and can provide information on the perceived level of pain (Craig et al., 1996; Ong et al., 1997).

4.2.5 Pain in birds

Pain in birds is an important animal welfare concern both clinically and in the poultry industry. Chickens in the poultry industry may experience both acute and chronic pain due to production practice. For example, beak-trimming involves cutting and/or cauterising the end of the chicken's beak. Birds have nociceptors for detecting noxious stimuli (Gentle, 1992). The beak is a well-innervated tissue and the process of cutting it may cause both acute and chronic pain (Cheng, 2006). Other potentially painful practices may include: leg shackling prior to slaughter, injuries of chickens during collection and transport of the birds (e.g. gathering the birds up for slaughter).

Both pet and wild birds are admitted to veterinary clinics due to injury or illness and may require pain relief following procedures. As birds seem to metabolise analgesic drugs differently to mammals, and at different rates, it is important to conduct reliable analgesic efficacy tests (Hoppes et al., 2003).

EEG responses to noxious stimuli have been used to explore potentially painful husbandry procedures as well as the ontological onset of awareness in mammalian species (Murrell and Johnson, 2006; Gibson et al., 2007; Diesch et al., 2009b, 2009a). The aim of the present study was to determine whether this model could be used for evaluating pain perception in chickens. That is, the aim was to investigate whether the chicken has an EEG response to noxious stimulation using standardised noxious inputs. If validated for chickens, this model could be used to investigate painful husbandry procedures used in the poultry industry such as beak trimming, leg shackling, and slaughter with/without stunning, as well as to test the efficacy of analgesic drugs used in clinical practice.

The aim of this study was to test whether noxious stimulation caused any identifiable changes in the EEG of anaesthetised chickens. The stimuli used were similar to those that have been used in previous mammalian studies. The second aim was to examine whether the intensity or mode of noxious stimulation was correlated with quantitative changes in the EEG.

4.3 Methods

4.3.1 Chickens

Two groups of chickens were used, 10 for the pilot study testing the effect of a toe clamp (Experiment 1) and 10 for testing the effects of four other stimuli (Experiment 2). The Experiment 1 chickens were male and the Experiment 2 chickens were female. All chickens were of the Hyline brown layer breed. At the time of the study, the Experiment 1 chickens were 5-6 weeks old and the Experiment 2 chickens were 7-8 weeks old. The chickens were obtained from a commercial hatchery and were kept in groups of 5-7 under controlled temperature and lighting conditions. Food (Ingham chick starter crumbles, Levin) and fresh water were freely available to the chickens. The chickens were kept on wood-shavings substrate with perches available. All procedures were approved by the Massey University Animal Ethics Committee (protocols 07/121 and 08/80).

The chickens in Experiment 1 were also subjects in the study described in chapter 5, which was conducted immediately after the pilot toe-clamp test described here. Experiment 2 was conducted after preliminary analysis of the pilot study results.

4.3.2 Anaesthesia

Anaesthesia was induced using either an induction chamber or a face mask. Halothane in oxygen was delivered to the chicken at 0.5-2% halothane in 2Lmin^{-1} of oxygen until the righting reflex was lost. This was used as the primary indicator of loss of consciousness.

The chicken's larynx was desensitised by applying 0.1 – 0.2mL of lignocaine local anaesthetic (Nopaine; Phoenix Pharm Distributers Ltd, Auckland, New Zealand) to the back of the throat and orotracheal intubation was performed using a 2.5mm non-cuffed endotracheal tube. The chicken was maintained under anaesthesia using a t-piece non-rebreathing anaesthetic circuit and was ventilated using an intermittent positive pressure ventilation (IPPV) system (V-valve ventilator, Vetronics, Bioanalytical Systems Inc, W La Fayette, IN, USA). An anaesthetic monitor (Hewlett Packard M1025B; Hewlett Packard, Hamburg, Germany) recorded respiration rate, inspired and end-tidal anaesthetic concentrations and end-tidal CO_2 concentrations. The chicken was placed on a water-heated blanket (T pump, Gayman Industries Inc, NY, USA) set to approximately 37°C to reduce heat loss. Heart rate and temperature were monitored throughout the experiment (Figure 4.1). End tidal halothane was kept stable at 0.95 $\pm$ 0.1% (range 0.85 – 1.05% across all chickens, variation $\pm$ 0.05% for each chicken).

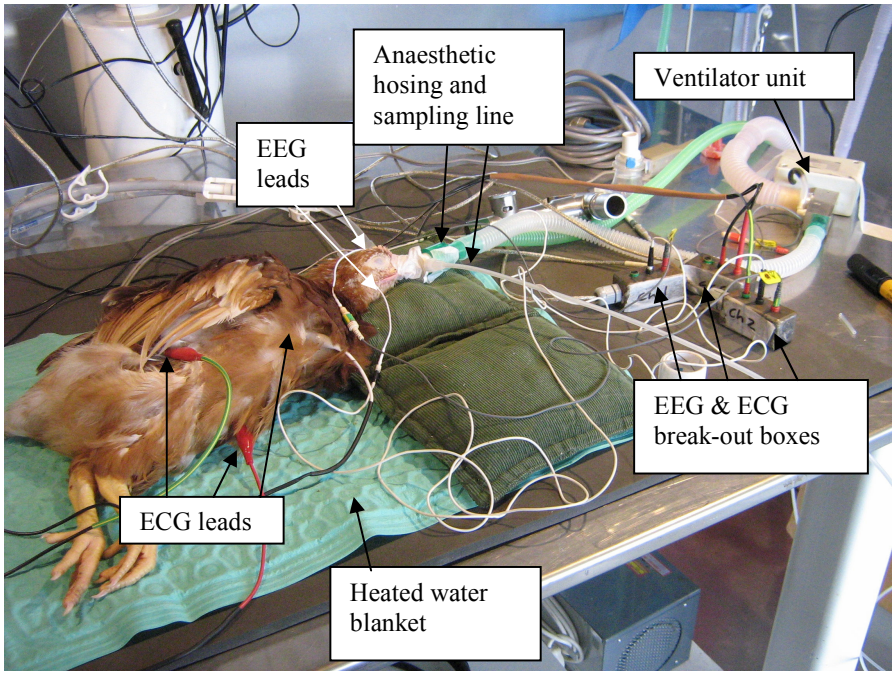


Figure 4.1 Experimental setup for measuring responses to noxious stimuli.

4.3.3 EEG and electrocardiogram (ECG) recording

For both experiments, the EEG was recorded using 27-gauge subcutaneous, stainless-steel needle electrodes (Viasys Healthcare, Surrey, England). These were positioned to record the EEG from the left and right sides of the brain using two channels in the chart recording software (Chart 5.5.6, ADInstruments Ltd, Sydney, Australia). The electrode positions were based on the montage for horses, described by Mayhew and Washbourne (1990). The electrode sites on the chickens were: lateral to the comb (non-inverting electrodes) and caudal to the external auditory meatus (inverting electrodes).

For Experiment 1 chickens there was a common non-inverting electrode for the left and right channels, and also an earth electrode located medio-dorsally on the chicken's neck at the base of the skull. Heart rate in Experiment 1 was monitored using a pulse oximeter, but was not recorded for analysis.

For Experiment 2, the chicken's ECG was recorded with electrode clips placed medio-cranially to the cranial end of the keel bone, 3cm lateral to the caudal end of the keel bone, and, the earth electrode, lateral to the pelvis. This last electrode served as a common earth for both ECG and EEG recording.

The EEG and ECG electrode cables fed into three identical break-out boxes, one for each channel, with each break-out box plugged into a physiological signal amplifier (Iso-Dam isolated physiological signal amplifier, World Precision Instruments, Sarasota FL, USA). The amplifiers provided a signal gain of 1000 and recorded frequencies between 0.1 Hz and 0.1 kHz, i.e. high-pass filters were

set at 0.1 Hz and low-pass filters were set at 0.1 kHz. Each amplifier fed into an analogue-to-digital converter (Powerlab, ADInstruments Ltd, Sydney, Australia), which digitised the input signals at 1000 points/second and displayed and stored them on an Apple personal computer using Chart recording software.

4.3.4 Treatments

In each experiment, once both anaesthesia and EEG were stable, 15 minutes of baseline EEG was recorded.

Experiment 1

After baseline recording, a toe-clamp stimulus was applied for 5s, the EEG recording continued throughout the stimulus and for 15 min afterwards (Table 4.1). These chickens were then kept under anaesthesia for use in a further experiment, described in Chapter 5.

Experiment 2

Four different noxious stimuli were applied to each chicken in this experiment. The stimuli were: a single thermal, mechanical or electrical stimulus and feather plucking consisting of the rapid removal of one feather followed 5 minutes later by the simultaneous removal of two feathers (Table 4.1). The original intention was to apply these four stimuli in a randomised order for each chicken. However, the electrical stimulation caused leg and body twitching which continued, in some chickens, throughout the rest of the experiment. Therefore, after observing this in the first three chickens, the electrical stimulus was given last to the seven remaining birds to prevent the muscular activity from affecting

the EEG responses to any stimulus applied subsequently. The other three stimuli were given in a random order.

(Table 4.1) Description of the noxious stimuli

	Stimulus	Description	Duration
Experiment 1	Mechanical	Pair of large haemostats (artery forceps) closed to the last ratchet to clamp the middle (longest) toe in the middle of the 2nd phalynx	5 s
Experiment 2	Mechanical	Pair of haemostats (artery forceps) closed to the last ratchet to pinch the skin just ventral to the chicken's vent	5 s
	Thermal	A metal rod heated to 55 ˚C applied to the skin on the body of the chicken, under the wing where there were no feathers	5 s
	Feather plucks (analysed as two separate stimuli)	Removal of feathers one from the breast cranial and lateral to the keel, and two simultaneously from the medial distal thigh 5 minutes later. (small contour/semiplume feathers)	< 1 s each time
	Electrical (delivered last)	Two silver/silver-chloride electrodes were placed subcutaneously in the chicken's lateral caudal thigh so that they were in close proximity to the lateral plantar nerve. The two electrodes were 1cm apart. A 50 V, 50 Hz stimulus was delivered across the electrodes.	2 s

The first of the four noxious stimuli was applied after the original 15 minutes of baseline recording. After the first stimulus the chicken was left for its EEG to re-stabilise for at least 15 minutes before the next stimulus was delivered. This procedure was repeated for each of the four stimuli so that the stimuli were applied no less than 15 minutes apart. The second feather removals were carried out 5 minutes after the first (see Table 1). After the fourth stimulus the chicken

was kept under anaesthesia and euthanised with an intravenous overdose of sodium pentabarbitone.

4.3.5 EEG Analysis

All the EEG recordings were stored on Apple personal computers and were analysed after the experiment was completed. All movement artefacts were excluded from the analysis of the EEG recording.

The EEG was analysed by Fast Fourier Transformation (FFT) using a purpose written software program (spectral analyser, C.B. Johnson, 2002), which carried out the FFT on each second of EEG. For each second, the FFT generates a frequency spectrum, which is a graphical representation of the contribution that each frequency makes to the power of the EEG waveform. Analysis of the EEG was based on the area under this frequency spectrum graph.

Variables derived from the frequency spectrum included: the *total power* of the EEG which was measured as the total area under the frequency spectrum; the *median frequency* which was the frequency below which half the total power was located; and the *95% spectral edge frequency* which was the frequency below which 95% of the total power was located (Murrell and Johnson, 2006).

To examine the effects of noxious stimuli for each chicken the mean "median frequency", "95% spectral edge frequency" and "total power" were calculated for: 30 seconds of baseline taken 1 minute prior to the each stimulus EEG and for seven 5-second EEG blocks following the stimulus extending to 40 seconds after

its application (Figure 4.2). Means were therefore calculated for each chicken at: baseline, during the stimulus, 5-10, 10-15, 15-20, 20-25, 25-30, 30-35, and 35-40 seconds after the stimulus. The mean value during the stimulus was only calculated when the EEG was not contaminated with artefacts at that time. The number of seconds over which the stimulus mean was calculated varied with the duration of the stimulus. The mean from 0-5 seconds after the stimulus was often excluded from the analysis because of contamination of the EEG with movement artefacts.

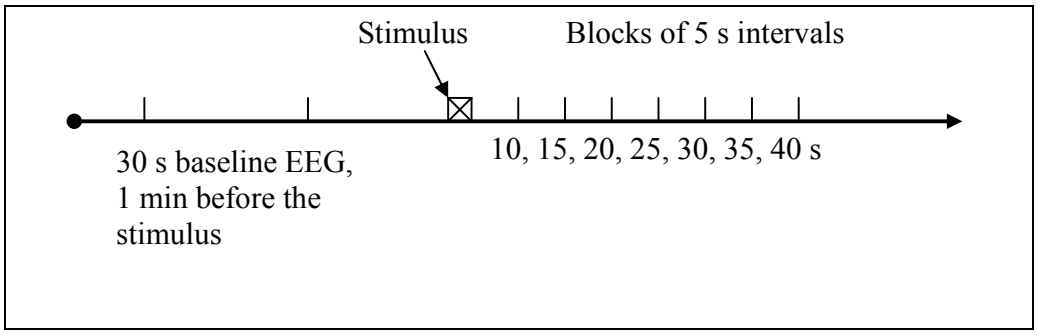


Figure 4.2 A diagram of how the EEG variables were calculated. To examine the effect of each noxious stimulus, the mean median frequency, 95% spectral edge frequency and total power were calculated over 5 seconds for each of seven time blocks following each stimulus. For each EEG variable, the mean of each block was compared to the mean calculated over 30 seconds of baseline EEG.

4.3.6 Adjustment to obtain results as percent change from baseline

The baseline values were standardized at 100% and the other values were converted to give a percent change from baseline to eliminate the skew caused by differing baseline values. To calculate the percent value the mean baseline value was calculated then each mean value (i.e. the means for 10, 15, 20 s etc after the stimulus) was divided by the baseline mean, and multiplied by 100.

4.3.7 Statistical analysis

All of the results were tested for normality and the results were found not to be normally distributed for some tests, and there were small numbers in some of the treatment groups. For each of the three EEG variables, a non-parametric repeated-measures ANOVA (Friedman's test) was used to test for an overall time effect caused by noxious stimulation. If a time effect was found then the Friedman's test was followed by Dunn's test to look at pair-wise comparisons between baseline and the seven time points following application of the stimulus (Figure 2)). The analysis was also repeated using a parametric test (repeated measures ANOVA and Dunnett's post hoc test), and the outcomes were very similar to the non-parametric test. All results were analysed using Graphpad Prism 5.01 for Windows (GraphPad Software, San Diego California, USA). Differences were considered significant at $P<0.05$.

4.3.8 Heart rate analysis

The heart rate results were analysed by calculating a baseline heart rate, which was the mean heart rate over a period of 10 seconds collected 1 min before the stimulus. The baseline heart rate was compared to the mean heart rate 0-10, 10-20, 20-30 and 30-40 s after the stimulus. A mixed model ANOVA with stimulus as a fixed effect and with repeated measures for time was used to analyse the heart rate results.

4.4 Results

4.4.1 Behaviour

Despite being under anaesthesia, many chickens showed movement responses to some of the noxious stimuli. The toe clamp and the electrical stimulus in particular often caused a limb withdrawal reflex. After the electrical stimulus many of the chickens twitched that limb for several minutes.

4.4.2 EEG Responses

The results from Channel 1 (left side of the brain) are presented below. Movement artefacts resulted in some of the EEG results being excluded from the analysis. Therefore there are not 10 chickens in every analysis; the number of chickens included in the analysis for each stimulus is stated in each of the results tables.

Experiment 1

Mechanical Stimulus – toe clamp

There was no effect of time on percent changes in median frequency ($F(8,48)=4.07$, $P=0.85$), 95% spectral edge frequency ($F(8,48)=4.38$, $P=0.82$) or total power ($F(8,48)=9.6$, $P=0.29$) following the mechanical toe clamp stimulus (Tables 4.2, 4.3 and 4.4). That is, there was no significant change from baseline at any time after application of the toe clamp stimulus.

Table 4.2. Mean (SEM) percentage changes in median frequency, relative to baseline values, for different noxious stimuli applied in Experiments 1 and 2. The value that differed significantly (p=0.05) from the baseline is marked with an asterisk. The values were scaled so that the baseline for each chicken = 100%.

Stimulus	baseline	stimulus	5	10	15	20	25	30	35	40
Toe-clamp (n=7)	100	105 (8)	-	116 (15)	105 (13)	108 (7)	106 (9)	103 (4)	105 (6)	105 (5)
Electrical (n=7)	100	-	-	**129 * (7)**	109 (5)	105 (5)	103 (8)	106 (4)	101 (5)	97 (3)
Feather 1 (n=8)	100	-	102 (4)	101 (5)	94 (5)	100 (4)	96 (3)	98 (4)	96 (3)	107 (5)
Feather 2 (n=8)	100	104 (5)	101 (4)	101 (3)	94 (4)	107 (3)	109 (3)	102 (3)	99 (3)	100 (5)
Skin pinch (n=6)	100	-	102 (9)	102 (3)	96 (5)	103 (5)	96 (4)	86 (6)	94 (6)	94 (2)
Thermal (n=9)	100	97 (3)	101 (3)	96 (4)	101 (4)	102 (4)	110 (6)	107 (4)	102 (5)	101 (4)

Table 4.3. Mean (SEM) percentage changes in 95% spectral edge frequency, relative to baseline values, for different noxious stimuli applied in Experiments 1 and 2. There were no significant differences between any of the values and the baseline. The values have been scaled so that the baseline for each chicken = 100%.

Stimulus	baseline	stimulus	5	10	15	20	25	30	35	40
Toe-clamp (n=7)	100	101 (1)	-	93 (9)	102 (2)	102 (2)	102 (2)	100 (1)	100 (1)	99 (1)
Electrical (n=7)	100	-	-	105 (1)	102 (1)	101 (1)	102 (2)	101 (1)	101 (1)	100 (2)
Feather 1 (n=8)	100	-	100 (1)	99 (0.5)	99 (1)	100 (1)	99 (1)	100 (1)	98 (1)	100 (1)
Feather 2 (n=8)	100	98 (1)	99 (1)	100 (1)	99 (1)	101 (1)	100 (1)	100 (1)	100 (1)	99 (1)
Skin pinch (n=6)	100	-	98 (1)	99 (1)	99 (1)	99 (1)	98 (1)	99 (1)	99 (1)	98 (1)
Thermal (n=9)	100	101 (1)	102 (1)	101 (1)	101 (1)	100 (1)	101 (1)	100 (1)	99 (2)	99 (1)

Table 4.4. Mean (SEM) percentage changes in total power, relative to baseline values, for different noxious stimuli applied in Experiments 1 and 2. There were no significant differences between any of the values and the baseline. The values have been scaled so that the baseline for each chicken = 100%.

Stimulus	baseline	stimulus	5	10	15	20	25	30	35	40
Toe-clamp (n=7)	100	101 (2)	-	104 (14)	115 (10)	108 (9)	106 (5)	105 (3)	102 (3)	102 (3)
Electrical (n=7)	100	-	-	101 (2)	109 (3)	108 (3)	104 (5)	103 (3)	107 (4)	108 (4)
Feather 1 (n=8)	100	-	99 (4)	99 (2)	97 (2)	99 (2)	99 (4)	100 (2)	102 (3)	98 (2)
Feather 2 (n=8)	100	97 (2)	98 (2)	97 (2)	100 (3)	94 (2)	96 (2)	98 (2)	96 (3)	97 (2)
Skin pinch (n=6)	100	-	101 (5)	102 (3)	101 (3)	97 (5)	102 (2)	105 (2)	103 (4)	100 (2)
Thermal (n=9)	100	102 (2)	100 (2)	98 (2)	99 (3)	99 (3)	94 (2)	99 (3)	104 (2)	104 (2)

Experiment 2

Electrical stimulus

Time had a significant effect on percent change in median frequency ($F(7,42)=18.1$, $P=0.0113$). Median frequency was significantly higher than baseline at 5-10 s after application of the electrical stimulus ($P<0.05$, Table 4.2, Figure 4.3). There were no significant changes in 95% spectral edge frequency ($F(7,42)=9.48$, $P=0.2203$, Table 4.3) or total power ($F(7,42)=8.86$, $P=0.2631$, Table 4.4).

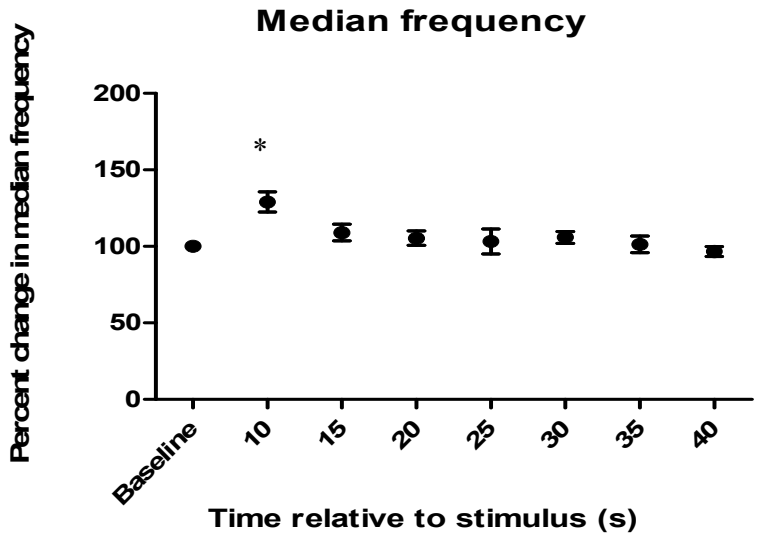


Figure 4.3. Percentage changes in mean (± SEM) median frequency relative to baseline for the electrical stimulus. The values were scaled so that the baseline point for each chicken equalled 100%. Values that differ significantly from baseline are marked with an asterisk ($P<0.05$).

Feather plucking

There was no effect caused by time after removal of the first feather for median frequency ($F(8,56)=6.482$, $P=0.5934$, Table 4.2), 95% spectral edge frequency ($F(8,56)=4.300$, $P=0.8291$, Table 4.3), or total power ($F(8,56)=2.377$, $P=0.9672$, Table 4.4). There was also no effect caused by time after the second feather removal for median frequency ($F(9,63)=12.0$, $P=0.2133$, Table 4.2), 95% spectral edge frequency ($F(9,63)=4.745$, $P=0.8559$, Table 4.3), or total power ($F(9,63)=8.182$, $P=0.5159$, Table 4.4).

Thermal stimulus

There was no effect caused by time on percentage changes in median frequency ($F(9,72)=11.79$, $P=0.2255$, Table 4.2), 95% spectral edge frequency ($F(9,72)=8.782$,

P=0.4577, Table 4.3), or total power (F(9,72)=18.62, P=0.0286, Table 4.4) following the thermal stimulus. That is, there was no significant change from baseline at any time after application of the thermal stimulus.

Mechanical stimulus – skin pinch

There was no effect caused by time on percentage changes in median frequency (F(8,40)=13.24, P=0.1037, Table 4.2), 95% spectral edge frequency (F(8,40)=5.600, P=0.6919, Table 4.3), or total power (F(8,40)=5.419, P=0.7120, Table 4.4) following the mechanical skin-pinch stimulus. That is, there was no significant change from baseline at any time after application of the skin-pinch stimulus.

Channel 2

The EEG recordings from the right side of the brain were analysed in the same way and revealed that very similar patterns were present in both hemispheres. The channel 2 results are presented in Appendix C.

4.4.3 Heart rate

The analysis identified significant effects for time ($F(4,25)=8.5$, $p<0.001$) and stimulus ($F(4,25)=4.7$, $p<0.05$). There was also an interaction effect ($F(16,25)=8.1$, $p<0.001$), which showed that the effect of time was dependent on which stimulus was being tested. The electrical stimulus caused an increase in heart rate both at 10-20 ($t=9.6$, $p<0.001$) and 20-30 seconds ($t=4.9$, $p<0.001$) after the stimulus. The heart rate did not differ from baseline by 20-30 or 30-40 s after the electrical stimulus ($p>0.05$). None of the other stimuli caused any significant changes in the heart rate (Figure 4.4).

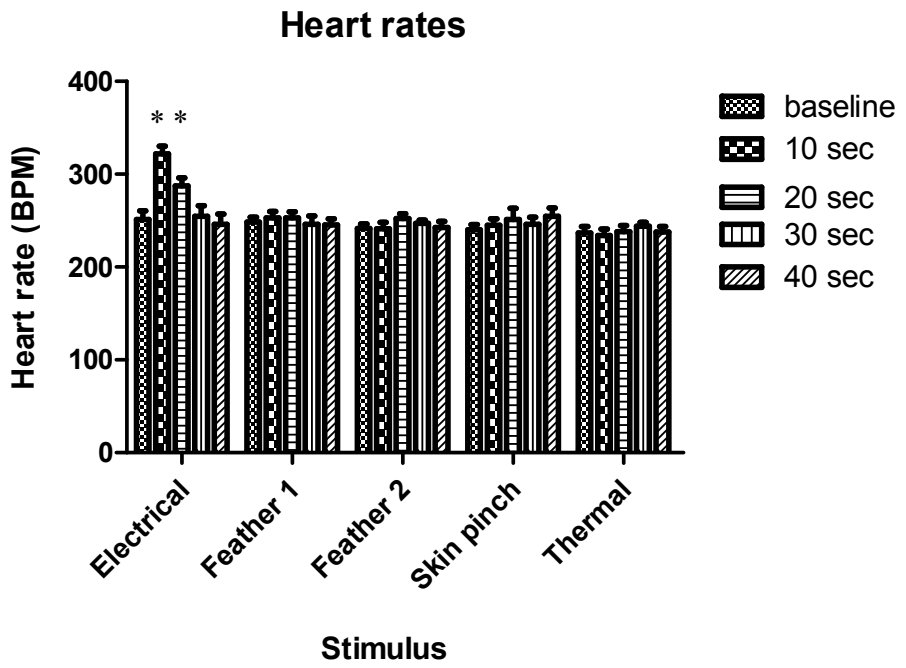


Figure 4.4. Mean heart rates during baselines and for the time 0-10, 10-20, 20-30 and 30-40 seconds following each of the noxious stimuli. The electrical stimulus was the only stimulus that resulted in a significant change in heart rate. Within each stimulus, values that differed significantly from baseline are marked with an asterisk ($P<0.05$).

4.5 Discussion

The aim of this study was to test whether noxious stimulation caused any identifiable changes in the EEG of anaesthetised chickens. The noxious stimuli caused no consistent changes in the chicken EEG variables. There was a small but significant increase in median frequency after the electrical stimulus; however, there were no changes in any of the EEG variables following the other noxious stimuli.

Although there was a change in the EEG following the electrical stimulus, that result should be interpreted with caution. Electrical stimulation causes non-specific activation of many afferent fibres and leads to both noxious and non-noxious input to the cerebral cortex (Murrell et al., 2007). Therefore the change in brain activity identified may or may not be related to processing of the noxious stimulus.

The absence of an EEG response to noxious stimuli in anaesthetised chickens is consistent with results from a previous study on the effects of a 50°C thermal stimulus and a comb pinch on conscious chickens. In that previous study there was also no response to either stimulus (Woolley and Gentle, 1987). Another study of conscious chickens found that feather removal caused a change in the EEG from a "high amplitude low frequency" drowsy resting state to a "low amplitude high frequency" pattern consistent with alertness (Gentle and Hunter, 1990). Therefore, it seems that any EEG response to noxious stimulation is inconsistent, and can be difficult to detect.

4.5.1 EEG responses to noxious stimulation in mammals

These results in chickens differ from those found in previous studies in mammals. There have been several studies investigating the effects of noxious stimulation on the mammalian EEG using the minimal anaesthesia model. For example, scoop dehorning of cattle without analgesia caused significant increases in both median frequency and 95% spectral edge frequency when compared to baseline values (before dehorning) and compared to values after dehorning when the cattle received a lignocaine analgesic ring-block (Gibson et al., 2007).

Dehorning also caused a decrease in total power of the EEG when compared to baseline or dehorning after administering a local anaesthetic (Gibson et al., 2007). Similarly, castration of ponies caused an increase in median frequency, but not in 95% spectral edge frequency (Murrell et al., 2003), while castration of lambs caused an increase in both median frequency and 95% spectral edge frequency (Johnson et al., 2009). Velvet antler removal in deer caused increases in median frequency and 95% spectral edge frequency and decreases in total power. These EEG changes were reduced when a lignocaine analgesic ring block was applied before antler removal (Johnson et al., 2005b).

It has been suggested that the decrease in total power seen in several studies may be a result of decreased adequacy of anaesthesia due to the noxious stimulus. Meanwhile, the increase in median frequency may reflect the processing of the noxious part of the stimulus (Murrell et al., 2007).

Although a change to high frequency, low-amplitude EEG is the more common response to pain, there may instead be a change to low frequency, high amplitude activity (Murrell and Johnson, 2006; Murrell et al., 2007). In some rat pups a tail clamp stimulus caused decreases in median (21-22 day old pups) or 95% spectral edge (12-14 and 21-22 day old pups) frequencies (Diesch et al., 2009b). Overall, previous mammalian studies seem to show that noxious stimuli can cause identifiable changes in the EEG. This was not the result observed in the present chicken EEGs.

4.5.2 Effect of pain on mammalian brain

In mammals, multiple areas in the cerebral cortex are involved in pain perception. These areas include the somatosensory cortices 1 and 2, the anterior cingulate gyrus and the insular cortex (Apkarian et al., 2005; Christmann et al., 2007). In accordance with this, painful stimuli seem to have widespread effects on brain activity. A localised stimulus can induce a change in general cortical functioning rather than just affecting localised brain regions (Ploner et al., 2006). It is likely that the widespread pain-induced changes in brain function can be attributed to the biological importance of pain. Pain must be able to attract an animal's attention to potential or actual tissue damage and elicit some form of reaction. This may be achieved by the extensive influence of pain on cerebral function (Ploner et al., 2006).

4.5.3 Heart rate responses in birds

There were increases in the chicken's heart rate both 10 and 20 seconds after the electrical stimulus. The other stimuli had no effect on the heart rate. Studies in anaesthetised ducks revealed increases in heart rate during noxious stimulation (Machin and Livingston, 2002). A study looking at the response of conscious chickens to noxious thermal or non-painful comb pinch stimuli detected increases in heart rate following the application of both stimuli (Woolley and Gentle, 1987). Another study reported an increase in heart rate in 62% of chickens following feather removal, but 20% of chickens had a decrease in heart rate and the remainder had irregular responses (Gentle and Hunter, 1990). In the present study, it is unclear why heart rate responses were not significant for three of the noxious stimuli. The heart rate response to the electrical stimulus may have been caused by an electrical effect on the heart. It is also possible that

any responses to the other stimuli were either very small or were too variable between birds for an overall effect to be detected.

4.5.4 Possible reasons for differences between the observed EEG responses of birds and mammals

There are a number of possible reasons why EEG responses to noxious stimuli may be detectable in mammals but not chickens, these are outlined below:

1. Intensity of stimulus

It is possible that the stimuli used in the study were not intense enough to generate a noticeable response in the EEG in birds. However, this seems unlikely. The stimuli were chosen because they were believed to be strong enough to elicit a response based on several lines of evidence.

The toe-clamp or tail-clamp has been used in many EEG studies on mammals, including those investigating the minimal alveoli concentration (MAC = the minimum amount of anaesthetic needed to prevent a movement response to noxious stimulation in 50% of subjects) (Ludders et al., 1988; Naganobu et al., 2000; Murrell et al., 2007; Diesch et al., 2009b). Furthermore, the chickens in these experiments often moved, showing a leg withdrawal reflex during or immediately after the toe-clamp was applied. This movement indicated that the stimulus was strong enough to cause central nervous system activation because the animal would have been close to MAC. Thus the EEG, at least in mammals, would have been likely to respond to the sensory input (Murrell and Johnson, 2006).

The electrical stimulus had also been used in previous studies in mammals (Murrell et al., 2007). The chickens also withdrew their leg either during or immediately after the electrical stimulation.

The thermal stimulus has been used before in mammals (Murrell et al., 2007) and the temperature used was considered hot enough to be noxious, but not hot enough to damage the skin of the chickens over the 5-second stimulus duration (Woolley and Gentle, 1987). It is possible that some of the heat was lost as the heated rod was taken from the water bath and carried to the chicken. However, the rod was applied to the skin under the chicken wing within 5 seconds of it being removed from the water bath.

The skin pinch was applied to an area that intuitively was thought to be sensitive. Certainly, the area around the chicken's vent seems sensitive. When one attempts to clean matted feathers from below the vent and tease out the mats chickens will struggle and vocalise in a manner that suggests they experience pain if the feathers are pulled or manipulated too greatly.

In accordance with this, *Feather removal* has been used as a noxious stimulus in previous work (Gentle and Hunter, 1990). Avian feathers are anchored in tissue that is supplied with sensory fibres and nerves that are sensitive to mechanical stimulation. The force required to remove feathers is far greater than that required to activate the mechanoreceptors and the feather plucking is likely to be painful (Gentle and Hunter, 1990). The sites on the chicken's leg and chest were selected because these are sites that seem to be particularly sensitive to feather removal in clinical cases (Massey University Vet Clinic, Wildlife Ward, personal communication, 2008).

Feather removal in conscious birds causes startle responses with agitated behaviours such as jumping, wing flapping and/or vocalisation. The continued removal of feathers caused the chickens to crouch with their head and tail down and their eyes partially closed (Gentle and Hunter, 1990).

2. Effect of anaesthesia on the EEG

As shown in Chapter 2, the anaesthetic agent and the depth of anaesthesia both affect the ability to record responses to noxious stimulation in the EEG. Noxious stimulus experiments on mammals use halothane at low end-tidal concentrations because it causes less depression of EEG activity than some other agents, such as isoflurane, sevoflurane and desflurane (Murrell and Johnson, 2006; Murrell et al., 2007). Therefore any changes in the EEG with noxious stimulation are easier to identify with halothane, compared to these other agents (Johnson and Taylor, 1998; Murrell et al., 2008). Similarly, in chickens, there was less depression of brain activity with halothane than with isoflurane or sevoflurane (see Chapter 2). It therefore seemed unlikely that halothane would obscure EEG responses to noxious stimulation in chickens.

3. Differences in brain anatomy

The avian brain differs structurally from the mammalian brain. It is possible that either the structure of the brain or the arrangement of the neurons affects how well the EEG is able to record changes in brain activity that occur as a result of noxious stimulation. A thorough comparison of avian and mammalian brains has been described previously (Butler et al., 2005; Butler and Cotterill, 2006) a brief summary of these differences is given below.

Despite some similarities between the brain regions of birds and mammals there are other distinct structures that are present in the mammalian brain, but not the avian brain (Butler et al., 2005). For example, the mammalian neocortex has a unique multi-layered structure that is not found in the avian brain (Butler et al., 2005). The mammalian neocortex has been a major focus in consciousness studies, partly because of its relative size in the mammalian brain, but also because of its extensive connectivity to, and formation of, apparently relevant networks and feedback loops involving the dorsal thalamus (Butler et al., 2005).

However, the absence of a particular brain structure in the avian brain compared to the mammalian brain does not necessarily imply that the associated function or processing capacity is also missing. Instead, that particular function may be carried out in another area of the avian brain (Butler et al., 2005; Butler and Cotterill, 2006).

To illustrate, the mammalian brain has a structure called the pallium, which comprises the dorsal part of the cerebrum (or telencephalon) and the rostral part of the forebrain. The mammalian pallium consists of a number of sub-structures including the hippocampal formation, olfactory cortex, claustrum, most of the amygdala and the neocortex (Butler et al., 2005). The avian brain also has a pallium, which contains some structures that are homologous to some of the mammalian areas (e.g. the hippocampal formation and the olfactory cortex). It is less clear how the other avian brain regions might resemble the mammalian claustrum and neocortex (Butler et al., 2005). The hippocampal region is located medially in the avian pallium, with the olfactory cortex located laterally. Between these structures are the hyperpallium (Wulst) and the dorsal ventricular ridge (collopallial subdivision). The hyperpallium and the dorsal ventricular

ridge have been described as being analogous to the mammalian neocortex and appear to be the sites that facilitate complex cognitive behaviours (Butler et al., 2005).

The positions of the hyperpallium and the dorsal ventricular ridge, relative to the positions of the EEG recording electrodes, may affect the success of distinguishing particular EEG responses to noxious stimuli. The hyperpallium is a relatively superficial structure although part of it does descend into the crevice between the left and right cerebral hemispheres. Similarly, the sections of the dorsal ventricular ridge (mesopallium, nidopallium and entopallium) are not wholly superficial structures and extend into deeper areas of the brain (see Appendix D) (Kuenzel and Masson, 1988; Butler et al., 2005; Jarvis et al., 2005; Butler and Cotterill, 2006).

As well as differences in brain anatomy, there are also differences in the anatomical arrangement of the neurons in the mammalian and avian neural circuits (Butler et al., 2005). The mammalian neocortex has a neural architecture with horizontal layers and pyramidal cells that are arranged into vertical columns (Butler et al., 2005). By contrast the neurons in the avian brain seem to have a more clustered (nuclear) arrangement. The dendrites of these neurons are projected in multiple dimensions and receive inputs from many directions (Butler et al., 2005). This clustered, multi-directional signal transfer may result in less localised changes in electrical activity in response to sensory input when compared to the mammalian brain. The EEG is sensitive to the dipoles which run through the layers of the cortex, but is less able to separate signals from multiple inputs (Apkarian et al., 2005). This may result in the absence of a response in EEG activity following noxious stimulation in birds

The electrode needles were positioned so that they transected the brain, in a similar way to the electrode arrangement in previous mammalian studies. However, the locations of regions within the brain and the arrangement of neurons within those regions are both different between birds and mammals. This may have been enough to affect the capacity of the EEG to record changes associated with nociception.

4. The possibility that birds do not perceive pain

The results of the current study may raise the question of whether birds actually perceive pain, and/or whether pain is processed in the higher brain regions of birds as it is in mammals. It seems very unlikely that chickens and other birds would not perceive pain or process it in higher brain areas, for two reasons. Firstly, birds have a nervous system that is sufficiently intricate to facilitate the presence of consciousness (Butler et al., 2005; Butler and Cotterill, 2006). Birds also show behavioural evidence for sophisticated cognitive processes that could only be the product of conscious thought processes (Butler et al., 2005). If an animal is conscious, then there is a strong likelihood of it having the capacity to perceive pain.

The second indicator that birds perceive pain is their responses to painful stimuli and analgesics. Avoidance behaviours including startles, jumping, wing-flapping and alarm vocalisations occur during noxious stimulation (Gentle and Hunter, 1990; Gentle, 1992). In addition, birds, including domestic chickens, are sensitive to the effects of common analgesics such as morphine (Hughes, 1990) and non-steroidal anti-inflammatory drugs (McGeown et al., 1999; Hocking et al., 2005). Lame broiler chickens show preference for feeds that contain analgesics,

which indicates that the chickens are feeling pain and that they have a preference for feeds that provide pain relief (Danbury et al., 2000).

4.5.5 Limitations to the study

There are some limitations to the use of the minimal anaesthesia model for recording EEG responses to noxious stimulation. For example, fluctuations in the depth of anaesthesia will affect the EEG recording (Murrell and Johnson, 2006). It was therefore very important to keep the end-tidal halothane concentration stable throughout the experiment. There can also be unexplained variation between individuals in their EEG spectra (Murrell et al., 2003). Some studies suggest that EEG recordings are not reliable or consistent enough to use as a measure of pain in the clinical context (Norman et al., 2008).

Electrophysiological measures such as the EEG provide accurate indicators of what is happening over time, but they are less suitable for providing information about what is happening in different regions of the brain. That is, the spatial representation of brain functioning is minimal (Christmann et al., 2007).

There are electrophysiological experiments which could be conducted to gather further information about potential changes in brain activity during nociception. One possible study could be to place the EEG electrodes at different locations on the head of the chicken. Different locations on the head may be more successful for detecting changes in brain activity. A second possible study would be to drill through the skull and position the electrodes in contact with the dura (the outside of the brain). Electrodes in closer proximity to the brain may be more sensitive to changes in brain activity during noxious stimulation. A third

investigation could position electrodes within the brain tissue. Electrodes could be placed at different locations and different depths within the brain. The brain activity recorded at different electrodes may provide an indication of where noxious stimuli are processed within the brain. The magnitude of any electrical response might also indicate whether or not subcutaneous EEG electrodes would be a feasible method of recording responses to noxious stimulation.

Without systematic criteria for electrode placement the series of experiments mentioned above could be very drawn-out and time-consuming. There are two techniques that could be used to direct these investigations. Firstly the investigations could focus on the hyperpallium and the dorsal ventricular ridge, which are believed to be involved in conscious processing (Butler et al., 2005). Use of a stereotaxic atlas would provide some coordinates for placing the electrodes (Kuenzel and Masson, 1988). A second method could be to use fMRI to record the brain activity of the chicken during noxious stimulation. The recording electrodes could then be positioned in relation to the areas that responded to stimulation during the fMRI.

There is some evidence that noxious stimulation causes muscular activity artefact in the EEG (Norman et al., 2008). However, many of the investigations into EEG responses to pain were conducted on animals under anaesthesia when muscular movements were limited. Furthermore, an investigation looking at rat EEG responses to noxious stimuli used cortically planted EEG electrodes. This methodology would have reduced the likelihood of EMG signal contamination, yet EEG changes were still identified following noxious stimulation (Murrell et al., 2007). These results indicate that not all EEG responses to noxious

stimulation are a result of muscle artefact and at least some responses can be attributed to changes in brain electrical activity.

4.6 Conclusions and suggestions for future work

There was a slight but significant increase in median frequency and an increase in heart rate following the electrical stimulus. However, none of the other noxious stimuli elicited changes in any of the EEG variables or the ECG of the chickens. These results suggest that the current EEG methodology cannot be used to measure pain in birds. There seem to be differences between birds and mammals which affect the characteristics of brain activity and/or make changes in this more difficult to detect using the EEG.

It would be valuable to identify the areas of the avian brain that process painful stimuli. The location of these areas may influence whether or not, or how well, their activity may be detected by the EEG electrodes. For example, if the areas that process pain are located deep within the avian brain, then the EEG may not pick up the changes in brain activity following a noxious stimulus. Imaging methods such as fMRI or PET would be helpful in determining which areas of the avian brain are involved in processing pain. For example, if the chicken could be kept anaesthetised in an fMRI or PET machine and have their brains scanned before, during and immediately after noxious stimulation, it may be possible to identify the brain areas that respond to, and process pain (Craig et al., 1996).

4.7 References

Apkarian, A. V., Bushnell, M. C., Treede, R. D., and Zubieta, J. K., 2005: Human brain mechanisms of pain perception and regulation in health and disease. *European Journal of Pain,* 9: 463-484.

Bromm, B. and Lorenz, J., 1998: Neurophysiological evaluation of pain. *Electroencephalography and clinical neurophysiology,* 107: 227-253.

Butler, A. B., Manger, P. R., Lindahl, B. I. B., and Arhem, P., 2005: Evolution of the neural basis of consciousness: a bird-mammal comparison. *BioEssays,* 27: 923-936.

Butler, A. B. and Cotterill, R. M. J., 2006: Mammalian and avian neuroanatomy and the question of consciousness in birds. *Biological Bulletin,* 211: 106-127.

Cheng, H., 2006: Morphopathological changes and pain in beak trimmed laying hens. *World's Poultry Science Journal,* 62: 41-52.

Christmann, C., Koeppe, C., Braus, D. F., Ruf, M., and Flor, H., 2007: A simultaneous EEG-fMRI study of painful electric stimulation. *NeuroImage,* 34: 1428-1437.

Craig, A. D., Reiman, E. M., Evans, A., and Bushnell, M. C., 1996: Functional imaging of an illusion of pain. *Nature,* 384: 258-260.

Danbury, T. C., Weeks, C. A., Chambers, J. P., Waterman-Pearson, A. E., and Kestin, S. C., 2000: Self-selection of the analgesic drug carprofen by lame broiler chickens. *The Veterinary Record,* 146: 307-311.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009a: Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Laboratory Animals,* 00: 1-9.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009b: Electroencephalographic responses to tail clamping in anaesthetized rat pups. *Laboratory Animals,* 43: 224-231.

Gentle, M. J. and Hunter, L. N., 1990: Physiological and behavioural responses associated with feather removal in *Gallus* var *domesticus*. *Research in Veterinary Science,* 50: 95-101.

Gentle, M. J., 1992: Pain in birds. *Animal Welfare,* 1: 235-247.

Gibson, T. J., Johnson, C. B., Stafford, K. J., Mitchinson, S. L., and Mellor, D. J., 2007: Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal,* 55: 152-157.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Hulls, C. M., Mitchinson, S. L., Stafford, K. J., Johnstone, A. C., and Mellor, D. J., 2009: Electroencephalographic responses of halothane-anaesthetised calves to slaughter by ventral-neck incision without prior stunning *New Zealand Veterinary Journal,* 57: 77-83.

Hawkins, M. G., 2006: The use of analgesics in birds, reptiles, and small exotic mammals. *Journal of Exotic Pet Medicine,* 15: 177-192.

Hocking, P. M., Robertson, G. W., and Gentle, M. J., 2005: Effects of non-steroidal anti-inflammatory drugs on pain-related behaviour in a model of articular pain in the domestic fowl. *Research in Veterinary Science,* 78: 69-75.

Hoppes, S., Flammer, K., Hoersch, K., Papich, M., and Paul-Murphy, J., 2003: Disposition and analgesic effects of fentanyl in white cockatoos (*Cacatua alba*). *Journal of Avian Medicine and Surgery,* 17: 124-130.

Hughes, R. A., 1990: Strain-dependent morphine-induced analgesic and hyperalgesic effects on thermal nociception in domestic fowl (*Gallus gallus*). *Behavioral Neuroscience,* 104: 619-624.

Jarvis, E. D., Gunturkun, O., Bruce, L., Csillag, A., Karten, H., Kuenzel, W., Medina, L., Paxinos, G., Perkel, D. J., Shimizu, T., Striedter, G., Wild, J. M., Ball, G. F., Dugas-Ford, J., Durand, S. E., Hough, G. E., Husband, S., Kubikova, L., Lee, D. W., Mello, C. V., Powers, A., Siang, C., Smulders, T. V., Wada, K., White, S. A., Yamamoto, K., Yu, J., Reiner, A., and Butler, A. B., 2005: Avian brains and a new understanding of vertebrate brain evolution. *Nature Reviews: Neuroscience,* 6: 151-159.

Johnson, C. B. and Taylor, P. M., 1998: Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. *British Journal of Anaesthesia,* 81: 748-753.

Johnson, C. B., Stafford, K. J., Sylvester, S. P., Ward, R. N., Mitchinson, S. L., and Mellor, D. J., 2005a: Effects of age on the electroencephalographic response

to castration in lambs anaesthetised using halothane in oxygen. *New Zealand Veterinary Journal,* 53: 433-437.

Johnson, C. B., Wilson, P. R., Woodbury, M. R., and Caulkett, N. A., 2005b: Comparison of analgesic techniques for antler removal in halothane-anaesthetised red deer (*Cervus elaphus*): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia,* 32: 61-71.

Johnson, C. B., Sylvester, S. P., Stafford, K. J., Mitchinson, S. L., Ward, R. N., and Mellor, D. J., 2009: Effect of age on the electroencephalographic response to castration in lambs anaesthetised with halothane in oxygen from birth to 6 weeks old. *Veterinary Anaesthesia and Analgesia,* 36: 273-279.

Kuenzel, W. J. and Masson, M., 1988: *A stereotaxic atlas of the brain of the chick (Gallus domesticus)*. Baltimore, Maryland, USA: The Johns Hopkins University Press.

Lee, S. J., Ralston, H. J. P., Drey, E. A., Partridge, J. C., and Rosen, M. A., 2005: Fetal pain: a systematic multidisciplinary review of the evidence. *Journal of the American Medical Association,* 294: 947-954.

Ludders, J. W., Mitchell, G. S., and Schaefer, S. L., 1988: Minimum anaesthetic dose and cardiopulmonary dose response for halothane in chickens. *American Journal of Veterinary Research,* 49: 929-932.

Machin, K. L. and Livingston, A., 2002: Assessment of the analgesic effects of ketoprofen in ducks anesthetised with isoflurane. *American Journal of Veterinary Research,* 63: 821-826.

Mayhew, I. G. and Washbourne, J. R., 1990: A method of assessing auditory and brainstem function in horses. *British Veterinary Journal,* 146: 509-518.

McGeown, D., Danbury, T. C., Waterman-Pearson, A. E., and Kestin, S. C., 1999: Effect of carprofen on lameness in broiler chickens. *The Veterinary Record,* 144: 668-671.

Mellor, D. J., Cook, C. J., and Stafford, K. J., 2000: Quantifying some responses to pain as a stressor. *In* Moberg, G. P. and Mench, J. A. (eds.), *The Biology of Animal Stress: basic principles and implications for welfare*. Wallingford, UK: CAB International, 171-198.

Molony, V. and Kent, J. E., 1997: Assessment of acute pain in farm animals using behavioral and physiological measurements. *Journal of Animal Science,* 75: 266-272.

Morris, J. P., Ong, R. M., O'Dwyer, J. K., Barnett, J. L., Hemsworth, P. H., Clarke, I. J., and Jongman, E. C., 1997: Pain-related cerebral potentials in response to acute painful electrical stimulation in sheep. *Australian Veterinary Journal,* 75: 883-886.

Morton, D. B. and Griffiths, P. H. M., 1985: Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment. *The Veterinary Record,* 116: 431-436.

Murrell, J. C., Johnson, C. B., White, K. L., Taylor, P. M., Haberham, Z. L., and Waterman-Pearson, A. E., 2003: Changes in the EEG during castration in horses and ponies anaesthetized with halothane. *Veterinary Anaesthesai and Analgesia,* 30: 138-146.

Murrell, J. C. and Johnson, C. B., 2006: Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29: 325-335.

Murrell, J. C., Mitchinson, S. L., Waters, D., and Johnson, C. B., 2007: Comparative effect of thermal, mechanical, and electrical noxious stimuli on the electroencephalogram of the rat. *British Journal of Anaesthesia,* 98: 366-371.

Murrell, J. C., Waters, D., and Johnson, C. B., 2008: Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. *Laboratory Animals,* 42: 161-170.

Naganobu, K., Fujisawa, Y., Odhe, H., Matsuda, Y., Sonoda, T., and Ogawa, H., 2000: Determination of the minimum anesthetic concentration and cardiovascular dose response for sevoflurane in chickens during controlled ventilation. *Veterinary Surgery,* 29: 102-105.

Norman, E., Rosen, I., Vanhatalo, S., Stjernqvist, K., Okland, O., Fellman, V., and Hellstrom-Westas, L., 2008: Electroencephalographic response to procedural pain in healthy term newborn infants. *Pediatric Research,* 64: 429-434.

Ong, R. M., Morris, J. P., O'Dwyer, J. K., Barnett, J. L., Hemsworth, P. H., and Clarke, I. J., 1997: Behavioural and EEG changes in sheep in response to painful acute electrical stimuli. *Australian Veterinary Journal,* 75: 189-193.

Ploner, M., Gross, J., Timmermann, L., Pollok, B., and Schnitzler, A., 2006: Pain suppresses spontaneous brain rhythms. *Cerebral Cortex,* 16: 537-540.

Woolley, S. C. and Gentle, M. J., 1987: Physiological and behavioural responses in the hen (*Gallus domesticus*) to nociceptive stimulation. *Comparative Biochemistry and Physiology A-Physiology,* 88A: 27-31.

Chapter 5. Effects of decapitation on the electroencephalogram of the chicken

"Rosie and Kestral"

Some of the findings from this chapter were presented at the 10th World Congress of Veterinary Anaesthesia in Glasgow, Scotland in 2009.

McIlhone, A. E., Beausoleil, N. J., Mellor, D. J., Mitchinson, S. L., and Johnson, C. B., 2009: Effect of decapitation on the electroencephalogram of anaesthetised chickens. *10th World Congress of Veterinary Anaesthesia*: p69 (Abstract).

5.1 Abstract

Decapitation is sometimes used as a method of killing chickens. The aim of this study was to investigate whether decapitation is a humane slaughter method. Two parts were addressed: 1) the length of time consciousness might persist after decapitation, and 2) whether the act of decapitation was noxious.

Fifteen chickens were maintained under anaesthesia with an end-tidal concentration of 0.9-1% halothane. Baseline EEG was recorded for 15 min before the chicken was decapitated. In both groups, the time to loss of EEG was calculated using two parameters: the time until the EEG became transitional (1/2 the original amplitude) and the time until the EEG became isoelectric (1/8 the original amplitude). Spectral analysis of the EEG generated the variables: median frequency, 95% spectral edge frequency and total power. These variables were used to compare the EEG before decapitation with the EEG following decapitation.

After decapitation it took an average of 35 s for the EEG to become transitional, and an average of 75 s for the EEG to become isoelectric. There were increases in both median frequency and 95% spectral edge frequency following decapitation ($P<0.05$). There was also a decrease in total power after decapitation ($P<0.05$).

It is unclear whether the changes in the EEG variables correspond to a response to noxious stimulation. However, it seems unlikely that consciousness is lost immediately at the time of decapitation. Due to the potential for pain perception during this time, decapitation seems to be an unsuitable method for killing chickens.

5.2 Introduction

Slaughter of animals is an important area of potential welfare compromise. Ideally the death of an animal should be rapid and painless, without suffering or distress. However there are many different slaughter methods and some are more humane than others. Decapitation is a method of killing animals that has been subject to several investigations trying to determine its humaneness. There are two main questions to ask about this killing method. Firstly, does the animal suffer pain during the slaughter method and before death, and secondly, how long does any potential suffering last for. This study will use the EEG to explore both questions, by examining how the EEG responds to decapitation, and also examining how long the EEG persists for after decapitation has occurred.

During slaughter, chickens are ideally stunned (rendered insensible) before being subjected to any potentially painful or distressing killing methods. However, sometimes pre-slaughter stunning is not possible or desirable, for example in emergency slaughter, research studies (Holson, 1992) or some forms of religious slaughter (Barnett et al., 2007).

In addition, killing methods considered to be relatively humane, such as anaesthetic overdose, are sometimes considered inappropriate for research purposes because the drugs can affect the biological systems that are being studied (Vanderwolf et al., 1988; Holson, 1992). For example, when tissues are being collected for research in neurochemistry, neuropharmacology or tissue culture (Bates, 2010). In such situations, decapitation has been used as a method of killing animals.

5.2.1 Brain activity after decapitation

Traditionally, decapitation was thought to be a humane method of killing animals because it was assumed that consciousness was lost as soon as the head was detached from the body. However, mounting evidence now suggests otherwise: that consciousness and pain perception may persist for some time after decapitation has occurred (Mikeska and Klem, 1975; Tidswell et al., 1987; Cartner et al., 2007). For example, studies of brain electrical activity have reported that EEG activity persists for approximately 13 seconds after decapitation (Mikeska and Klem, 1975). The 1986 report of the American Veterinary Medical Association (AVMA) took note of Mikeska and Klem's findings (1975) and recommended that animals be sedated or lightly anaesthetised before decapitation (AVMA, 1986).

Previous studies have measured the brain activity in mammals following decapitation (Mikeska and Klem, 1975; Tidswell et al., 1987). However, it has been argued that there is insufficient evidence to support the notion that consciousness persists following decapitation (Vanderwolf et al., 1988; Holson, 1992). Yet these arguments mainly suggest different interpretations of the EEG results and do not provide any strong evidence for the absence of consciousness after decapitation either. For example, after decapitation of rats there was a change in the EEG to high frequency activity, which has been likened to that which occurs following head trauma, thus suggesting a reduction or loss of consciousness (Allred and Berntson, 1986). However, this observation was not substantial enough evidence to support the proposal that consciousness is lost immediately after decapitation. High frequency EEG patterns can also indicate a response to noxious stimulation in mammals (Murrell and Johnson, 2006; Gibson

et al., 2007) or lightening of anaesthesia in chickens (Chapter 1) or mammals (Johnson et al., 1994; Johnson and Taylor, 1998; Murrell et al., 2008).

5.2.2 Distinguishing states of consciousness and welfare

While isoelectric EEG traces almost certainly indicate an unconscious state, it is not so easy to determine when an animal is conscious; many EEG waveforms are ambiguous (Bates, 2010). Disagreements over whether an animal is conscious seem to be based on interpretation of EEG waveforms rather than the EEG results (appearance) per se. That is, the character of the EEG is not disputed, but different authors have differing interpretations of what that EEG waveform reflects in terms of brain function (Mikeska and Klem, 1975; Allred and Berntson, 1986; Holson, 1992; Bates, 2010; Van Rijn et al., 2011).

The debate in the literature about the humaneness of decapitation of laboratory rats is still unresolved (Bates, 2010). The 2007 AVMA Guidelines on Euthanasia listed decapitation, including that of birds, as conditionally acceptable as a method of harvesting undamaged and uncontaminated brain tissues (i.e. if the benefits of the study are considered to outweigh the perceived costs of decapitation). However, the report also mentioned that the interpretation of brain activity after decapitation was still open to debate (AVMA, 2007). The Australian and New Zealand Council for the Care of Animals in Research and Teaching (ANZCCART) guidelines for euthanasia list decapitation as "acceptable with reservations" for rodents and "unacceptable" for birds (ANZCCART, 2001). In contrast, in some slaughter codes decapitation is listed (along with cervical dislocation) as a poultry slaughter method that does not require prior stunning (SCARM, 2002).

An exception to the ANZCCART guideline is the case of incubating chicks. The guidelines suggest that decapitation is acceptable for the pre-hatched chick in the first half of incubation, after which time it is suggested that the chick can perceive pain (ANZCCART, 2001).

5.2.3 Decapitation of chickens

Previous studies have also explored the EEG response to decapitation of chickens (Gregory and Wotton, 1986). These studies found that brain activity was not lost instantaneously at the time of decapitation and that it took around 14 s for the spontaneous EEG to reduce by 50% and 32 s for the EEG activity to decrease to less than 5% of the baseline activity (Gregory and Wotton, 1986). It is not clear what time consciousness might be lost following decapitation thus it is still unclear whether or not decapitation is a humane method of killing animals.

5.2.4 Study objective

The aim of this study was to evaluate the potential for brain activity that could support consciousness following decapitation. The results from this study could be used to assess the humaneness of decapitation for use in chickens, but could also be applied, at least partially to assess decapitation in other species such as rodents. In this study, EEG measures were used to examine activity in the chicken brain in order to address two issues: firstly, how long the EEG persists after the time of decapitation; and secondly, the state of brain activity after decapitation and whether it is indicative of consciousness and/or pain perception.

There was a notable limitation to this experiment. As discussed in Chapter 4, the chicken EEG does not appear to have a consistent, detectable response to noxious stimulation. In previous studies on pain perception during slaughter, certain changed in EEG variables were considered indicative of responses to noxious stimulation (Gibson et al., 2007). Thus the EEG could be used to determine whether slaughter methods such as ventral neck incision was a noxious experience for cattle(Gibson et al., 2009a; Gibson et al., 2009b; Gibson et al., 2009d, 2009c).

The failure of the chicken EEG respond to standardised noxious stimuli limited the information that could be gained from an EEG study on the potential noxiousness of decapitation. The current study was conducted using the chickens from the pilot study (Experiment 1) described in Chapter 4, and was therefore undertaken before the lack of response to noxious stimulation became apparent. Nevertheless, although some EEG information may be lacking, the EEG duration after decapitation could still be measured.

5.3 Methods

5.3.1 Chickens

Fifteen male chickens of the Hyline brown strain were used, 10 of these were the same chickens used in Experiment 1 of Chapter 4. The chickens were obtained from a commercial hatchery as day-old chicks and were raised in a group under controlled temperature and lighting conditions, which were adjusted as the chicks grew older. Food (Ingham chick starter crumbles, Levin) and fresh water were available to the chicks *ad-libitum*. The chickens were kept on wood-shavings substrate with perches available. The chickens were 5-6 weeks old at the time of the study. All procedures were approved by the Massey University Animal Ethics Committee (protocol 07/121)

5.3.2 Anaesthesia and experimental setup

Anaesthesia was induced using an induction chamber. Halothane in oxygen was delivered to the chicken at 0.5-2% halothane in 2Lmin^{-1} of oxygen until it lost its righting reflex, which was the primary indicator for loss of consciousness.

The chicken's larynx was desensitised by applying 0.1 – 0.2mL of lignocaine local anaesthetic (Nopaine; Phoenix Pharm Distributers Ltd, Auckland, New Zealand) to the back of the throat and orotracheal intubation was performed using a 2.5mm non-cuffed endotracheal tube. The chicken was then maintained under anaesthesia using a t-piece non-rebreathing anaesthetic circuit and ventilated using an intermittent positive pressure ventilation (IPPV) system (V-valve ventilator, Vetronics, Bioanalytical Systems Inc, W La Fayette, IN, USA). An anaesthetic monitor (Hewlett Packard M1025B; Hewlett Packard, Hamburg, Germany) recorded respiration rate, end tidal CO_2 concentrations, and inspired

and end-tidal anaesthetic concentrations. End tidal halothane was kept stable at 0.95 ± 0.1% (range 0.85 – 1.05% across all chickens, variation ± 0.05% for each chicken). The chicken was placed on a water-heated blanket set to approximately 37°C to reduce heat loss. Heart rate and temperature were also monitored throughout the experiment (Figure 5.1).

The chicken's head and neck were placed through the opening of a small-animal guillotine (purpose built in the Massey University Fundamental Sciences workshop) so that the blade of the guillotine was positioned over the mid-point of the chicken's neck. The chicken's head was secured to a stereotaxic frame which held the head still as the chicken was guillotined.

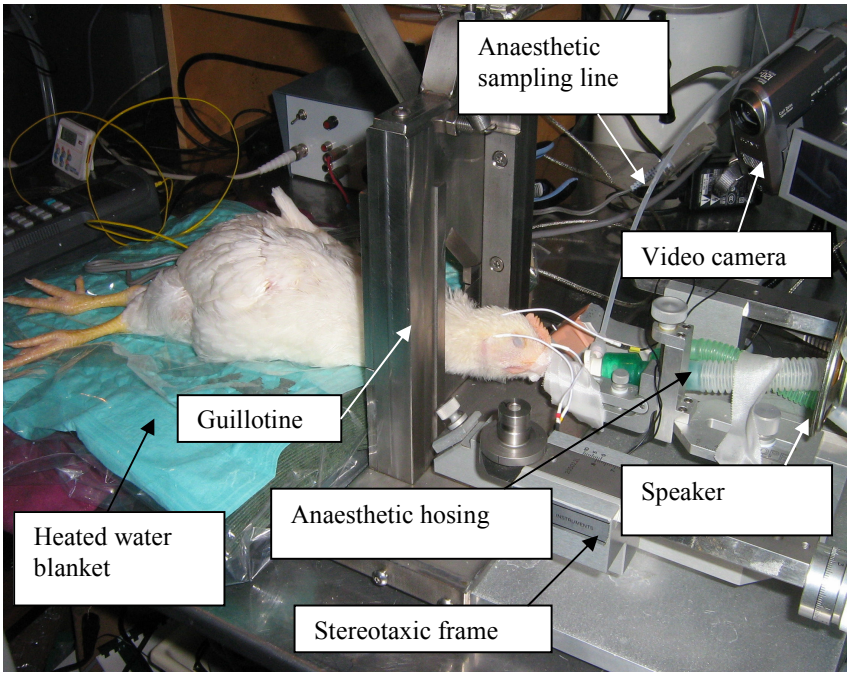


Figure 5.1 Setup for recording EEG responses to noxious stimulation and decapitation. The endotracheal tube was secured to the chicken's beak with tape. The anaesthetic hosing and the chicken's beak were also attached firmly to the stereotaxic frame.

5.3.3 Behaviour recording

The behaviour of the chicken during and following decapitation was recorded on a video camera that was synchronised with the EEG. After the experiment was completed the video was examined and the various behaviours (i.e. eye-opening, throat movements) were recorded.

5.3.4 EEG recording

The EEG was recorded using 27-gauge subcutaneous, stainless-steel needle electrodes (Viasys Healthcare, Surrey, England). These were positioned to record the EEG from the left and right sides of the brain using two channels on the chart recorder. The electrode positions were based on the montage for horses, described by Mayhew and Washbourne (1990). The electrode sites on the chickens were: lateral to the comb (non-inverting electrodes), caudal to the external auditory meatus (inverting electrodes) and an earth electrode medio-dorsally on the chicken's neck at the base of the skull. There was a single (common) non-inverting electrode for the left and right channels.

The EEG cables fed into two identical break-out boxes, one for each channel, with each break-out box plugged into a physiological signal amplifier (Iso-Dam isolated physiological signal amplifier, World Precision Instruments, Sarasota FL, USA). The amplifiers provided a signal gain of 1000 times and recorded frequencies between 0.1 Hz and 0.1 kHz, i.e. high-pass filters were set at 0.1 Hz and low-pass filters were set at 0.1 kHz. Both amplifiers fed into two analogue-to-digital converters (Powerlab, ADInstruments Ltd, Sydney, Australia). One AD converter digitised the EEG signal at 1000 points/second and stored it on an

Apple personal computer. The second AD converter was set up with another computer to record auditory evoked potentials.

Once both the anaesthesia and EEG were stable, 15 mins of baseline EEG was recorded.

5.3.5 Noxious stimulus

After 15 mins of baseline was recorded, a toe-clamp noxious stimulus was applied to 10 of the 15 chickens; these will be referred to here as "Group 1". The toe-clamp was applied to the middle segment of the chicken's middle toe using a pair of large artery forceps (haemostats) closed to the last ratchet. The clamp was applied for 5 s and then removed. The EEG results relating to noxious stimulation in the Group 1 chickens are presented in Chapter 4. "Group 2" refers to the five chickens that did not receive the toe-clamp stimulus.

5.3.6 Decapitation

After noxious stimulation, Group 1 chickens were left for 15-20 min to allow the anaesthesia and EEG to restabilise, and then a second 15 min baseline EEG was recorded. At the end of the baseline period, the chicken was decapitated using the small animal guillotine. The EEG was recorded until the signal was isoelectric on both EEG channels.

The five chickens that were not first given a noxious stimulus (Group 2) had only one 15 min baseline EEG recording made prior to decapitation. The rest of the protocol, including electrode set up and anaesthesia, was the same as for the Group 1 chickens.

5.3.7 Auditory Evoked Potentials

Auditory stimuli were delivered at time points throughout the experiment which included: baseline, after the noxious stimulus and before and after decapitation. The auditory stimulus was a clicking noise that was emitted from a speaker positioned approximately 15cm from the chicken's head. The click repeated at random intervals a total of 3300 times during each delivery. Each click had a duration of 2.5 ms, an amplitude of 3 volts (V) and a delay minimum of 5 ms.

The evoked potential signal was recorded on a separate computer from that recording EEG. The AD converter of the second computer was also linked to the AD converter of the first computer. This setup allowed the recording of a marker in a separate channel of the chart recorder so that the timing of the auditory stimulus delivery could be located in the EEG signal after the recording was completed. Unfortunately, something went amiss with the timing of the recordings of the auditory evoked potentials and they were not collected properly, therefore no analysis was done with them.

5.3.8 Recording time to loss of active EEG after decapitation

After decapitation the EEG gradually changed form from active, to transitional to isoelectric. For each chicken the time taken for the EEG to change to each state was recorded. The active EEG was that which occurred during the baseline period. Transitional EEG was defined as EEG with an amplitude that was 1/2 that of the active EEG. Isoelectric EEG was defined as EEG with an amplitude that was 1/8 that of the active EEG.

The time taken for the EEG to become transitional or isoelectric was compared between Groups 1 and 2 using Mann-Whitney tests in Graphpad Prism 5.01 for Windows (GraphPad Software, San Diego California, USA).

5.3.9 EEG frequency analysis

The EEG was analysed by Fast Fourier Transformation (FFT) using a purpose written software program (spectral analyser, C.B. Johnson, 2002), which carried out the FFT on each second of EEG. The FFT generated a frequency spectrum, which was a graphical representation of the contribution that each frequency made to the power of the EEG waveform. Analysis of the EEG was based on the area under this frequency spectrum graph.

Variables derived from the frequency spectrum included the following: the *total power* of the EEG which was measured as the total area under the frequency spectrum, the *median frequency* which was the frequency below which half the total power was located and the *95% spectral edge frequency* which was the frequency below which 95% of the total power was located (Murrell and Johnson, 2006). All movement artefacts were removed from the results before analysis.

To examine the effect of decapitation the mean "median frequency", "95% spectral edge frequency" and "total power" were calculated from: 30 s of baseline EEG taken 1 min prior to decapitation, and for eleven 10-second EEG blocks following decapitation extending to 110 s following decapitation (Figure 5.2). Means were therefore calculated for each chicken at: baseline, 0-10, 10-20,

20-30, 30-40, 40-50, 50-60, 60-70, 70-80, 80-90, 90-100 and 100-110 s after decapitation with each time point representing a 10-second average.

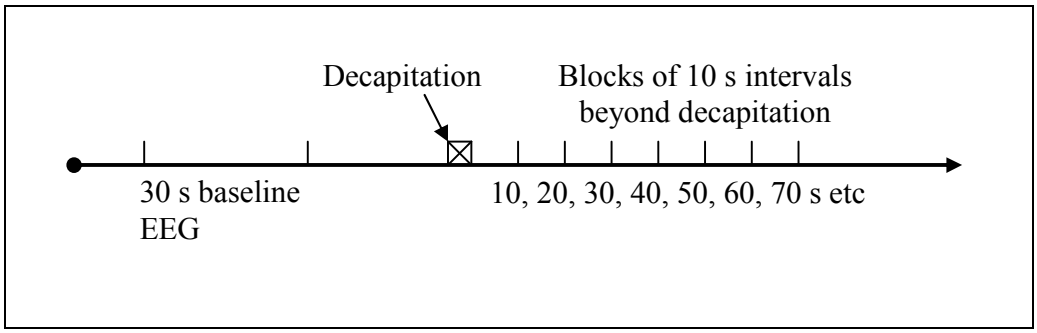


Figure 5.2. To examine the effect of decapitation, the mean median frequency, 95% spectral edge frequency and total power were calculated over 10 s for each of the 11 blocks after decapitation. The baseline EEG was then compared with 10 s EEG intervals following decapitation.

5.3.10 Adjustment to obtain results as percent change from baseline

The baseline values were standardised at 100% and the other values were converted to give a percent change from baseline to eliminate the skew caused by differing baseline values. To calculate the percent value the mean baseline value was calculated then each mean value (i.e. the means for 10, 20, 30 s etc after decapitation) was divided by the baseline mean, and multiplied by 100.

5.3.11 Statistical analysis

The statistical analyses on the FFT variables were conducted using SAS (SAS 9.1.3, SAS Institute Inc., Cary, NC, USA). All of the results were tested for normality. The total power results were log transformed to better approximate a normal distribution. A mixed model ANOVA was used on each of the EEG

variables with Group (previous noxious stimulus or none) as a fixed effect and repeated measures for time. If a time effect was found, post hoc testing with a Bonferroni adjustment for multiple comparisons was used to compare time intervals (i.e. baseline and the time points following decapitation).

Channel 1 and 2 were both used in the same analysis in order to make up a usable sample size. After decapitation, movement artefacts in the EEG recording sometimes made the EEG unusable. The EEG frequencies appeared very similar on both sides of the brain, but sometimes movement artefacts were present on one side but not the other. Results from channels 1 (left side) and 2 (right side) were therefore pooled to make a larger sample size when the EEG was usable in one channel but not the other. That is, each chicken only contributed one channel to the results, but the EEG could have been from either the left or the right side of the brain (Table 5.1). If the EEG was usable from both the left and right side then one side was selected randomly. Combining the EEG results from the two channels was justified in that when both channels were available from the same bird, the responses to decapitation were very similar.

Table 5.1. Source of EEG in FFT analysis of results, the chickens not listed here were excluded from the FFT analysis due to high levels of movement artefact in the recording

Chicken number	EEG channel used	Side of brain
1	1	Right
2	2	Left
4	2	Left
5	2	Left
7	1	Right
8	2	Left
9	1	Right
11	2	Left
13	1	Right
14	2	Left
15	1	Right

5.4 Results

5.4.1 Behaviour

Many of the chickens moved after decapitation. Although the head was secured, eye-opening and throat movements (e.g. twitching, swallowing and gasping) were observed (Table 5.2). In 12 of the 15 chickens the eye opened at the time of decapitation. In 7 chickens the eye opened (or re-opened) after a delay of 43 to 61 s. Throat movements started between 2 and 20 s after decapitation and persisted for a mean of 35 s. It was also common for the neck stump to curl backwards (towards the body) after decapitation. The chicken's body also moved; there was wing-flapping and limb-kicking.

Table 5.2. Table displaying the times after decapitation that the chickens' eyes opened and closed, as well as the times that throat movements started and stopped. Times are in seconds after decapitation. Chickens 6, 10, 13 and 15 all opened their eyes a second time, both times are therefore shown and included in the mean value. Note, only one eye could be observed and this was not consistently left or right.

Chicken	Eye observed	Eye opened	Eye closed	Duration	Throat start	Throat stop	Duration
1	Left	43	95	52	14	38	24
2	Left	0	6	6	17	74	57
3	Left	61	97	36	14	51	37
4	Left	0	21	21	17	41	24
5	Left	0	11	11	20	51	31
6	Left	0	6	6	12	111	99
6 re-open	Left	55	89	34			
7	Left	0	5	5	18	44	26
8	Right	44	94	50	17	38	21
9	Left	0	6	6	15	39	24
10	Right	0	5	5	13	43	30
10 re-open	Right	43	85	42			
11	Right	0	8	8	2	36	34
12	Right	0	5	5	4	39	35
13	Right	0	5	5	12	42	30
13 re-open	Right	48	77	29			
14	Right	0	8	8	3	34	31
15	Right	0	5	5	13	38	25
15 re-open	Right	44	84	40			
Mean (SEM)		**18 (5)**	**37 (9)**	**20 (4)**	**13 (1)**	**48 (5)**	**35 (5)**

5.4.2 Time to loss of active EEG after decapitation

After decapitation the EEG gradually changed state from an active EEG to transitional EEG (1/2 amplitude of active EEG) and then eventually became isoelectric (1/8 amplitude of active EEG) (Figure 5.3).

a) Active EEG

b) Transitional EEG

c) Isoelectric EEG

Figure 5.3. Examples of active, transitional and isoelectric EEG recordings

Active EEG was present after decapitation for between 9 and 78 s (mean of 39 (8) s) for Group 1 and between 22 and 57 s (mean of 27 (8) s) for Group 2. The EEG then changed to transitional EEG. The EEG became isoelectric after a mean

duration of 77 (2) s for Group 1 and 69 (2) s for Group 2 (Table 5.3). No significant differences were detected between Groups 1 and 2 for the period from decapitation to the onset of transitional EEG (Mann-Whitney U = 18.50, P>0.05) or for the period from decapitation to the onset of isoelectric EEG (Mann-Whitney U = 10.50, P>0.05).

Table 5.3. Times taken for the EEG to become transitional and isoelectric following decapitation, time is in seconds (s)

Chicken number	Time to transitional	Time to isoelectric
1	21	75
2	9	85
3	59	79
4	30	72
5	78	85
6	63	90
7	20	78
8	16	70
9	65	71
10	27	67
Mean (SEM) Group 1	**39 (8)**	**77 (2)**
11	22	62
12	24	76
13	21	69
14	10	67
15	57	73
Mean (SEM) Group 2	**27 (8)**	**69 (2)**
Mean (SEM) Overall	**35 (6)**	**75 (2)**

Eye-opening occurred during active and transitional EEG activity and some chickens still had their eyes open during the isoelectric phase (Figure 5.4).

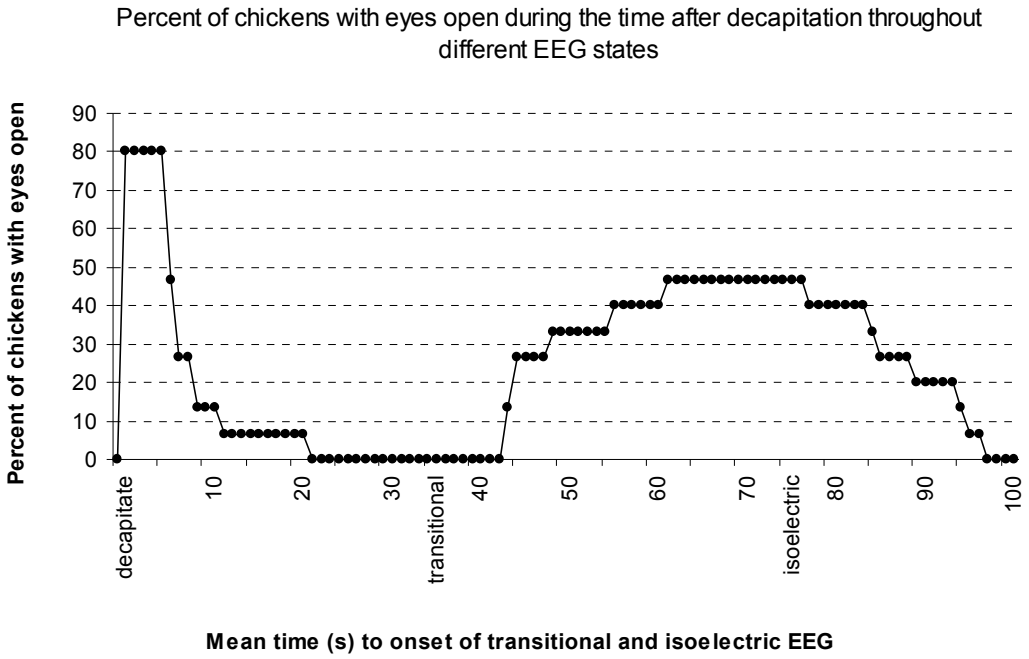


Figure 5.4. Percentage of chickens that had their eyes open following decapitation. The x-axis shows time in seconds (s) and also the EEG status following decapitation, based on the mean time to transitional and isoelectric EEG calculated for all 15 chickens.

5.4.3 EEG results from Groups 1 and 2

The sample size for Group 2 was very small with only 5 chickens. Furthermore, some of the EEG traces from chickens in Group 2 were unusable due to high levels of artefact. As a result, there were times when only 1 chicken contributed to the mean values that were used in the analysis of the 10 second time intervals following decapitation. These results were analysed along with Group 1 in a mixed model ANOVA analysis, to determine whether there were any differences between the two groups and also to test whether the FFT variables after decapitation differ from the baseline values. The results that were available from

Group 1 and Group 2 are shown in Figures 5.5 to 5.7. Although there appeared to be some differences between Groups 1 and 2, the small sample size of Group 2 makes it difficult to draw strong conclusions.

5.4.4 Normality

The median frequency, 95% spectral edge frequency and total power results were all tested for normality. Both median and spectral edge frequency results fitted the requirements for a normal distribution. The total power results needed to be log-transformed to meet the requirements for normal distribution.

5.4.5 Median frequency

The mixed model ANOVA revealed no significant effect of Group on median frequency ($F(1,9)= 1.80$, $P= 0.2129$) but there was a significant time effect ($F(1,9)=9.47$, $p<0.0001$). There was also an interaction between Group and time ($F(1,9)=3.86$, $P=0.0002$), suggesting that any differences in median frequency between groups was dependent on time, and/or the effect of time was different between groups.

In Group 1, an increase in median frequency, was apparent in the results after 10-30 s, but became significantly different from baseline 50 s after decapitation. Median frequency was significantly higher than baseline until 80 s after decapitation for Group 1, after which it began to decrease. At 90 s the values were no longer significantly different from baseline (Figure 5.5).

Although the trends in median frequency appeared to be similar in Groups 1 and 2, Group 2 values following decapitation did not differ significantly from

baseline. However this may have been due to the small sample size. The decrease in median frequency back towards baseline seemed to occur sooner in Group 2 than in Group 1. Groups 1 and 2 were significantly different from each other from 90 to 110 s after decapitation.

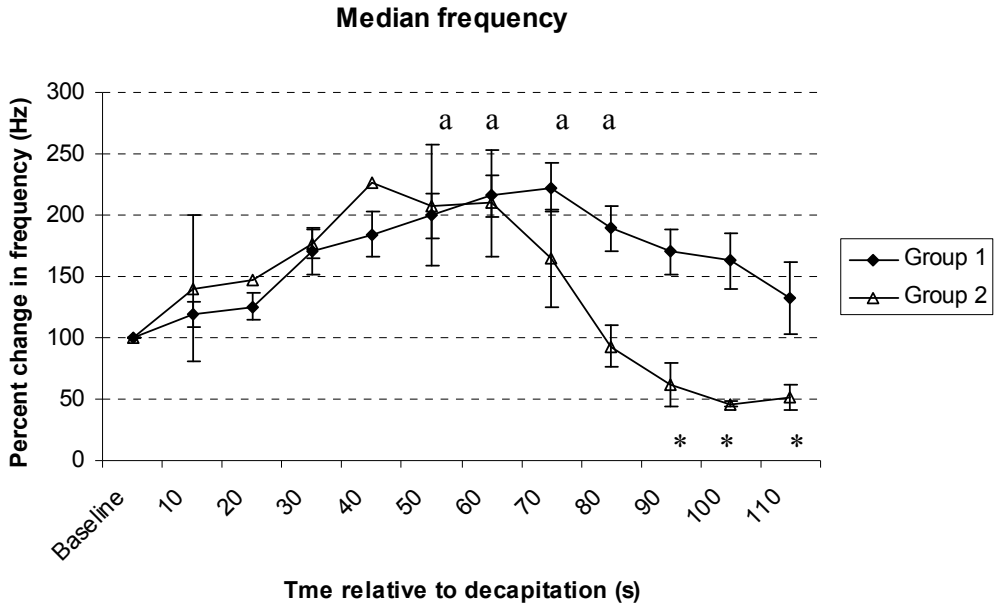


Figure 5.5. Mean (± SEM) percentage change in median frequency following decapitation for chickens from Groups 1 and 2. The results have been scaled so that the baseline values = 100%. a = Group 1 value significantly different from baseline ($P<0.05$), * = Group 1 and 2 significantly different from each other ($P<0.05$). Baseline EEG was collected 30 s prior to decapitation. Decapitation occurred at time 0, and values at time 10 s are the average of time points 0-10 s after decapitation.

5.4.6 95% Spectral edge frequency

There was no significant effect of Group on 95% Spectral edge ($F(1,9)= 4.89$, $P=0.0544$). There was a time effect ($F(1.9)=5.21$, $P<0.0001$), but there was also a significant interaction between Group and time, again suggesting that the effect of time was different between groups, or that the group effect differed between times ($F(1,9)=3.82$, $P=0.0002$).

In Group 1, there was an increase in 95% spectral edge frequency 10-20 s after decapitation. The increase became significantly different from baseline after 40 s (Figure 5.6). The values were significantly higher than baseline until 80 s after decapitation, at which time they were no longer significantly different from baseline.

The general trends for the 95% spectral edge frequency were similar for Groups 1 and 2. However, as for median frequency, there were no significant differences between baseline and any time points after decapitation in Group 2. The decrease in 95% spectral edge frequency appeared to occur sooner in Group 2 than in Group 1. The Group 2 values were significantly lower than the Group 1 values at 90 and 100 s after decapitation (Figure 5.6).

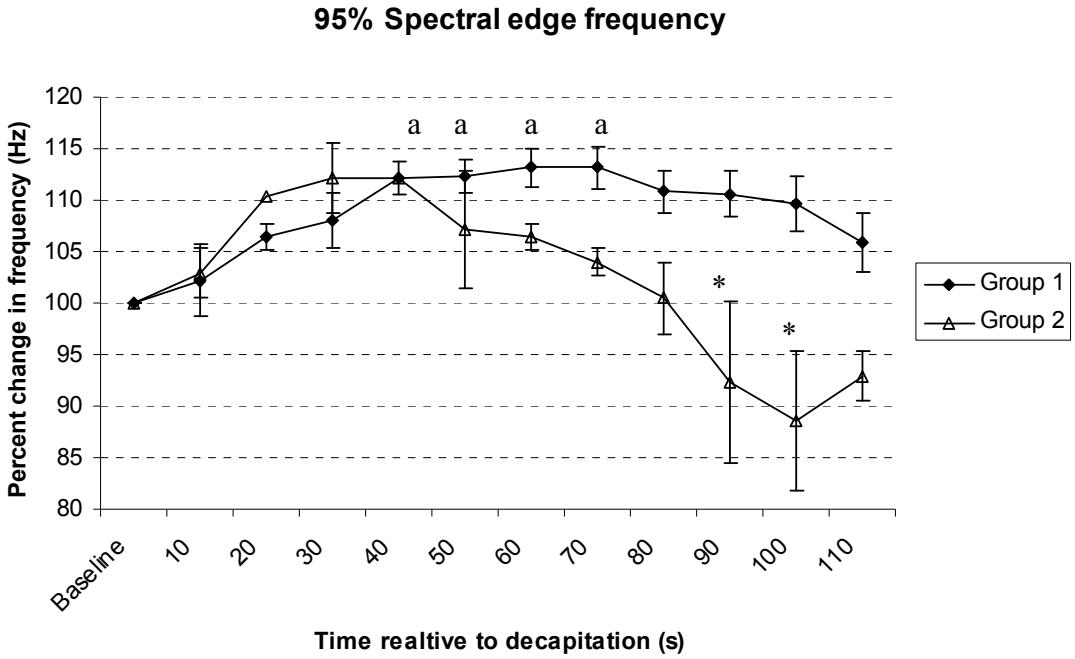


Figure 5.6. Mean (± SEM) percentage change in 95% spectral edge frequency following decapitation for chickens from Groups 1 and 2. The results have been scaled so that the baseline values = 100%. a = Group 1 value significantly different from baseline ($P<0.05$), * = Group 1 and 2 significantly different from each other ($P<0.05$). Baseline EEG was collected 30 s prior to decapitation. Decapitation occurred at time 0, and values at time 10 s are the average of time points 0-10 s after decapitation.

5.4.7 Total power (log transformed)

There was an effect of time on log transformed total power ($F(1,9)=16.73$, $P<0.0001$), but no effect of Group ($F(1,9)=0.04$, $P=0.8506$). There was also no significant interaction between group and time ($F(1,9)=0.76$, $P=0.6736$), so any changes with time were similar between the two groups.

The total power of the EEG was numerically variable during the first 30s after decapitation. There was a decrease in total power which became significantly lower than baseline by 90 s and 100 s after decapitation in Groups 1 and 2 respectively (Figure 5.7).

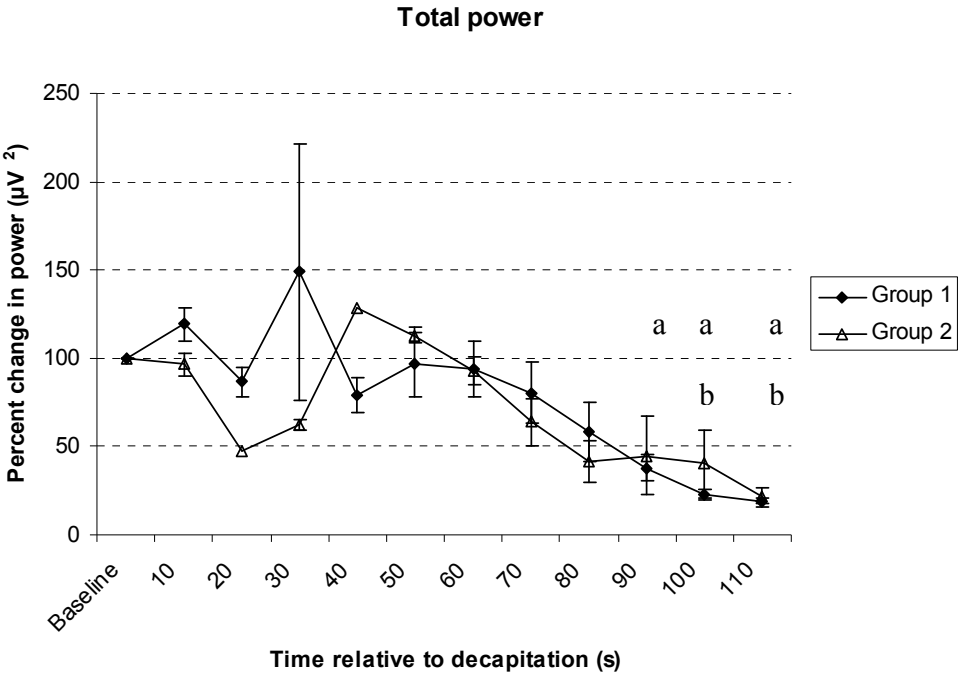


Figure 5.7. Mean (± SEM) percentage change in total power following decapitation for chickens from Groups 1 and 2. The results have been scaled so that the baseline values = 100%. a = Group 1 value significantly different from baseline, b = Group 2 value significantly different from baseline ($P<0.05$). Baseline EEG was collected 30 s prior to decapitation. Decapitation occurred at time 0, and values at time 10 s are the average of time points 0-10 s after decapitation.

5.5 Discussion

5.5.1 Summary of main findings

The results show that after decapitation it took a mean of 35 (6) s for the EEG to become transitional, and a mean of 75 (2) s for the EEG to become isoelectric. After decapitation median frequency and 95% spectral edge frequency increased significantly above baseline from 40-80 s in Group 1, but these values did not change significantly from baseline in Group 2. For both groups there was a significant decrease in total power by 90 to 110 s after decapitation.

The EEG was isoelectric from 60 to 70s after decapitation onwards. After this point the FFT analysis could no longer provide useful information because the signal to noise ratio of the waveform was too small. Therefore, time-points beyond approximately 70 s post decapitation should be interpreted with caution as they may have been affected by the very small EEG signal and could be offset by background noise in the waveform.

It therefore appears that brain activity capable of supporting consciousness (if the chickens were not anaesthetised) may be present for approximately 35 s following decapitation, after 35 s the EEG becomes transitional and consciousness is likely to be lost at this time. The onset of isoelectric EEG approximately 75 s after decapitation is a strong indicator that consciousness is lost by this point.

5.5.2 Comparison to previous work

Much of the literature discussing the humaneness of decapitation has focused on laboratory rodents (Mikeska and Klem, 1975; Holson, 1992; Bates, 2010). Other studies have examined slaughter methods in farm species (Tidswell et al., 1987; Gibson et al., 2009b; Gibson et al., 2009d). Therefore, in this discussion the results from the chickens used in the current study will largely be compared with results from those mammalian studies. Comparisons with other chicken studies will be made when there is previous literature available.

Behaviour

As with the chickens used here, eye-opening and gasping movements were observed in decapitated lambs (Tidswell et al., 1987). Mouth movements have also been observed in decapitated rats (Van Rijn et al., 2011). In the chickens, eye opening occurred during active, transitional and isoelectric EEG, so this may have been a reflex response to the incision and may or may not be associated with consciousness (in an un-anaesthetised animal).

5.5.3 Characteristics of loss of brain activity in mammals and birds

Although measures of EEG amplitude or frequency have been used to estimate possible endpoints for loss of consciousness (Lambooy and Spanjaard, 1981; Velarde et al., 2002; Raj and O'Callaghan, 2004), these endpoints are not definitive. During and after slaughter the EEG changes from a baseline of active (conscious or normal but anaesthetised) EEG and finishes as isoelectric EEG. This sequence has been found in mammalian species (Lambooy and Spanjaard, 1981; Gibson et al., 2009a; Gibson et al., 2009b) as well as in chickens in the current study. An isoelectric EEG indicates undoubted loss of consciousness, but

the change from active EEG to this waveform is not always instantaneous (Lambooy and Spanjaard, 1981; Gregory and Wotton, 1986). Often there is a phase located between active and isoelectric EEG, where it is less clear whether the animal is conscious or not (Gibson et al., 2009b).

One approach to identifying the loss of an EEG that is compatible with consciousness is to determine when the EEG became 'transitional' i.e. the signal had a wave amplitude that was half the size of the active EEG (Lambooy and Spanjaard, 1981; Gibson et al., 2009c). Transitional EEG is apparently unlikely to be associated with consciousness (Gibson et al., 2009c), but this is difficult to validate. It is hard to know whether consciousness is lost when the EEG is reduced by 50% or 40% or 60%. Instead it may be that consciousness is reduced as the EEG progresses from active to isoelectric EEG and may be lost at some point during transitional EEG.

5.5.4 Time to loss of consciousness in chickens

The time to loss of EEG, as indicated by time to transitional and isoelectric EEG, was longer for the current chickens (mean of 35 s to transitional and 75 to isoelectric) than has been previously reported for this species. A previous study reported that after decapitation it took approximately 14 s for the spontaneous EEG activity to reduce by 50% and 32 s for the activity to decrease to less than 5% of the baseline activity (Gregory and Wotton, 1986). In addition, the chicken's brain remained responsive to visual stimuli (visual evoked potentials) for about 30 s before the response dropped in activity to 50% (Gregory and Wotton, 1986). The time to loss of EEG after decapitation was apparently longer than the time to loss of EEG following cardiac arrest, but shorter than the durations to loss of EEG

following various neck/vessel cut methods of slaughter (Gregory and Wotton, 1986).

Based on the current study, it is not possible to say precisely when the chicken loses brain activity that would be associated with consciousness because there are no definitive indicators of consciousness. However, the EEG responses measured in this study do not appear to indicate immediate loss of brain activity at the time of decapitation, i.e. the EEG is not immediately isoelectric. If consciousness was lost during transitional EEG, which begins about 35 s after decapitation, then there is potential for the chicken to experience pain or distress during this period. From an animal welfare point of view, this would be an unacceptably long time. Based on studies in cattle it appears that the act of severing the neck is strongly noxious (Gibson et al., 2009a; Gibson et al., 2009b; Gibson et al., 2009c), therefore, there is a strong likelihood that decapitation of a chickens head would also be noxious. In view of the possibility that chickens were not instantly rendered insensible by decapitation it would be advisable to stun them before slaughter by this method (Gregory and Wotton, 1986).

5.5.5 Time to loss of brain activity in other species

Other species have different durations to loss of brain activity after decapitation. In mice, the EEG amplitude decreased significantly 15-20 s after decapitation and the amplitude of visual evoked potentials decreased significantly by 10-15 s after decapitation (Cartner et al., 2007). In lambs the EEG amplitude following decapitation was very similar to the amplitude beforehand, it decreased in amplitude after approximately 8 s and was isoelectric by 20 s (Tidswell et al., 1987).

Derr (1991) reports a series of calculations that suggest that blood pressure and thus consciousness are lost from the rat brain 2.7s after decapitation. However, this conclusion was not supported by direct experimental evidence such as EEG recordings, which tend to indicate longer durations to loss of brain activity (i.e. closer to 14 s) (Mikeska and Klem, 1975; Holson, 1992; Cartner et al., 2007). More recently, EEG power in rats was reported to decreases by 50% on average 5.5 s after decapitation. However, the power of the 13-100Hz frequencies was reduced more quickly, decreasing by 50% in 3.7 s, which was closer to Derr's 1991 estimate (Van Rijn et al., 2011). The question of how long consciousness persists for after decapitation therefore remains unresolved.

The two studies performed on chickens (Gregory and Wotton, 1989; Gregory and Wotton, 1990) and the current study all seem to indicate that there is a protracted period after decapitation during which brain activity may still support consciousness. The situation in mammals may be more variable and perhaps warrants further studies to clarify the duration of this period.

For the current study using chickens, the next question was to address whether the chickens might be experiencing pain between the time of decapitation and when they lose consciousness.

5.5.6 Changes in the EEG after decapitation

The current study of chickens identified statistically significant changes in the EEG following decapitation, including an increase in median and spectral edge frequencies for Group 1 chickens and a decrease in total power for both Groups.

Neither of the two previous studies of chicken slaughter reported the nature of the EEG frequencies after decapitation or slaughter (Gregory and Wotton, 1986; Gregory and Wotton, 1990). Therefore, when possible, the interpretation of the current results is made with reference to mammalian studies. However, this had limitations due to the difference between avian and mammalian EEG responses discussed in Chapter 4.

Changes in the EEG similar to those observed in the current study have been observed in rats following decapitation. In one study using rats, the EEG changed to what was called low-voltage fast activity (LVFA or activation) following decapitation. After the period of activation which lasted 6-29 s (mean 13s) the rat's EEG gradually lost amplitude and the frequency decreased until the EEG became isoelectric after 19-46 s (mean 27s) (Mikeska and Klem, 1975). The researchers stated that the EEG evidence strongly suggests not only that brain activity remains, but also that this brain activity could indicate the presence of consciousness, at least initially, after decapitation (Mikeska and Klem, 1975). The EEG activation following decapitation in these rats was interpreted as indicating conscious awareness of pain and distress caused by the procedure (Mikeska and Klem, 1975).

5.5.7 High frequency activity

Some other reports have challenged the interpretation of the low voltage fast activity (LVFA) that can occur after decapitation (Allred and Berntson, 1986; Holson, 1992), and stated that because this type of activity occurred in both conscious and anaesthetised animals it was not evidence of consciousness (Holson, 1992). However, this activated EEG seems to be similar to the EEG

waveform that has been observed during noxious stimulation in anaesthetised mammals, suggesting that it reflects processing of noxious stimuli (Murrell and Johnson, 2006; Gibson et al., 2007).

The median frequency, 95% spectral edge frequency and total power variables provide an alternative method to LVFA for reporting on the changes in voltage and frequency that occur in the EEG (Bates, 2010). As discussed in Chapter 4, a number of studies have found increases in median frequency and 95% spectral edge frequency and decreases in total power during noxious stimulation (Murrell and Johnson, 2006). During noxious stimulation the brain is described as being in an activated or desynchronised state. The neurons' polarisation and firing falls out of phase so fewer neurons are firing in synchrony. The EEG voltage, and thus power, is therefore lower, but, because the neurons are firing out of phase, the EEG wave frequency is faster (higher) (Bates, 2010). The product of this neural activity is LVFA which equates to an increase in median and 95% spectral edge frequencies, and a decrease in total power (Murrell and Johnson, 2006).

It has also been suggested that the sensory input associated with decapitation overcomes the effect of anaesthesia (Klem, 1987), leading to an arousal of the brain rather than loss of function. The EEG power of rats that were decapitated under anaesthesia was very similar to that of rats that were conscious prior to decapitation (Van Rijn et al., 2011). This observation seems to support the theory that decapitation causes activation of the EEG and potentially the occurrence of consciousness and pain perception at least initially after the procedure.

Therefore, the activated EEG seen following decapitation in rats may be attributed to a response to noxious stimulation (Bates, 2010). Because we failed to find significant responses to noxious stimulation in the chicken EEG (Chapter 4), it is unclear whether the EEG changes observed after decapitation in the current study were associated with noxious stimulation. A number of different explanations for the EEG responses to decapitation reported above are possible.

5.5.8 Explanations for EEG changes following decapitation but not previous noxious stimuli

1. Magnitude of stimulus

It is possible that the intensity of noxious stimulation associated with decapitation was greater than that elicited by the stimuli use in Chapter 4 and other noxious stimulus experiments on chickens and that it therefore elicited a measurable EEG response (Woolley and Gentle, 1987; Gentle and Hunter, 1990)). However, as discussed in Chapter 4, it seems unlikely that the lack of EEG response was due to the noxious stimuli in that study being insufficient in intensity. The stimuli used were considered to be strongly noxious, and were of similar intensity to stimuli that had elicited EEG responses in mammals (Murrell et al., 2007; Diesch et al., 2009b, 2009a). Furthermore, some of the stimuli, particularly the toe clamp and the electrical stimulus, were followed by body movements such as leg kicking or withdrawal. Therefore, it seems unlikely that the EEG response to decapitation simply reflected a more intense noxious stimulus

2. Other physiological changes

It is possible that the EEG changes elicited by decapitation resulted from physiological changes other than those linked to nociception. For example, decapitation severed the blood supply to the brain and that may have affected cerebral activity and therefore the recorded electrical activity. However, results from studies of cattle slaughter showed that although blood loss affected the EEG, it did not cause high frequency activation (Gibson et al., 2009a) as was indicated here by the increases in median and spectral edge frequencies.

Ventral neck incision in anaesthetised cattle caused increases in 95% spectral edge and median frequencies indicating more high frequency activity and a response to noxious stimulation. The change in total power was biphasic, initially increasing, possibly due to movement artefacts, followed by a decrease, attributed to the response to noxious stimulation, followed by loss of cortical function (Gibson et al., 2009b). These changes in the EEG signal were associated with cutting the neck tissues and were not seen when only the blood vessels were cut, which supports the theory that the activated EEG represents a response to noxious stimulation rather than a response to loss of blood supply to the brain (Gibson et al., 2009a).

It therefore seems unlikely that the high frequency EEG occurring immediately after decapitation in the chicken resulted from blood loss. The decrease in total power seen after the period of activation resembles the change seen in the cattle EEG following vessel incision (Gibson et al., 2009a) and presumably results from loss of brain electrical activity. Reports of neck cut studies in chickens do not include comment on whether or not there was an activated EEG response to neck incision (Gregory and Wotton, 1986).

3. Effect of removing spinal effects of anaesthesia

As discussed in Chapter 2, anaesthesia is largely mediated through effects on the spinal cord. Anaesthetic requirements for animals increase if delivery of the anaesthetic is isolated to the brain and not delivered to the spinal cord (Antognini and Schwartz, 1993). Therefore, if the spinal component of anaesthesia was removed – i.e. by severing the spinal cord during decapitation, this may have removed the inhibitory effects of spinally mediated anaesthesia, which might have had an activating effect on the brain electrical activity.

Although decapitation may remove the spinal effects of anaesthesia in the chicken, it is difficult to say whether this effect has the same influence in the mammalian EEG. When the spinal cord of sheep was severed using the punctilla method the EEG amplitude was unchanged until the neck blood vessels were severed 130s later (Tidswell et al., 1987). The interpretation of these results suggests that severing the spinal cord does not cause immediate loss of consciousness (Tidswell et al., 1987).

In a previous study using rats, EEG activation occurred after decapitation yet the rats used had not been anaesthetised and were only under the effects of a muscle relaxant at the time of decapitation (Mikeska and Klem, 1975). Therefore in that study the EEG activation could not have been an effect caused solely by the removal of the spinal effects of anaesthesia. This suggests that in mammals some of the EEG response to decapitation may indicate a response to noxious stimulation.

4. Electromyogram (EMG)

Some of the high frequency activity occurring after decapitation may have been caused by muscular activity. Muscular contractions generate electrical activity which can be recorded using an electrode setup that is similar to that of the EEG recording electrodes. The muscular electrical activity is called the electromyogram or EMG (Cusick and Peters, 1973). Some noxious stimuli are accompanied by muscular activity that may generate EMG artefacts in the EEG (Norman et al., 2008). These artefacts can be present in the 10-30 Hz range, making them difficult to distinguish from the EEG signal (Norman et al., 2008).

However, EEG responses to noxious stimuli have been observed in animals where the electrodes were cortically implanted (Murrell et al., 2007) and EEG responses to decapitation have been identified in rats given muscle relaxants (Mikeska and Klem, 1975), indicating that EMG was not the only source of activation in the EEG waveform during noxious stimulation. In the present experiment muscular movements were intermittent and created higher amplitude waves than the EEG. The there was also a video recording of the movements of the chicken's head. Therefore, segments of EEG that contained EMG could be identified and removed from the analysis so they did not contaminate the results.

5.5.9 Difference between Groups 1 and 2

Differences between the two Groups of chickens in their EEG response to decapitation may relate either to the prior application of the toe clamp, or to the different durations of anaesthesia before decapitation.

The different durations of anaesthesia may have affected the responses to decapitation of the two Groups in two ways. Firstly, as already mentioned, the removal of the anaesthetic effect on the spinal cord may have caused activation of the EEG (Antognini and Schwartz, 1993). Secondly, the chickens being anaesthetised may have prolonged the time that EEG persisted after decapitation (Gregory and Wotton, 1986) by slowing brain metabolism including oxygen utilisation, thereby allowing the EEG activity to persist for a longer period of time after decapitation (Klem, 1987; Vanderwolf et al., 1988).

The chickens in Group 1, as well as having received a prior noxious stimulus, had been anaesthetised for a longer period of time than those in Group 2. This was because after the noxious stimulus was given the chickens were allowed to re-stabilise and a second baseline was recorded before they were decapitated. If anaesthesia slows brain metabolism and prolongs the duration of EEG after decapitation (Gregory and Wotton, 1986; Klem, 1987) then the longer duration of anaesthesia for Group 1 compared to Group 2 might have slowed brain metabolism more, thereby allowing the EEG to persist for a longer period after decapitation of the chickens in Group 1.

However, this effect may have been confounded by the prior administration of a noxious stimulus to the chickens in Group 1 but not in Group 2. It is therefore not clear whether the difference between Groups 1 and 2 was due to the duration of anaesthesia or to some effects of prior noxious stimulation (although the lack of EEG response to the toe-clamp perhaps makes this unlikely). Having both groups anaesthetised for the same duration of time would have allowed differentiation of these possible effects. However, a study using rats demonstrated that the order of application of different noxious stimuli had no

effect on the magnitude of the EEG response to noxious stimulation (Murrell et al., 2007). Extrapolating this observation to the chicken, it may be suggested that the prior noxious stimulation was not likely to have affected the chicken's EEG response to decapitation. The slight differences between Groups 1 and 2 in how the median frequency and spectral edge frequency responded to decapitation are more likely to be a result of the different durations of anaesthesia.

5.5.10 Future work

From the present study, it is not obvious whether the brain activation after decapitation is associated with pain processing or with changes in the depth of anaesthesia due to severance of the spinal cord. There are studies that could be conducted to gather more information. For example, a spinal nerve blockade could be applied to assess whether blocking spinal cord signals has an activating effect on the EEG.

To examine whether any part of the response is related to pain a local/spinal analgesic could be applied to the neck region before decapitation. If the EEG then shows no changes after decapitation this would support the notion that the changes seen in the current study were linked to nociception.

Finally, the use of muscular relaxants prior to decapitation could reduce the possibility of EMG artefacts affecting the EEG results i.e. administering muscle relaxants prior to decapitating the chicken.

5.5.11 Implications of work

There are a number of other slaughter methods, besides decapitation, which do not cause instantaneous loss of brain activity. Visual evoked responses were not initially affected by neck dislocation and it took between 105 and 245 s for the evoked response to be lost in chickens (Gregory and Wotton, 1990). Time to loss of 50% of EEG activity in chickens following neck cut varied between 19 and 180 s depending on which vessels were cut (Gregory and Wotton, 1986). The time until EEG was 5% of the original activity varied between 60 and 233 s after the incision (Gregory and Wotton, 1986). In slaughter of cattle it can take 30-80 s for the EEG to become isoelectric following a neck cut without prior stunning (Bager et al., 1992) and durations as long as 192 s for anaesthetised cattle have been reported for other studies (Gibson et al., 2009b). With such long time periods between application of the slaughter method and probable loss of consciousness on animal welfare grounds it is advisable to stun the animal before slaughter.

Stunning

In slaughter methods where consciousness is not lost immediately it would be advisable to stun the animal before slaughter. In chickens, stunning using a concussive method appears to cause more rapid loss of brain activity than many of the slaughter methods alone (Gregory and Wotton, 1990). Electrical stunning is also used, but the level of success varies depending on the current and voltage used (Raj et al., 2006).

A non-penetrative captive bolt in cattle is likely to rapidly render the animal unconscious and does not appear to be accompanied by any EEG response associated with noxious stimulation (Gibson et al., 2009d). This same form of

stunning also appears to abolish EEG responses to noxious stimulation following ventral neck incision (Gibson et al., 2009c).

5.6 Conclusions

There are two main components to consider when assessing the humaneness of decapitation. There is the question of whether the animal remains conscious after decapitation and for how long and secondly, whether the animal perceives pain during any period of consciousness following decapitation. From the current results, these questions cannot be answered definitively. The EEG of chickens after decapitation seemed to resemble an activated state with increases in median and 95% spectral edge frequencies. In anaesthetised mammals these EEG changes often indicate a response to noxious stimulation and the potential for pain perception if the animal were conscious. However, previous experiments in birds (Chapter 4) found no EEG responses to noxious stimulation. Therefore it is unjustified to say that the EEG response here was a response to noxious sensory input associated with decapitation.

Instead it is possible that decapitation removed the inhibitory effects of spinal anaesthesia, which may have resulted in increased cerebral activity immediately following decapitation. If these results do indicate the removal of spinal anaesthesia then it is possible the chickens were aroused from anaesthesia. If consciousness is lost during transitional EEG, as has been suggested (Gibson et al., 2009c), then there is potential for consciousness to persist for at least 30 s after decapitation in chickens. If severance of the neck is noxious, this is a long period of time for the chicken to consciously experience the pain and distress associated with decapitation.

These results also have implications for mammalian studies. If it were to be confirmed that the active EEG seen in chickens was due to the removal of the spinal anaesthesia then it is possible that similar effects may occur in mammals. However, in previous studies of non-anaesthetised rats increases in EEG frequencies were observed after decapitation (Mikeska and Klem, 1975). Therefore the effects of decapitation on the rat brain may not be solely attributable to the effects of anaesthesia and may be a response to noxious stimulation.

In situations where there is uncertainty about whether or not the animal may be conscious welfare guidelines should be written to give the animal the benefit of the doubt (Tidswell et al., 1987). The current results suggest that chickens may be conscious for a protracted period following decapitation. If decapitation is noxious for chickens, as is suggested for mammals, then it is possible that chickens experience pain or distress during this time. This would suggest that decapitation is an inappropriate method to use for killing chickens.

5.7 References

Allred, J. B. and Berntson, G. G., 1986: Is euthanasia of rats by decapitation inhumane? *Journal of Nutrition,* 116: 1859-1861.

Antognini, J. F. and Schwartz, K., 1993: Exaggerated anesthetic requirements in the preferentially anesthetized brain. *Anesthesiology*, 79: 1244-1249.

ANZCCART, 2001: Euthanasia of animals used for scientific purposes. *In* Reilly, J. S. (ed.). Adelaide: Australian and New Zealand Council for the Care of Animals in Research and Teaching.

AVMA, 1986: Report of the AVMA panel on euthanasia. *Journal of the American Veterinary Medical Association,* 188: 252-268.

AVMA, 2007: *AVMA Guidelines on euthanasia*. American Veterinary Medical Association, Schaumburg, IL.

Bager, F., Braggins, T. J., Devine, C. E., Graafhuis, A. E., Mellor, D. J., Tavener, A., and Upsdell, M. P., 1992: Onet of insensibility at slaughter in calves: effects of electroplecticseizure and exsanguination on spontaneous electrocortical activity and indices of cerebral metabolism. *Research in Veterinary Science,* 52: 162-173.

Barnett, J. L., Cronin, G. M., and Scott, P. C., 2007: Behavioural responses of poultry during kosher slaughter and their implications for the birds' welfare. *Veterinary Record,* 160: 45-49.

Bates, G., 2010: Humane issues surrounding decapitation reconsidered. *Journal of the American Veterinary Medical Association,* 237: 1024-1026.

Cartner, S. C., Barlow, S. C., and Ness, T. J., 2007: Loss of cortical function in mice after decapitation, cervical dislocation, potassium chloride injection, and CO_2 inhalation. *Comparative Medicine,* 57: 570-573.

Cusick, C. J. and Peters, J. J., 1973: Electromyographic, electroencephalographic, and behavioural changes during the onset of erect posture in newly hatched chicks. *Poultry Science,* 53: 1456-1462.

Derr, R. F., 1991: Pain perception in decapitated rat brain. *Life Sciences,* 49: 1399-1402.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009a: Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Laboratory Animals,* 00: 1-9.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009b: Electroencephalographic responses to tail clamping in anaesthetized rat pups. *Laboratory Animals,* 43: 224-231.

Gentle, M. J. and Hunter, L. N., 1990: Physiological and behavioural responses associated with feather removal in *Gallus* var *domesticus*. *Research in Veterinary Science,* 50: 95-101.

Gibson, T. J., Johnson, C. B., Stafford, K. J., Mitchinson, S. L., and Mellor, D. J., 2007: Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal,* 55: 152-157.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Chambers, J. P., Stafford, K. J., and Mellor, D. J., 2009a: Components of electroencephalographic responses to slaughter in halothane-anaesthetised calves: effects of cutting neck tissues compared with major blood vessels. *New Zealand Veterinary Journal,* 57: 84-89.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Hulls, C. M., Mitchinson, S. L., Stafford, K. J., Johnstone, A. C., and Mellor, D. J., 2009b: Electroencephalographic responses of halothane-anaesthetised calves to slaughter by ventral-neck incision without prior stunning *New Zealand Veterinary Journal,* 57: 77-83.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Mitchinson, S. L., Stafford, K. J., and Mellor, D. J., 2009c: Amelioration of electroencephalographic responses to slaughter by non-penetrative captive-bolt stunning after ventral-neck incision in halothane-anaesthetised calves. *New Zealand Veterinary Journal,* 57: 96-101.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Mitchinson, S. L., Stafford, K. J., and Mellor, D. J., 2009d: Electroencephalographic responses to concussive non-penetrative captive-bolt stunning in halothane-anaesthetised calves. *New Zealand Veterinary Journal,* 57: 90-95.

Gregory, N. G. and Wotton, S. B., 1986: Effect of slaughter on the spontaneous and evoked activity of the brain. *British Poultry Science,* 27: 195-205.

Gregory, N. G. and Wotton, S. B., 1989: Effect of electrical stunning on somatosensory evoked potentials in chickens. *British Veterinary Journal,* 145: 159-164.

Gregory, N. G. and Wotton, S. B., 1990: Comparison of neck dislocation and percussion of the head on visual evoked responses in the chicken's brain. *The Veterinary Record,* 126: 570-572.

Holson, R. R., 1992: Euthanasia by decapitation: evidence that this technique produces prompt, painless unconsciousness in laboratory rodents. *Neurotoxicology and Teratology,* 14: 253-257.

Johnson, C. B., Young, S. S., and Taylor, P. M., 1994: Analysis of the frequency spectrum of the equine electroencephalogram during halothane anaesthesia. *Research in Veterinary Science,* 56: 373-378.

Johnson, C. B. and Taylor, P. M., 1998: Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. *British Journal of Anaesthesia,* 81: 748-753.

Klem, W. R., 1987: Correspondence (decapitation). *Laboratory Animal Science,* 37: 148-151.

Lambooy, E. and Spanjaard, W., 1981: Effect of the shooting position on the stunning of calves by captive bolt. *The Veterinary Record,* 109: 359-361.

Mayhew, I. G. and Washbourne, J. R., 1990: A method of assessing auditory and brainstem function in horses. *British Veterinary Journal,* 146: 509-518.

Mikeska, J. A. and Klem, W. R., 1975: EEG evaluation of the humaneness of asphyxia and decapitation euthanasia of the laboratory rat. *Laboratory Animal Science,* 25: 175-179.

Murrell, J. C. and Johnson, C. B., 2006: Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29: 325-335.

Murrell, J. C., Mitchinson, S. L., Waters, D., and Johnson, C. B., 2007: Comparative effect of thermal, mechanical, and electrical noxious stimuli on the electroencephalogram of the rat. *British Journal of Anaesthesia,* 98: 366-371.

Murrell, J. C., Waters, D., and Johnson, C. B., 2008: Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. *Laboratory Animals,* 42: 161-170.

Norman, E., Rosen, I., Vanhatalo, S., Stjernqvist, K., Okland, O., Fellman, V., and Hellstrom-Westas, L., 2008: Electroencephalographic response to procedural pain in healthy term newborn infants. *Pediatric Research,* 64: 429-434.

Raj, A. B. M. and O'Callaghan, M., 2004: Effect of amount and frequency of head-only stunning currents on the electroencephalogram and somatosensory evoked potentials in broilers. *Animal Welfare,* 13: 159-170.

Raj, A. B. M., O'Callaghan, M., and Knowles, T. G., 2006: The effects of amount and frequency of alternating current used in water bath stunning and of slaughter methods on electroencephalograms in broilers. *Animal Welfare,* 15: 7-18.

SCARM, 2002: *SCARM report: Primary industries standing committee model code of practice for the welfare of animals: livestock at slaughtering establishments*. SCARM, Victoria, Australia.

Tidswell, S. J., Blackmore, D. K., and Newhook, J. C., 1987: Slaughter methods: electroencephalographic (EEG) studies on spinal cord section, decapitation and gross trauma of the brain in lambs. *New Zealand Veterinary Journal,* 35: 46-49.

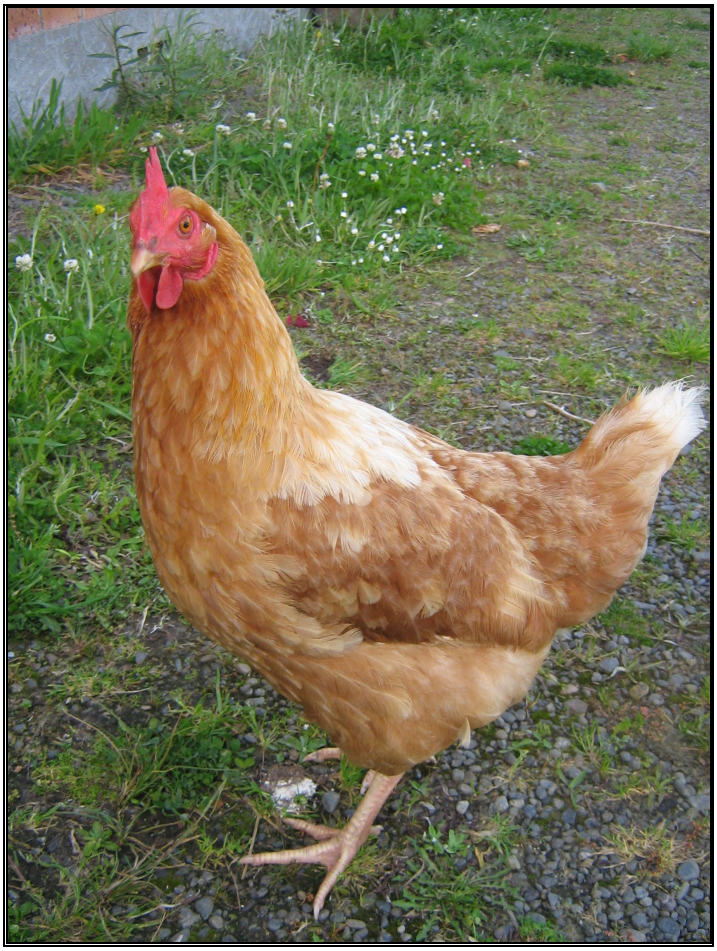
Van Rijn, C. M., Krijnen, H., Menting-Hermeling, S., and Coenen, A. M. L., 2011: Decapitation in rats: latency to unconsciousness and the "wave of death". *Public Library of Science One (online),* 6: e16514-.

Vanderwolf, C. H., Buzaki, G., Cain, D. P., Cooley, R. K., and Robertson, B., 1988: Neocortical and hippocampal electrical activity following decapitation in the rat. *Brain Research,* 451: 340-344.

Velarde, A., Ruiz-de-la-Torre, J. L., Rosello, C., Fabrega, E., Diestre, A., and Manteca, X., 2002: Assessment of return to consciousness after electrical stunning in lambs. *Animal Welfare,* 11: 333-341.

Woolley, S. C. and Gentle, M. J., 1987: Physiological and behavioural responses in the hen (*Gallus domesticus*) to nociceptive stimulation. *Comparative Biochemistry and Physiology A-Physiology,* 88A: 27-31.

Chapter 6. General discussion



"Sopwith"

6.1 Four areas of the thesis

This thesis explored four areas of brain electrical activity in chickens. The first study examined the effects of four different anaesthetics on the EEG and how the EEG varied between the different anaesthetics at different concentrations. The second study explored the development of brain activity in chicks during incubation and after hatching and revealed changes in the EEG at different stages of development. The third study sought to examine the effect of noxious stimulation on the EEG and revealed that, unlike the mammalian EEG, the chicken EEG does not appear to change in response to noxious sensory input. The fourth study revealed the changes that occur in the EEG following decapitation and provides an indication of how long brain activity persists for after decapitation.

6.1.1 Effect of anaesthesia on the chicken EEG

Anaesthetics are an important part of mammalian EEG studies that examine responses to noxious stimulation. Animals need to be "lightly" anaesthetised in order for the EEG to maintain its ability to change in response to noxious stimulation (Murrell and Johnson, 2006). In mammals, halothane is the anaesthetic of choice for these experiments, because in comparison to isoflurane and sevoflurane, it causes less suppression of brain activity (Johnson and Taylor, 1998; Murrell et al., 2008).

In Chapter 2 an experiment using halothane, methoxyflurane, isoflurane and sevoflurane found that the chicken EEG responded to the different anaesthetics in a manner similar to that observed in mammals. Furthermore, the chicken's EEG also responded to the changes in anaesthetic depth in a similar way to that

seen in mammals. Halothane has been the anaesthetic of choice for several mammalian studies recording EEG responses to noxious stimulation (Johnson et al., 2005; Murrell and Johnson, 2006; Gibson et al., 2007). Based on these results it seemed that halothane is an appropriate anaesthetic to use in experiments exploring the impact of noxious stimuli on the EEG of chickens. The results also suggest that the observed lack of EEG response to noxious stimulation (Chapter 4) was unlikely to have been caused by the effects of the anaesthetic.

6.1.2 Onset of consciousness

One objective of this thesis was to investigate the onset of consciousness in chicks before and after hatching. The onset of consciousness has been examined in mammalian species that are born in different states of maturity i.e. rats (relatively immature) (Diesch et al., 2009a), wallabies (extremely immature) (Diesch et al., 2009b). These investigations used the EEG to assess brain activity at different ages and estimate the potential for consciousness; the patterns of EEG development appear similar between species although the timing of the onset of activity differed relative to birth/hatching. An interesting comparison has recently been drawn between mammals and birds regarding the onset of consciousness before and after birth or hatching (Mellor and Diesch, 2007). The study in Chapter 3 sought to gather further information regarding the timing of the onset of consciousness in chicks.

The results of Chapter 3 represent an almost continuous record of the development pattern of the EEG before and after hatching. In comparison to previous studies on the development of EEG in chicks, the EEG was described using modern and quantitative spectral analytical methods. The EEGs revealed

that the onset of electrical activity was interspaced with isoelectric EEG when it first appeared at about day 13 of incubation. The voltage and frequency of the EEG increased until day 16-17 of incubation, suggesting increased neural activity. However, as the chick approached the time of hatching the EEG activity was reduced in both frequency and amplitude. This decrease in amplitude may be attributed to a limitation in the oxygen that is available to the chick before it cracks open the egg shell and hatches (Vince et al., 1975; Tazawa, 1980; Kraaier et al., 1988; Ozaki et al., 1995).

Overall, the results were inconclusive with regard to the onset of consciousness in chickens. Based on the evidence available, it is unlikely that the undisturbed chick would be conscious until after hatching. The onset of conscious is likely to be a gradual process that occurs after the chick has hatched. The EEGs recorded during incubation could be compared with those recorded after hatching, but a limiting factor was that the hatched chicks were either asleep or recovering from anaesthesia at the time the EEG was recorded. Therefore, comparisons between hatched chicks and incubating chicks could not be made with reference to consciousness.

To further elucidate the onset of consciousness in chicks, investigations should examine processing of sensory input before and after hatching. This may provide more information about the perceptual capacity of birds during development. For example studies using rats and wallaby joeys have used EEG responses to noxious stimuli to examine the point at which these mammals may become sensitive to pain (Diesch et al., 2009a, 2009b). It was therefore reasoned that it would be useful to conduct a similar study on chicks, to determine when,

either before or after hatching, the chick EEG becomes responsive to noxious sensory inputs.

6.1.3 Noxious stimuli

Before examining EEG responses to noxious stimulation in chicks at different ages, it was necessary to conduct a pilot study to determine whether the chicken EEG generated a detectable response to noxious stimulation (Chapter 3). The pilot study showed that no changes in the EEG were detectable following a toe-clamp stimulus. Therefore a follow up study was undertaken which aimed to record EEG responses to several forms of noxious stimulation in chickens. The only response seen in the EEG was a small and transient increase in median frequency following electrical stimulation. None of the other stimuli generated any changes in the EEG variables.

Based on these two studies, it appeared that the EEG was not useful to evaluate chicken brain responses to noxious stimulation. Therefore, it was not possible to use this noxious stimulus model to investigate the development of sensory processing and the onset of cerebral electrical responsiveness to noxious stimulation in chicks of different ages. It is interesting to consider why there was no EEG response to noxious stimulation in chickens, when the equivalent stimuli generate EEG responses in mammals. There are a number of possible interpretations, including the following: that birds do not perceive pain, which seems unlikely given birds' cognitive skills and also their behavioural responses to noxious inputs; that the stimuli were not intense enough, which also seems unlikely because they were similar in intensity to stimuli that elicited EEG responses in mammals; that the anaesthetic may have affected the results, which

again seems unlikely because halothane seems to act similarly in birds compared to mammals, as outlined in Chapter 2; and, finally, the most plausible possibility, that the differences between avian and mammalian brain/neural anatomy limits the effectiveness of the EEG for detecting changes in brain activity in birds.

6.1.4 Decapitation

Decapitation was the final area of investigation described here (Chapter 5). The EEG showed marked changes after decapitation with increases in both median frequency and spectral edge frequency and a gradual decrease in total power until the EEG was isoelectric. The EEG persisted for a mean of 35 s until it became transitional and 75 s until it became isoelectric after decapitation. These results suggest that brain activity and consciousness were not lost immediately and that there was a protracted period during which pain and suffering may occur after decapitation. Decapitation as a method of killing chickens could therefore be associated with significant welfare compromise.

The changes in median frequency and spectral edge frequency occurring after decapitation are open to interpretation. Similar EEG changes were observed in response to noxious stimulation in mammals. However, as shown in Chapter 4, the chicken EEG was virtually unresponsive to noxious stimulation, so there is no strong evidence to suggest that these changes after decapitation were a response to noxious stimulation.

It is possible that these EEG changes were a consequence of removing the spinal effects of anaesthesia. Anaesthesia is strongly mediated by effects of anaesthetics on the spinal cord (Antognini, 1997). Decapitation of these chickens, i.e. severing

the spinal cord, may have removed the suppressive effects caused by the anaesthetised spinal cord, which might have resulted in the observed changes in brain activity.

6.2 Apparent discrepancy between response to noxious stimulus and response to decapitation

There seems to be a discrepancy between the results from the noxious stimulus experiment and the results from the anaesthesia and decapitation experiments. No consistent EEG response to noxious stimulation was observed during the study presented in Chapter 4, yet the measured characteristics of the chicken EEG changed in response to varying depths and types of anaesthesia and also to decapitation. The chicken EEG therefore appears to detect some changes in brain activity, but only in certain circumstances.

The varying results may be explained as follows. Anaesthetics might be more global than noxious stimulation in their effects on the chicken brain, creating more obvious changes in EEG activity. At present, the areas of the avian brain that respond to and process pain neural signals are unknown, although it is clear that the neuroanatomy differs between birds and mammals (Butler and Cotterill, 2006). If the areas of the brain which process pain have less widespread and intense electrical effects than the regions that are affected by anaesthesia, this may explain the discrepancy between the results of the different experiments described here.

6.3 Assessment of methodology

6.3.1 Interpretation of the EEG results

The EEG records spontaneous brain electrical activity to provide an indication of gross brain function. However, there are limits to the interpretation of results collected using the EEG. There are no set markers in EEG parameters defining a conscious EEG and an unconscious EEG. Even the use of terms such as *intermediate EEG* or *transitional EEG* is limited because they do not represent specific functional states of brain activity between that reflected by a normal EEG and an isoelectric state. Transitional EEG, for example, is defined as the stage when EEG amplitude is half that of a normal EEG, but this criterion has not been shown definitively to mean anything other than consciousness is *likely* to be absent when the brain is in this state (Lambooy and Spanjaard, 1981; Gibson et al., 2009a; Gibson et al., 2009b).

6.3.2 FFT variables

The EEG variables median frequency, 95% spectral edge frequency and total power provide quantitative descriptions of how the EEG waveform is changing (Murrell and Johnson, 2006). Changes in these variables can be related to altered levels of brain functioning, for example loss of high frequency activity causing a decrease in median frequency could be related to increased depth of anaesthesia (Simons et al., 1989; Otto, 2008). An important quality of this analysis is that the measures are quantitative and can be analysed statistically.

However, it is not clear what changes in brain processing occur to cause an increase in either higher frequency or lower frequency brain activity. It would be

useful to have a better understanding of the physiological mechanisms that are underlying these changes.

6.3.3 EEG electrode needles

There were positive and negative attributes of the electrode needles used in these studies. These attributes have been discussed in the experimental Chapters 3 and 4. Briefly, on the positive side, the needles had a very fine gauge and were minimally invasive, being inserted under the skin; they were therefore not likely to have caused significant noxious stimulation to any of the chickens. A negative aspect is that in the developmental studies the needles were not securely fastened to the chicks so the electrodes movement caused significant artefacts in the EEG recordings. More securely anchored electrodes would have reduced some of these artefacts. Likewise, hatched chicks had to be anaesthetised for electrode placement which compromised the attempts to compare EEGs of conscious hatched chicks with those of incubating chicks.

Use of more invasive electrodes, for example cortically implanted electrodes, may have reduced contamination of the EEG recordings by EMG artefacts that were found in the older chicks (day 18-20 of incubation and hatched chicks) as well as in the noxious stimuli and decapitation studies. However, in the very young chicks, prior to about day 17 of incubation, there was no firm skull structure (the cartilage was not yet ossified) so cortically implanted electrodes may not have helped reduce artefacts.

More invasive electrodes also have disadvantages. They may present a greater risk of disrupting the brain activity, and the implanting of cortical electrodes may

be more noxious, which would require use of analgesics that may affect baseline brain activity. The chicks would also have needed recovery time after the electrode placement it would be difficult to get EEG from a very newly hatched chick.

Furthermore, in the anaesthesia studies there was a strong desire to re-home the chickens to a free-range lifestyle after the study. Using more invasive electrodes seemed to increase the likelihood of complications with the study and thereby could have compromised the opportunity to re-home the chickens.

6.3.4 Electrode placement

The electrode sites used in these experiments were derived from previous mammalian studies; the sites were originally developed for use in horses (Mayhew and Washbourne, 1990). Due to the differences in brain anatomy between birds and mammals, these sites may not have been appropriate for use in chickens e.g. the placement of the electrodes may not have been correct for recording changes in response to noxious stimulation. In mammals it appears that the electrodes are in close enough proximity to brain regions that process pain to allow detection of changes in the activity in these regions (Gibson et al., 2007). Without prior knowledge of how or where noxious events are processed in the chicken's brain it was difficult to know the best position for EEG electrodes.

It was thought that the chosen EEG electrode sites would be suitable to detect whole brain activity, as has been done in several mammalian species. The electrode sites effectively transect the brain as they do in mammals. Visually, the

chicken EEGs recorded in these studies appeared very similar to unpublished baseline EEG recordings collected in the same laboratory from anaesthetised cattle, dogs, rats, deer, horses and pigs (personal observation). This similarity would suggest that the EEG was recording similar neural processes in each species.

Given the superficial position of the electrodes, it seems likely that the structures generating the EEG are close to the brain surface (Klem, 1969). Yet the arrangement of neurons in the brain differs between birds and mammals (Butler et al., 2005). So despite an apparent similarity between the EEGs, and despite the fact the EEG may have been recording from superficial structures in both groups, specific qualities of the EEG signal (such as electrical activity during noxious stimulation) may actually differ between birds and mammals.

Minimal anaesthesia model

The minimal anaesthesia model has been very successful for recording EEG responses to noxious stimulation in anaesthetised mammals. Unfortunately the model does not seem to work in the same way in chickens. While the EEG responded to changes in anaesthesia and exhibited developmental changes and responses to decapitation, it was not suitable for detecting responses to noxious sensory input. Assuming that the chicken brain does respond to noxious stimuli, an alterative method for assessing pain processing in birds would be useful.

6.4 Further studies

There are a number of areas in which it would be beneficial to conduct further research.

6.4.1 Developmental studies

The spontaneous EEG only provided limited information on the functional development of the chicken brain. It would be useful to determine the extent to which various sensory inputs are interpreted in the brains of chicks of different ages. The noxious stimulus model could not be used here, but there are other methods for examining sensory processing. For example, evoked potentials (auditory, somatosensory or visual) can be used to detect which regions, within the nervous system, are used to process the stimulus (Mayhew and Washbourne, 1990). Information being processed by the brain is generally passed through several areas. When an animal receives a sensory input the groups of neurons or areas within the brain that act in interpreting the stimulus are activated in succession. A record of the brain activity as the animal processes the sensory input would therefore show a series of electrical potential bursts, each indicating the activation of a group of neurons. The neural response to the stimulus is called an evoked potential (Mayhew and Washbourne, 1990). The first spikes in the evoked potential are likely to represent the activity of neural groups early in the neural pathway and reflect basic primary processing of the sensory information. The later spikes may indicate more diverse activity and higher processing of the signal (Murrell and Johnson, 2006).

Measurement of evoked potentials, for example, from chicks of different stages of development could provide more information about the state of the chick's

nervous system (Sedlacek, 1976). Comparing the evoked potential waveform to that occurring in a conscious/mature chick may give an indication of the extent to which the stimuli are being processed by the developing chick brain.

It would also be helpful to gather EEG recordings from conscious freely behaving chicks, for the purpose of comparison to EEG recordings from before hatching. The current study was unable to record EEG from conscious chicks because of movement artefacts. Cortically implanted electrodes may reduce the amount of artefact, although cortical electrodes have other limitations. The cortical electrodes would need to be placed while the chick was under anaesthesia and with the use of analgesics. The chick would then need time to recover from the procedure. This protocol would put constraints on how quickly after hatching a recording could be collected. In the study in Chapter 3, the needle electrodes were placed on the chick and the EEG was being recorded within minutes of hatching, which would not be possible with cortical electrodes. An alternative would be to place the electrodes before the chick hatched. However, this could cause greater disturbance to the chick and could potentially stimulate the chick before it hatches, resulting in an artificially active EEG.

6.4.2 Noxious stimuli

The lack of an EEG response to noxious stimulation in chickens is an interesting finding. It may reflect a difference between mammals and birds in the way pain-related impulses are processed in the nervous system. As the EEG method used to assess pain processing in mammals has proven to be ineffective in chickens, it would be useful to find an alternative method. Imaging studies using technology such as fMRI or PET could be used to identify which areas of the

chicken brain respond to noxious sensory input. Once this information has been collected it may be possible to apply it to find another, simpler, method of recording responses to noxious stimuli in chickens. Having a model similar to the minimal anaesthesia model that could be used in birds would be useful for pain studies in avian species, for example for testing analgesics, exploring painful husbandry procedures and looking at the development of pain perception in young birds.

6.4.3 Decapitation

Information from additional studies could help to interpret the present decapitation results. For example, giving a spinal block of local anaesthetic (e.g. lidocaine) to a bird under general anaesthesia may help to determine whether severing the spinal cord and interrupting its influence on the depth of anaesthesia causes an increase in brain activity such as was observed here.

Administering local analgesia to the chickens before decapitation may reveal whether or not the observed response in the EEG is related to noxious stimulation. If analgesia given before the decapitation prevented the observed changes in median frequency and 95% spectral edge frequency, this may indicate that these changes were associated with noxious stimulation

6.5 Conclusions

This series of experiments reveals some interesting differences between bird and mammalian brain activity. Both species have similar responses to anaesthesia, which seemed to justify the use of halothane for further noxious stimulation studies. The onset of EEG activity in immature/young chicks showed a similar

trend to previous studies of both mammals and birds. However the information provided by spontaneous EEG activity was limited in terms of what it could reveal about consciousness.

There was no consistent EEG response to noxious stimulation in chickens, which contrasts with the responses seen in mammals. This lack of response may be related to differences in brain anatomy between birds and mammals. On the other hand there were marked changes in the chicken's EEG after decapitation. Without any prior evidence for EEG responses to noxious stimulation it is difficult to suggest what the likely reasons for these EEG changes were. The EEG results indicate that the active brain activity, and potentially consciousness, could persist for approximately 35 s after decapitation, which suggests that decapitation may not be a humane method for killing conscious chickens.

It would be worthwhile conducting further investigations to explore how and where sensory inputs related to pain are processed in the avian brain. Such information would be useful in developing methods for assessing pain in birds.

6.6 References

Antognini, J. F., 1997: The relationship among brain, spinal cord and anaesthetic requirements. *Medical Hypotheses,* 48: 83-87.

Butler, A. B., Manger, P. R., Lindahl, B. I. B., and Arhem, P., 2005: Evolution of the neural basis of consciousness: a bird-mammal comparison. *BioEssays,* 27: 923-936.

Butler, A. B. and Cotterill, R. M. J., 2006: Mammalian and avian neuroanatomy and the question of consciousness in birds. *Biological Bulletin,* 211: 106-127.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009a: Electroencephalographic responses to tail clamping in anaesthetized rat pups. *Laboratory Animals,* 43: 224-231.

Diesch, T. J., Mellor, D. J., Johnson, C. B., and Lentle, R. G., 2009b: Developmental changes in the electroencephalogram and responses to a noxious stimulus in anaesthetized tammar wallaby joeys (*Macropus eugenii eugenii*). *Laboratory Animals,* 00: 1-9.

Gibson, T. J., Johnson, C. B., Stafford, K. J., Mitchinson, S. L., and Mellor, D. J., 2007: Validation of the acute electroencephalographic responses of calves to noxious stimulus with scoop dehorning. *New Zealand Veterinary Journal,* 55: 152-157.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Hulls, C. M., Mitchinson, S. L., Stafford, K. J., Johnstone, A. C., and Mellor, D. J., 2009a: Electroencephalographic responses of halothane-anaesthetised calves to slaughter by ventral-neck incision without prior stunning *New Zealand Veterinary Journal,* 57: 77-83.

Gibson, T. J., Johnson, C. B., Murrell, J. C., Mitchinson, S. L., Stafford, K. J., and Mellor, D. J., 2009b: Amelioration of electroencephalographic responses to slaughter by non-penetrative captive-bolt stunning after ventral-neck incision in halothane-anaesthetised calves. *New Zealand Veterinary Journal,* 57: 96-101.

Johnson, C. B. and Taylor, P. M., 1998: Comparison of the effects of halothane, isoflurane and methoxyflurane on the electroencephalogram of the horse. *British Journal of Anaesthesia,* 81: 748-753.

Johnson, C. B., Wilson, P. R., Woodbury, M. R., and Caulkett, N. A., 2005: Comparison of analgesic techniques for antler removal in halothane-anaesthetised red deer (*Cervus elaphus*): electroencephalographic responses. *Veterinary Anaesthesia and Analgesia,* 32: 61-71.

Klem, W. R., 1969: *Animal electroencephalography*. London: Academic Press Inc.

Kraaier, V., Van Huffelen, A. C., and Wieneke, G. H., 1988: Quantitative EEG changes due to hypobaric hypoxia in normal subjects. *Electroencephalography and clinical neurophysiology,* 69: 303-312.

Lambooy, E. and Spanjaard, W., 1981: Effect of the shooting position on the stunning of calves by captive bolt. *The Veterinary Record,* 109: 359-361.

Mayhew, I. G. and Washbourne, J. R., 1990: A method of assessing auditory and brainstem function in horses. *British Veterinary Journal,* 146: 509-518.

Mellor, D. J. and Diesch, T. J., 2007: Birth and hatching: key events in the onset of awareness in the lamb and chick. *New Zealand Veterinary Journal,* 55: 51-60.

Murrell, J. C. and Johnson, C. B., 2006: Neurophysiological techniques to assess pain in animals. *Journal of Veterinary Pharmacology and Therapeutics,* 29: 325-335.

Murrell, J. C., Waters, D., and Johnson, C. B., 2008: Comparative effects of halothane, isoflurane, sevoflurane and desflurane on the electroencephalogram of the rat. *Laboratory Animals,* 42: 161-170.

Otto, K. A., 2008: EEG power spectrum analysis for monitoring depth of anaesthesia during experimental surgery. *Laboratory Animals,* 42: 45-61.

Ozaki, H., Watanabe, S., and Suzuki, H., 1995: Topographic EEG changes due to hypobaric hypoxia at simulated high altitude. *Electroencephalography and clinical neurophysiology,* 94: 349-356.

Sedlacek, J., 1976: Acoustic and somatosensory evoked responses in the brain hemispheres of chick embryos. *Physiologia Bohemoslovaca,* 25: 103-108.

Simons, A. J. R., Boezeman, E. H. J. F., and Pronk, R. A. F., 1989: Automatic EEG monitoring of anaesthesia. *In* Jones, J. G. (ed.), *Bailliere's Clinical Anaesthesiology: International Practice and Research, Vol.3 number 3*. London: Bailliere Tindall, 623-646.

Tazawa, H., 1980: Oxygen and CO_2 exchange and acid-base regulation in the avian embryo. *American Zoologist,* 20: 395-404.

Vince, M., Misson, B. H., and Freeman, B. M., 1975: Blood gas partial pressures and the onset of lung ventilation in the chick embryo. *Comparative Biochemistry and Physiology,* 51A: 457-463.

Appendix A

Channel 2 results from Chapter 2

A.1 Halothane

A repeated-measures ANOVA was used to test for an overall effect of concentration on median frequency, 95% spectral edge frequency. If a significant concentration effect was found, the ANOVA was followed by post-hoc testing with a Bonferroni adjustment for multiple comparisons. The results for total power using halothane were not normally distributed. The analysis was therefore conducted using the non-parametric Friedman's test followed by the post-hoc test using Dunn's adjustment for multiple comparisons. All results were analysed using Graphpad Prism 5.01 for Windows (GraphPad Software, San Diego California, USA). Differences were considered significant at $P<0.05$.

A.1.1 Median frequency

There was a significant effect of halothane concentration on median frequency ($F(4,44) = 14.68$, $P<0.0001$). As halothane concentration increased, median frequency tended to decrease, with the median frequency at 2 MAC being significantly lower than at 1 MAC. In agreement with this, the median frequency tended to increase as the halothane concentrations was decreased. The median frequency was lower with the halothane concentration at 2 MAC compared to 1 MAC. However, the change in median frequency was not always significant as the concentration changed between MAC multiples 2 and 1.5 or 1.5 and 1 (Figure A.1).

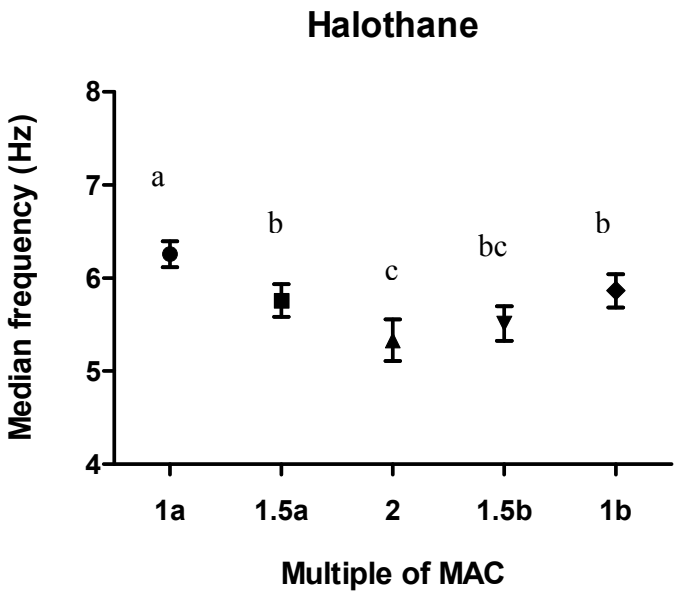


Figure A.1. Changes in the mean median frequency in response to altered concentration of halothane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $p<0.05$.

A.1.2 95% spectral edge frequency

Halothane concentration also had a significant effect on 95% spectral edge frequency ($F(4,44) = 7.27$, $P<0.001$). The 95% spectral edge frequency was lower at halothane concentrations of 1.5 MAC than at 1 MAC. At 2 MAC the mean spectral edge frequency was not significantly different to at 1 or 1.5 MAC (Figure A.2).

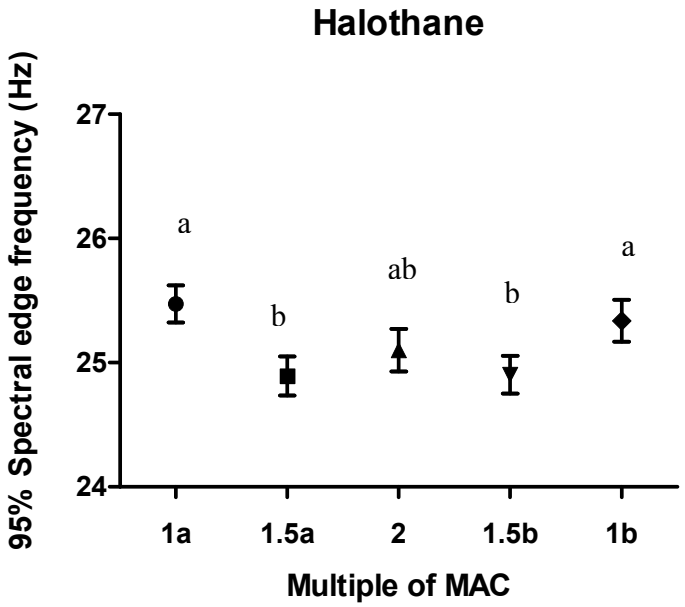


Figure A.2. Changes in the mean 95% spectral edge frequency in response to altered concentration of halothane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $p<0.05$.

A.1.3 Total power

Total power was significantly affected by changes in halothane concentration ($F(4,44) = 39.13$, $P<0.0001$). There was a trend for total power to increase as the concentration of halothane increased and for total power to decrease as the halothane concentration decreased. Total power was significantly higher at halothane concentrations of 1.5 and 2 MAC than at 1 MAC (Figure A.3).

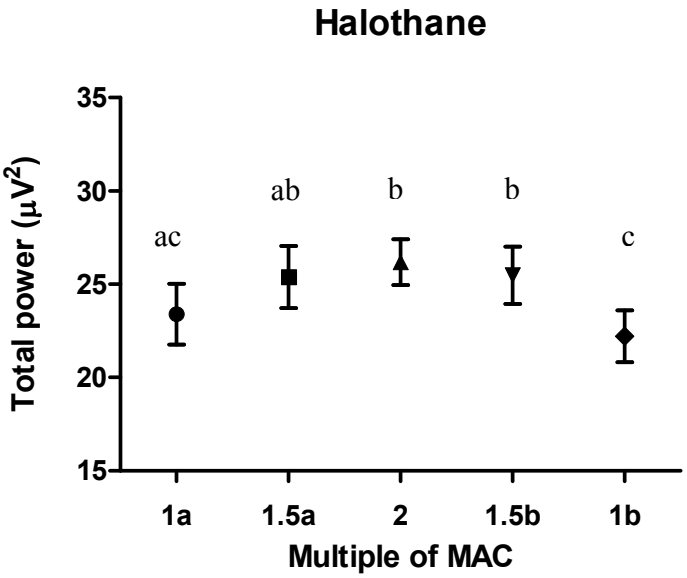


Figure A.3. Changes in the mean total power in response to altered concentration of halothane. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $p<0.05$.

A.2 Methoxyflurane

A repeated-measures ANOVA was used to test for an overall effect of concentration on total power. If a significant concentration effect was found, the ANOVA was followed by post-hoc testing with a Bonferroni adjustment for multiple comparisons. The results for median frequency and 95%spectral edge using methoxyflurane were not normally distributed. The analyses for these variables were therefore conducted using the non-parametric Friedman's test followed by the post-hoc test using Dunn's adjustment for multiple comparisons. All results were analysed using Graphpad Prism 5.01 for Windows (GraphPad Software, San Diego California, USA). Differences were considered significant at $P<0.05$.

A.2.1 Median frequency

The methoxyflurane concentration did not have a significant effect on median frequency ($F(2,20) = 5.64$, $P=0.06$). There appears to be an increase in median frequency as the methoxyflurane concentration is reduced, but these changes are not statistically significant (Figure A.4).

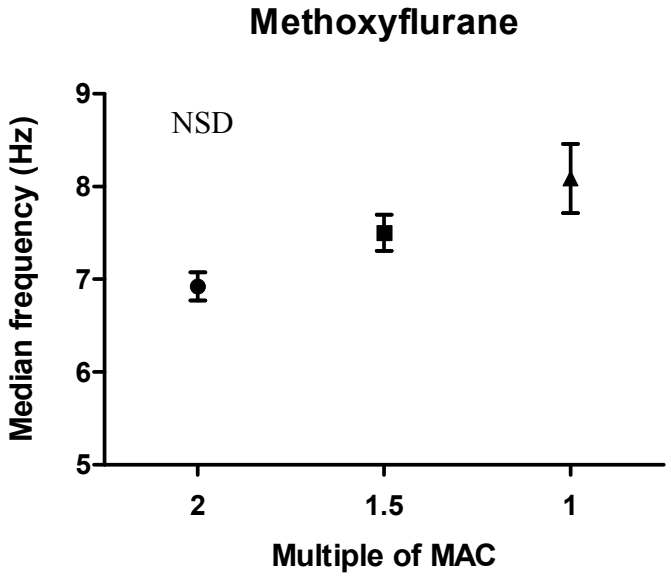


Figure A.4. Changes in the mean median frequency in response to altered concentration of methoxyflurane. Note: the y-axis does not start at zero; error bars represent SEM. There were no significant differences between the median frequency values at the different methoxyflurane concentrations.

A.2.2 95% Spectral edge frequency

A decrease in methoxyflurane concentration from 2 to 1 MAC caused a significant increase in 95% spectral edge frequency ($F(2,20) = 9.455$, $P=0.0065$) (Figure A.5).

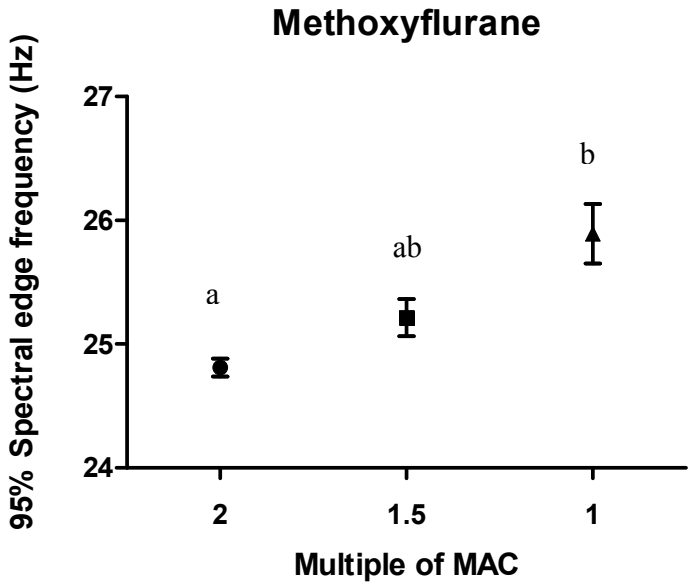


Figure A.5. Changes in the mean 95% spectral edge frequency in response to altered methoxyflurane concentrations. Note: the y-axis does not start at zero; error bars represent SEM; shared letters indicate values are not significantly different. Differences are significant at $p<0.05$.

A.2.3 Total power

Total power appeared to increase when the methoxyflurane concentration decreased from 1.5 to 1 MAC but this change in total power was not significant ($F(2,20) = 3.53$, $P=0.0487$) (Figure A.6).

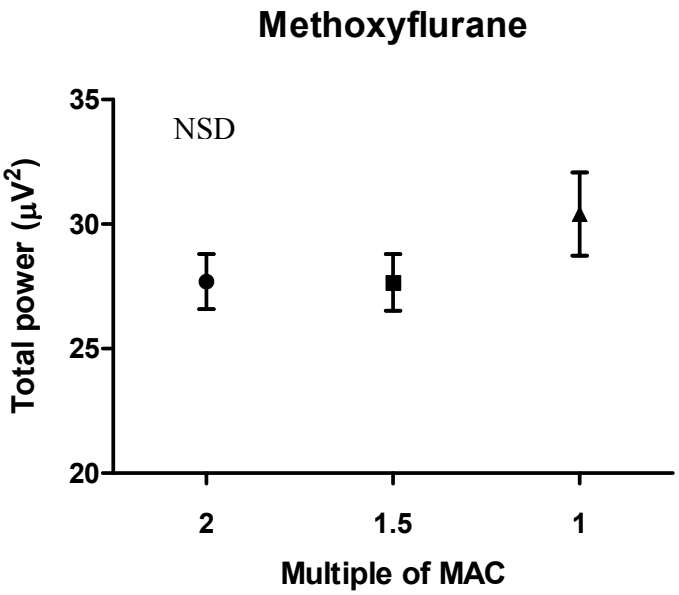


Figure A.6. Changes in the mean total power in response to altered methoxyflurane concentrations. Note: the y-axis does not start at zero; error bars represent SEM. There were no significant differences between the total power values at the different methoxyflurane concentrations.

Appendix B

Extended graphs from Chapter 3

The following bar graphs show the percent of the recording that could be identified as one of three EEG states (Figure B.1), as well as the proportions of active (Figure B.2), intermediate (Figure B.3) and isoelectric EEG (Figure B.4) at the different time-points during the recordings. These graphs show the variation between the time-points throughout the EEG recording, the means across these time points were presented in the Results of Chapter 3 (Figures 3.3-3.6).

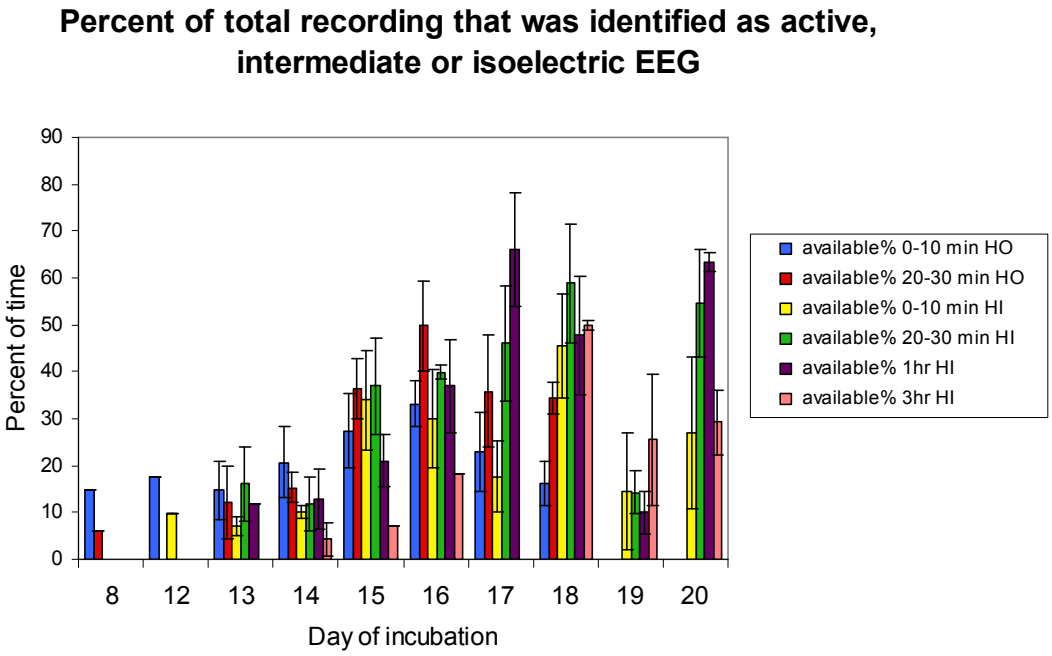


Figure B.1 Percent of time EEG was present in recording from chicks at different stages of incubation and at different time points during the recording. Error bars indicate standard errors. HO = head out of the egg, HI = head inside the egg.

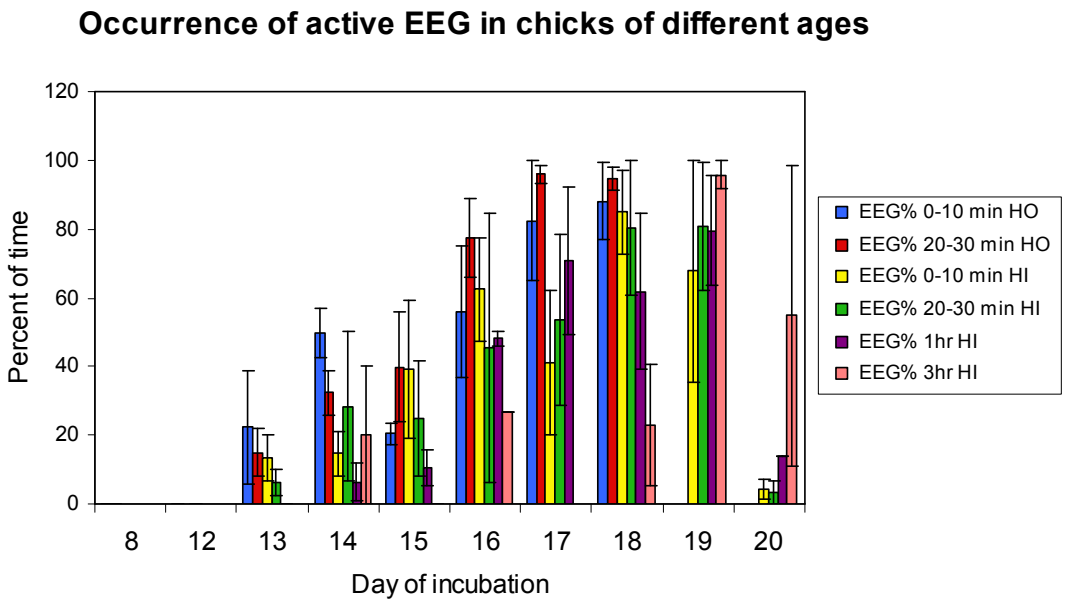


Figure B.2 Percent of time active EEG was present in recording from chicks at different stages of incubation and at different time points during the recording. Error bars indicate standard errors. HO = head out of the egg, HI = head inside the egg.

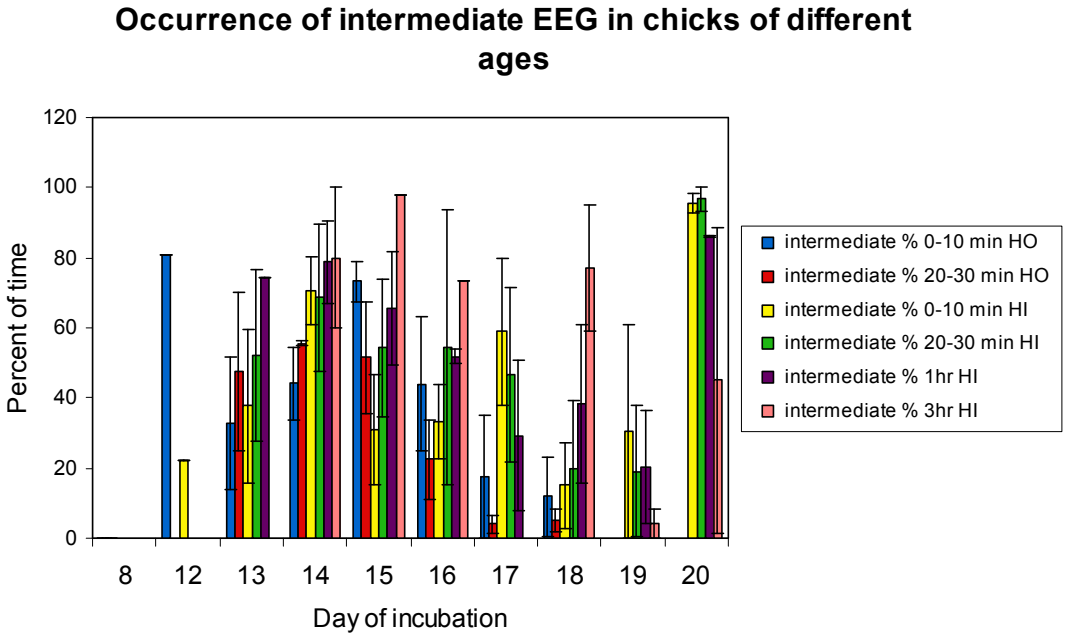


Figure B.3 Percent of time the recording was intermediate in chicks at different stages of incubation and at different time points during the recording. Error bars indicate standard errors. HO = head out of the egg, HI = head inside the egg.

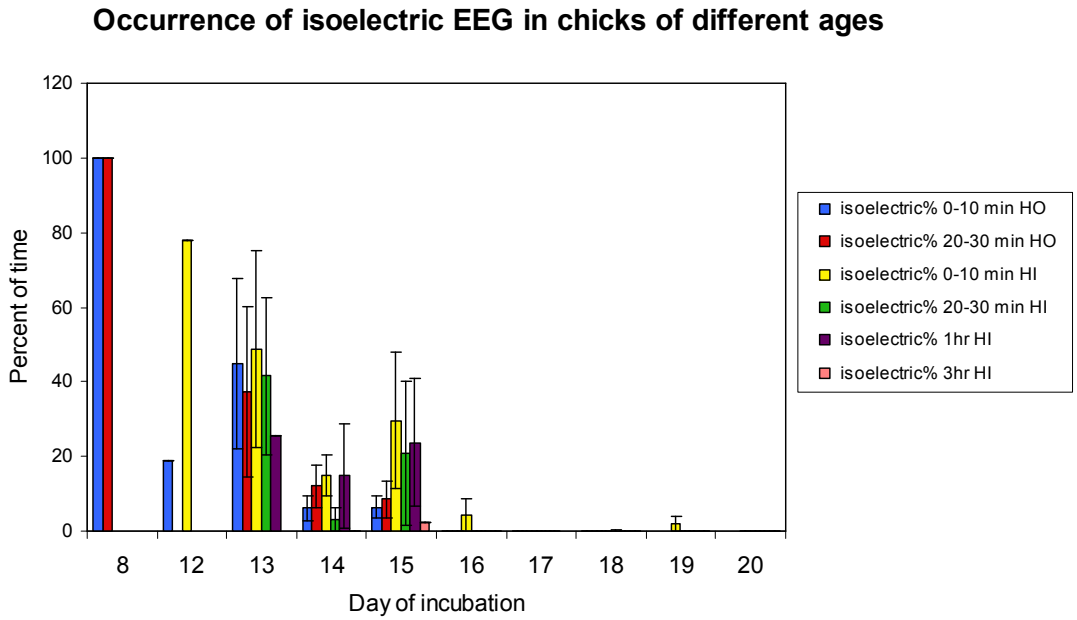


Figure B.4 Percent of time the recording was isoelectric in chicks at different stages of incubation and at different time points during the recording. Error bars indicate standard errors. HO = head out of the egg, HI = head inside the egg.

Appendix C

Channel 2 results from Chapter 4

C.1 Statistical analysis

The results were found not to be normally distributed for some tests, and there were small numbers in some of the treatment groups. For each of the three EEG variables, a non-parametric repeated-measures ANOVA (Friedman's test) was used to test for an overall time effect caused by noxious stimulation. If a time effect was found then the Friedman's test was followed by Dunn's test to look at pair-wise comparisons between baseline and the seven time points following application of the stimulus (Figure C.2)). The analysis was also repeated using a parametric test (repeated measures ANOVA and Dunnett's post hoc test), and the outcomes were very similar to the non-parametric test.

C.1.1 Experiment 1

Mechanical Stimulus – toe clamp

There was no effect of time on percent changes in median frequency ($F(7,49)=6.5$, $P=0.4827$), 95% spectral edge frequency ($F(7,49)=2.18$, $P=0.9492$) or total power ($F(7,49)=11.33$, $P=0.1247$) following the mechanical toe clamp stimulus (Tables C.1, C.2 and C.3). That is, there was no significant change from baseline at any time after application of the toe clamp stimulus.

C.1.2 Experiment 2

Electrical stimulus

Time had a significant effect on percent change in 95% spectral edge frequency ($F(7,35)=22.06$, $P=0.0025$). 95% Spectral edge frequency was significantly higher than baseline at 5-10 s after application of the electrical stimulus ($P<0.05$, Table C.2). There were no significant changes in median frequency ($F(7,35)=11.84$, $P=0.1058$, Table C.1) or total power ($F(7,35)=3.889$, $P=0.7925$, Table C.3).

Feather plucking

There was no effect caused by time after removal of the first feather for median frequency ($F(8,72)=3.84$, $P=0.8713$, Table C.1), 95% spectral edge frequency ($F(8,72)=4.4$, $P=0.8194$, Table C.2), or total power ($F(8,72)=10.32$, $P=0.2433$, Table C.3). There was also no effect caused by time after the second feather removal for median frequency ($F(8,64)=5.926$, $P=0.6555$, Table C.1), 95% spectral edge frequency ($F(8,64)=5.63$, $P=0.6886$, Table C.2), or total power ($F(8,64)=3.615$, $P=0.8901$, Table C.3).

Thermal stimulus

There was no effect caused by time on percentage changes in median frequency ($F(9,72)=9.655$, $P=0.3792$, Table C.1) or total power ($F(9,72)=16.05$, $P=0.0658$, Table C.3) following the thermal stimulus. Time had a significant effect on percent change in 95% spectral edge frequency ($F(9,72)=19.52$, $P=0.0211$, Table C.2). 95% Spectral edge frequency was significantly higher than baseline at 20-25 s after application of the thermal stimulus ($P<0.05$, Table C.2).

Mechanical stimulus – skin pinch

There was no effect caused by time on percentage changes in median frequency ($F(8,40)=12.22$, $P=0.1416$, Table C.1), 95% spectral edge frequency ($F(8,40)=3.772$, $P=0.8771$, Table C.2), or total power ($F(8,40)=9.689$, $P=0.2875$, Table C.3) following the mechanical skin-pinch stimulus. That is, there was no significant change from baseline at any time after application of the skin-pinch stimulus.

Table C.1. Mean (SEM) percentage changes in median frequency, relative to baseline values, for different noxious stimuli applied in Experiments 1 and 2. The values were scaled so that the baseline for each chicken = 100%.

Stimulus	baseline	stimulus	5	10	15	20	25	30	35	40
Toe-clamp (n=7)	100			120 (14)	113 (11)	112 (10)	98 (5)	101 (7)	106 (4)	100 (4)
Electrical (n=6)	100	93 (12)	-	132 (13)	120 (8)	113 (6)	108 (7)	110 (5)	102 (7)	-
Feather 1 (n=10)	100	-	101 (5)	105 (3)	103 (3)	103 (3)	99 (4)	104 (4)	99 (3)	104 (4)
Feather 2 (n=9)	100	-	100 (4)	96 (5)	96 (3)	101 (5)	100 (3)	99 (3)	94 (4)	101 (6)
Skin pinch (n=6)	100	-	104 (5)	106 (3)	104 (6)	106 (7)	98 (3)	98 (4)	88 (7)	97 (4)
Thermal (n=9)	100	97 (3)	95 (5)	99 (4)	102 (5)	103 (4)	111 (4)	104 (5)	103 (4)	97 (3)

Table C.2. Mean (SEM) percentage changes in 95% spectral edge frequency, relative to baseline values, for different noxious stimuli applied in Experiments 1 and 2. The values that differed significantly (p=0.05) from the baseline are marked with an asterisk. The values have been scaled so that the baseline for each chicken = 100%.

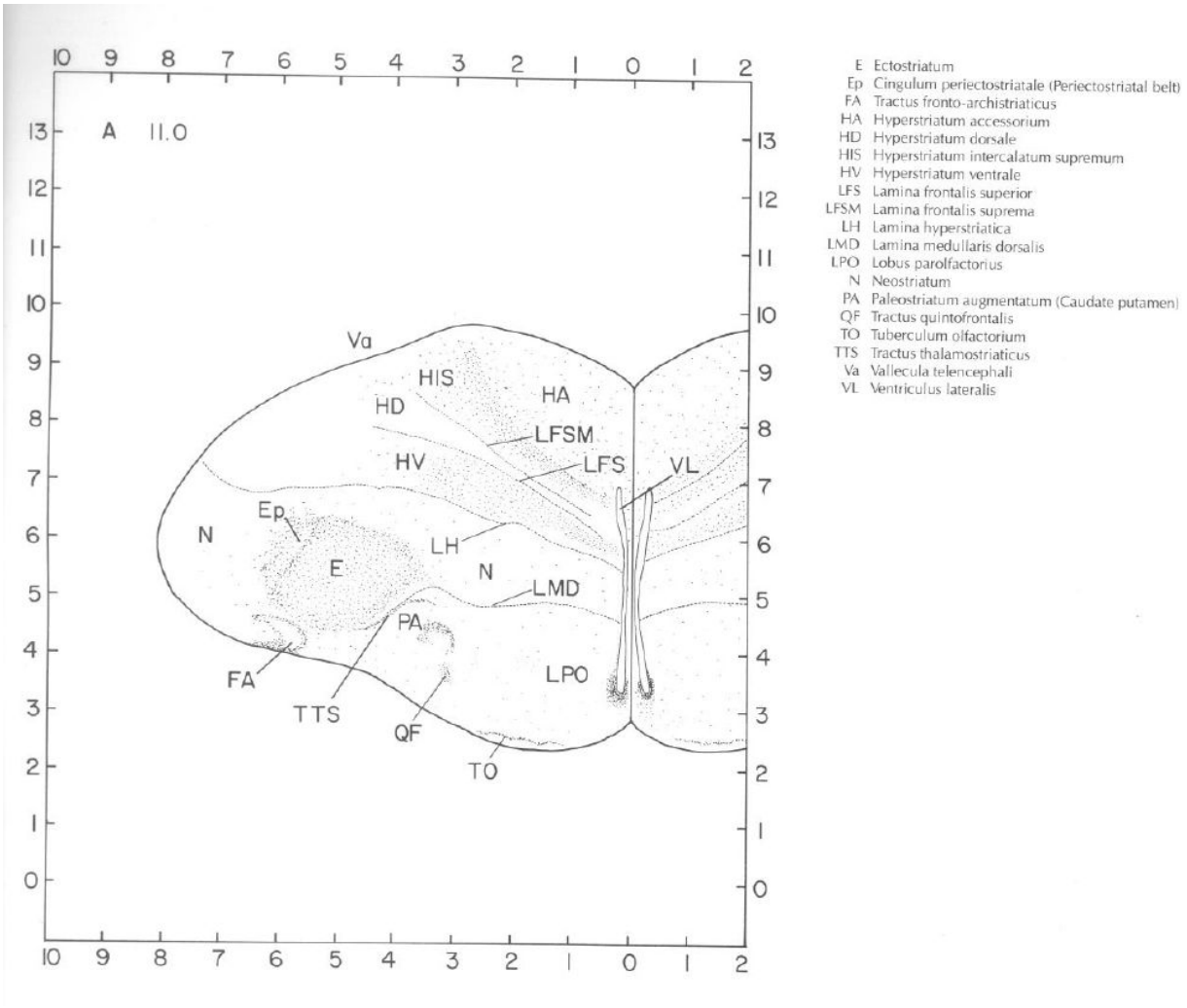
Stimulus	baseline	stimulus	5	10	15	20	25	30	35	40
Toe-clamp (n=7)	100			101 (2)	102 (2)	100 (0.7)	99 (2)	101 (1)	101 (1)	101 (0.9)
Electrical (n=6)	100	97 (2)		**105* (1)**	102 (1)	103 (1)	102 (1)	103 (0.8)	101 (1)	
Feather 1 (n=10)	100		99 (0.9)	100 (0.6)	101 (1)	100 (1)	101 (0.9)	100 (0.7)	99 (0.6)	100 (0.8)
Feather 2 (n=9)	100		101 (0.5)	100 (0.8)	100 (0.6)	100 (1)	101 (0.4)	101 (0.6)	99 (0.7)	100 (1)
Skin pinch (n=6)	100		100 (1)	99 (0.6)	99 (1)	101 (0.8)	100 (1)	100 (1)	99 (1)	100 (1)
Thermal (n=9)	100	100 (0.9)	100 (0.8)	102 (0.8)	100 (1)	100 (1)	**103* (0.4)**	100 (0.6)	100 (1)	98 (0.9)

Table C.3. Mean (SEM) percentage changes in total power, relative to baseline values, for different noxious stimuli applied in Experiments 1 and 2. There were no significant differences between any of the values and the baseline. The values have been scaled so that the baseline for each chicken = 100%.

Stimulus	baseline	stimulus	5	10	15	20	25	30	35	40
Toe-clamp (n=7)	100			113 (8)	104 (7)	99 (6)	107 (4)	103 (4)	100 (3)	102 (3)
Electrical (n=6)	100.0	107 (5)		102 (1)	104 (4)	106 (3)	98 (4)	99 (2)	106 (4)	
Feather 1 (n=10)	100		98 (3)	97 (2)	(95) (1)	98 (2)	99 (3)	95 (2)	97 (3)	98 (2)
Feather 2 (n=9)	100		100 (1)	99 (2)	101 (2)	101 (2)	99 (2)	98 (2)	99 (3)	101 (2)
Skin pinch (n=6)	100		99 (4)	102 (3)	98 (4)	98 (4)	98 (2)	106 (3)	106 (3)	97 (3)
Thermal (n=9)	100	102 (1)	100 (3)	98 (2)	99 (1)	104 (2)	94 (2)	101 (1)	101 (2)	104 (2)

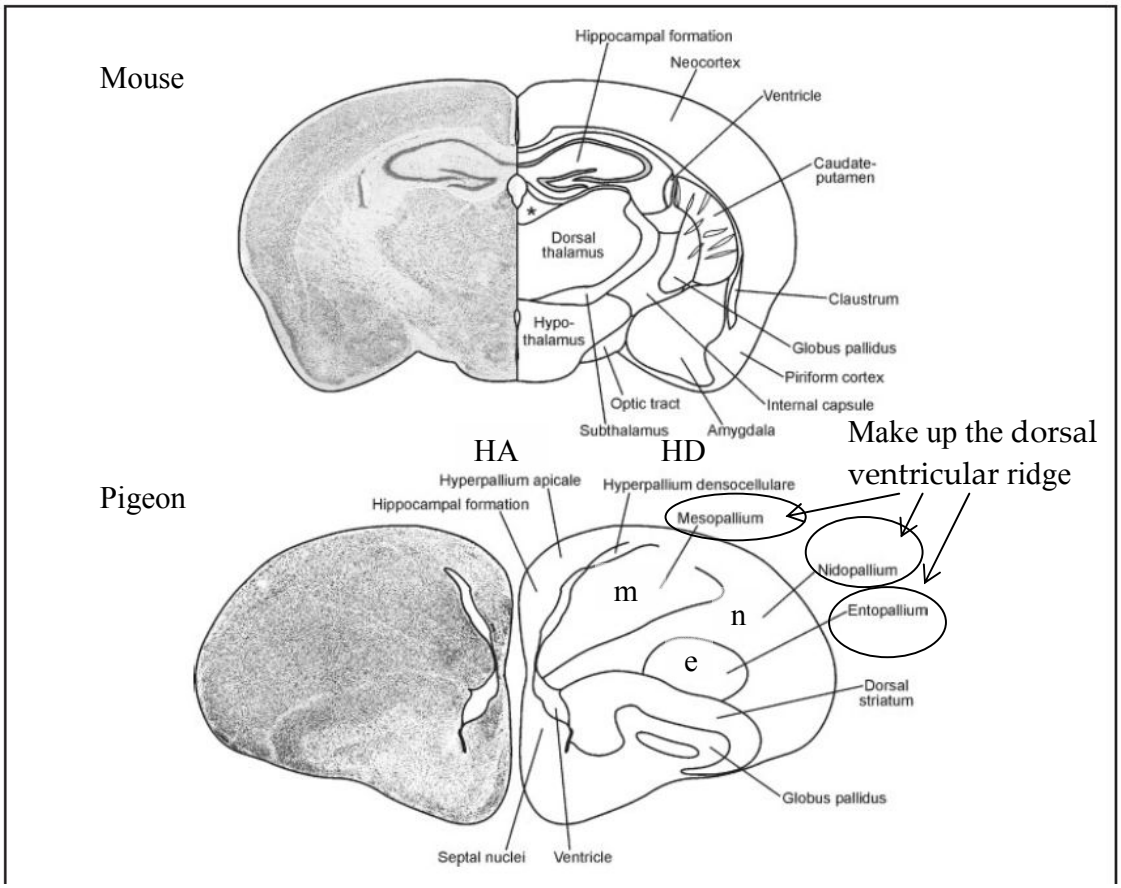
Appendix D

Comparison of Bird vs. Mammalian brain anatomy



Structures in this image are labelled using the old convention. For example, using the new names, the labels would be: N = nidopallium, HA = hyperpallium apicale, HD = hyperpallium densocellulare, HV = mesopallium ventrale, and E = endopallium.

Picture from: Kuenzel, W. J. and Masson, M., 1988: *A stereotaxic atlas of the brain of the chick (Gallus domesticus)*. Baltimore, Maryland, USA: The Johns Hopkins University Press. Page 48.



The three circled labels, corresponding to m, n and e, are the mesopallium, nidopallium and entopallium respectively, which make up the dorsal ventricular ridge discussed in the text. In the bird brain, HA = hyperpallium apicale, HD = hyperpallium densocellulare

Image from: Butler, A. B., Manger, P. R., Lindahl, B. I. B., and Arhem, P., 2005: Evolution of the neural basis of consciousness: a bird-mammal comparison. *BioEssays,* 27: 923-936. Page 928

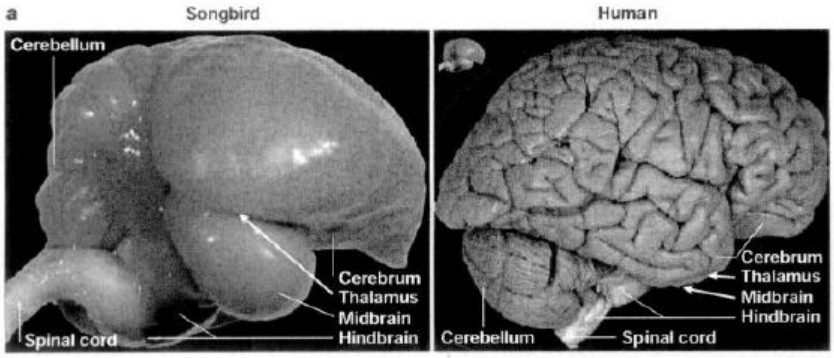
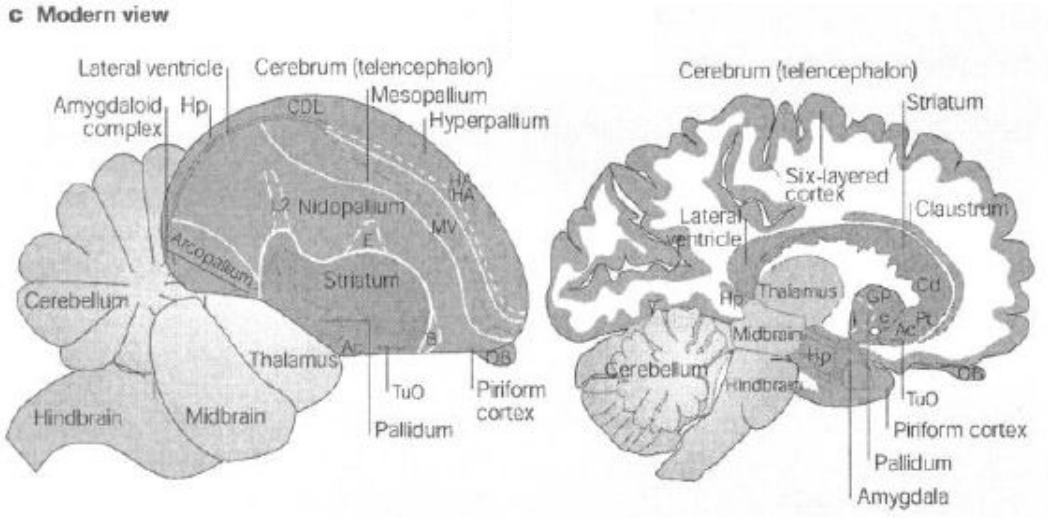
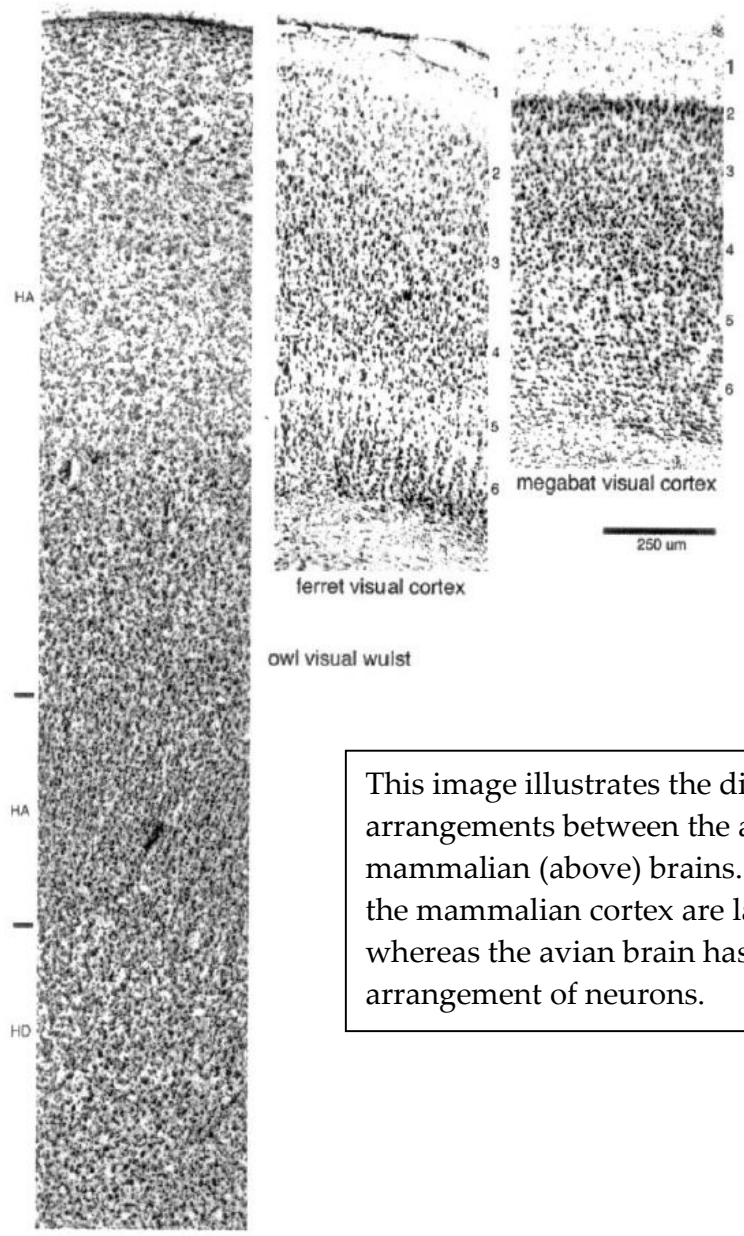


Figure 1 | **Avian and mammalian brain relationships. a** | Side view of a songbird (zebra finch) and human brain to represent avian and mammalian species. In this view, the songbird cerebrum covers the thalamus; the human cerebrum covers the thalamus and midbrain. Inset (left) next to the human brain is the zebra finch brain to the same scale. Human brain image reproduced, with permission, courtesy of John W. Sundsten, Digital Anatomist Project



c | Modern consensus view of avian and mammalian brain relationships according to the conclusions of the Avian Brain Nomenclature Forum. Solid white lines are lamina (cell-sparse zones separating brain subdivisions). Large white areas in the human cerebrum are axon pathways called white matter. Dashed grey lines divide regions that differ by cell density or cell size; dashed white lines separate primary sensory neuron populations from adjacent regions. Abbreviations where different from **b**: E, entopallium; B, basorostralis; HA, hyperpallium apicale; Hp, hippocampus; IHA, interstitial hyperpallium apicale; MV, mesopallium ventrale.

Pictures from: Jarvis, E. D., Gunturkun, O., Bruce, L., Csillag, A., Karten, H., Kuenzel, W., Medina, L., Paxinos, G., Perkel, D. J., Shimizu, T., Striedter, G., Wild, J. M., Ball, G. F., Dugas-Ford, J., Durand, S. E., Hough, G. E., Husband, S., Kubikova, L., Lee, D. W., Mello, C. V., Powers, A., Siang, C., Smulders, T. V., Wada, K., White, S. A., Yamamoto, K., Yu, J., Reiner, A., and Butler, A. B., 2005: Avian brains and a new understanding of vertebrate brain evolution. *Nature Reviews: Neuroscience,* 6: 151-159. Page 153.



This image illustrates the difference in neural arrangements between the avian (left) and mammalian (above) brains. The 6 layers of the mammalian cortex are labelled above, whereas the avian brain has a different arrangement of neurons.

Figure 3. Photomicrographs of the visual lemnopallium in the barn owl (*Tyto alba*), common ferret (*Mustela putorious*) and eastern tube-nosed megabat (*Nyctimene robinsoni*). In the owl, the photomicrograph depicts a coronal section stained for Nissl substance (localized in cell bodies) through the visual hyperpallium, the Wulst (pial surface to the top of the page). Note the thick outer layer termed the hyperpallium apicale (HA), the high cell density of the nucleus interstitialis hyperpallii apicalis (IHA), and the deepest portion termed the hyperpallium densocellulare (HD) (see Ref. 41 for teminology). This region of the Wulst, forms what are termed pseudolayers,[79] which are best thought of as flattened and stretched out nuclei rather than true layers as is evident in the cerebral cortex of the ferret and megabat pictured here. This architecture contrasts with the typical 6-layered cerebral cortex found in the primary visual cortex of mammals.

Picture from: Butler, A. B., Manger, P. R., Lindahl, B. I. B., and Arhem, P., 2005: Evolution of the neural basis of consciousness: a bird-mammal comparison. *BioEssays,* 27: 923-936. Page 929.