# Characterising the responses of farm mammals to a thoracic squeeze and the relationship to tonic immobility

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# ABSTRACT

Applying compression to the thorax of newborn farm mammals causes immobility accompanied by reduced responsiveness. Traditionally, this response was proposed to arise in neonatal foals due to the 'thoracic squeeze' mimicking the compression occurring during birth. Recent findings regarding the responses to the squeeze suggest a link to Tonic Immobility (TI). TI is a temporary and reversible state of reduced responsiveness and immobility with characteristic physiological changes. It is elicited by the collective actions of handling and sustained physical contact with additional pressure (restraint) and may be facilitated by inversion. TI is reported in young and adult animals of numerous species. The aim of this thesis was to examine whether responses to the thoracic squeeze are consistent with TI. First, behavioural responses to a squeeze were explored in lambs, with a focus on determining whether responses persisted beyond the neonatal period. Cortisol responses of healthy neonatal piglets to the squeeze were then investigated to explore similarities in Hypothalamic-Pituitary-Adrenal responses between the squeeze and TI. The final study examined electroencephalographic (EEG) responses of healthy neonatal piglets to a thoracic squeeze under light anaesthesia, to determine whether the squeeze causes changes in brain activity or exerts anti-nociceptive effects such as those reported during TI. The results demonstrated that responses to a thoracic squeeze persist beyond the neonatal period in lambs, and responses are generalised across multiple mammalian species. Furthermore, cortisol responses of piglets to a thoracic squeeze followed a similar pattern to that previously observed during TI in other species. Also consistent with some TI studies, the initial handling and restraint required to apply the squeeze appeared to induce the cortisol response in piglets. No inferences could be made about the effects of a thoracic squeeze on state of awareness in neonatal piglets, or the squeeze's effect on nociception due to methodological limitations. Nevertheless, the results of this research support the hypothesis that the thoracic squeeze may be classified as a stimulus for inducing TI. Further work is required to characterise the effects of the squeeze on awareness and nociception and to explore the affective experiences of animals subjected to the squeeze.

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*"L'avenir, tu n'as point à le prévoir mais à le permettre."*
**- Antoine de Saint-Exupéry**

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**Holdsworth S.E.,** Kells N.J., Vallée E., Ward N., Mellor D.J., Beausoleil N.J. Evaluating the behavioural responses of healthy newborn calves to a thoracic squeeze. *Animals* 12, 840, 2022

Beausoleil N. J., **Holdsworth S. E.,** Lehmann H. Avian nociception and pain. In: *Sturkie's Avian Physiology*. Pp 223-231. Elsevier, 2022

# CHAPTER 1
# General Overview of Thesis

## 1.1 THESIS STRUCTURE

The thesis presents an extensive review of the literature relevant to the thoracic squeeze, and tonic immobility as a potential mechanism (Chapter 2). As part of the literature review, responses to the thoracic squeeze were compared with what is currently understood about the manifestations of tonic immobility, and knowledge gaps were identified that would enable clear conclusions to be made about the classification of the thoracic squeeze as a stimulus for inducing tonic immobility. Following the literature review, this thesis presents the results of three separate experimental studies (Chapters 3-5), each of which are formatted according to specific guidelines of the peer-reviewed journals they were prepared for or submitted to for publication. A general discussion is presented after the experimental chapters (Chapter 6).

Experiment 1 provided a first look at the behavioural responses of lambs of multiple ages to application of a thoracic squeeze. The premise behind this experiment was based on the theory that a special birth-related mechanism is responsible for the behaviours observed after application of a thoracic squeeze. There is currently no evidence to support this theory, and its conditions around birth suggest that the behaviours observed during the squeeze would theoretically become extinct with age. If this is the case, then another mechanism, such as tonic immobility, could be explored. The results of this paper were submitted for publication in *Applied Animal Behaviour Science* and is currently under review.

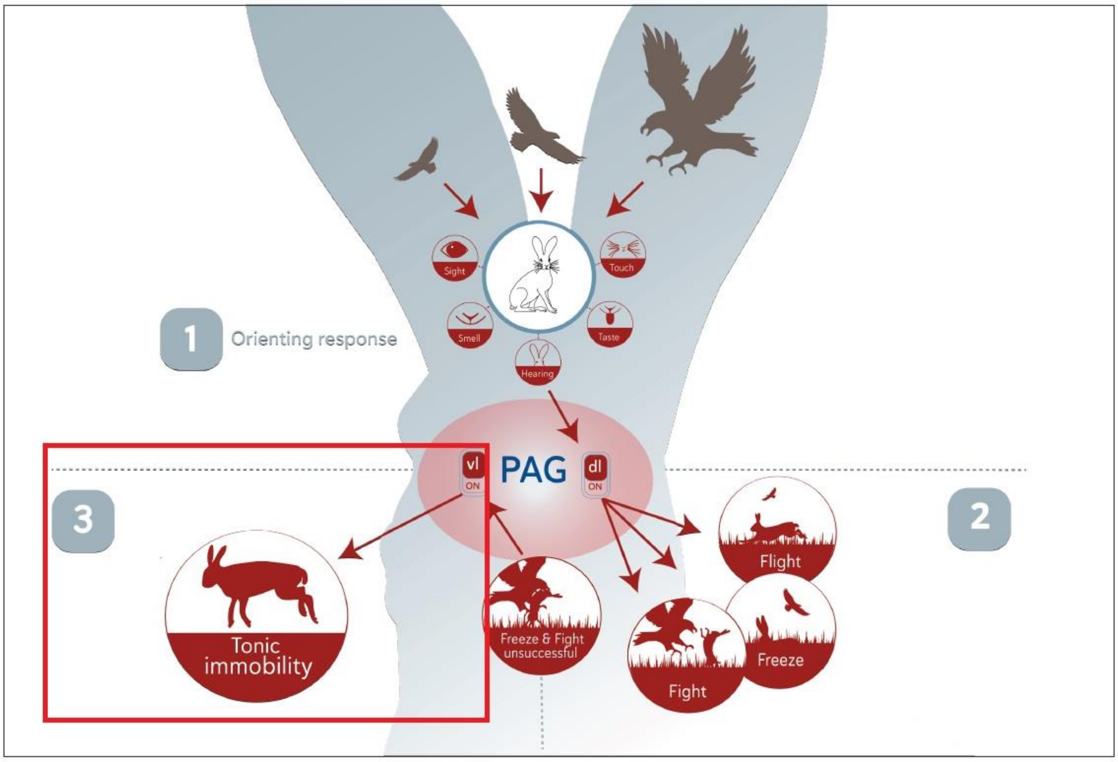
Experiment 2 focused on evaluating Hypothalamic-Pituitary-Adrenal (HPA) responses of healthy neonatal piglets to a thoracic squeeze. The study aimed to investigate how cortisol responses to a squeeze compared to those observed during tonic immobility. The results of this study are formatted for submission to a journal.

Experiment 3 aimed to provide information about the brain states of healthy neonatal piglets during a thoracic squeeze, and investigated whether nociceptive responses during the squeeze are diminished, similarly to what has been previously observed during tonic immobility. Little is known about the quality of reduced responsiveness observed during the squeeze and what implications this has for the use of the thoracic squeeze in industry settings. The results of the experiment are presented in Chapter 5.

The general discussion provides an overview of the experimental results, and discusses the implications of these results in the application of the thoracic squeeze technique in farm mammals. It also addresses the overall research question using evidence provided by the experimental chapters. Lastly, methodological developments and considerations are discussed in relation to the techniques and methods used in the three experiments.

# CHAPTER 2

# 2 Literature review



## 2.1 Introduction

In several species of precocial mammals, squeezing the chest by looping a fabric rope around the thorax and pulling it tight causes collapse into lateral recumbency and immobility for as long as the squeeze is held (Toth *et al.* 2012). Removal of this squeeze results in a rapid return to normal behaviour (Toth *et al.* 2012). In recent years, this 'thoracic squeeze' technique has been proposed as a therapy for neonatal farm animals that show a delay or impairment in the expression of normal behaviour after birth. It is proposed to act by inducing a 'sleep-like state' that inhibits the experience of both pain and stress (Toth *et al.* 2012; Aleman *et al.* 2017; Mellor 2017). In several species of farm animals, squeezing these 'dummy' newborns has reportedly resulted in the rapid onset of normal behaviour enabling them to thrive after birth (Aleman *et al.* 2017). While behavioural responses to a thoracic squeeze have been studied in a range of mammalian species, little is known about the mechanisms underlying the responses.

The literature contains similar reports of immobility and reduced responsiveness across a range of vertebrate and invertebrate taxa. Reports include the use of methods for restraint and handling during procedures in the veterinary and agricultural industries (Ong 1993). This 'tonic immobility' (TI) phenomenon is thought to be one of four sequential responses to a detected threat or stressor (Marx *et al.* 2008). TI manifests in a range of behavioural and physiological changes, governed by specific neural pathways as part of the cascade of defensive responses to a stressor (Gallup 1977; Marx *et al.* 2008). It is elicited by physical contact that triggers a shift into immobility, behavioural quiescence, and reduced responsiveness to external stimuli (Gilman *et al.* 1950).

Both the thoracic squeeze and TI share numerous similarities, and recent studies on the thoracic squeeze have suggested a potential connection between the squeeze and TI. However, in order to explore the link between the two, it is first necessary to understand what TI is, how it manifests, and what neural mechanisms are responsible for eliciting the response in such a wide range of species and ages. In particular, a thorough understanding of the mechanisms underlying TI provides necessary context for comparisons made between the thoracic squeeze and TI and informs the current knowledge gaps in the thoracic squeeze literature that need further investigation. For instance, the release of cortisol and the inhibition of pain are features of TI that have been demonstrated to be involved in the thoracic squeeze response (Toth *et al.* 2012), though their actions and timing are not well characterised. As such, there is a need to determine whether application of a thoracic squeeze causes a cortisol (stress) response and whether animals experience analgesia during the squeeze.

## 2.2 Reduced responsiveness and immobility in farmed mammalian species after application of a thoracic squeeze

The thoracic squeeze is a technique that involves applying sustained pressure to the thorax of neonatal farm animals to cause immobility and behavioural quiescence (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). The squeeze is applied by looping a rope or inflating a cuff around the chest. To date, the thoracic squeeze has been recorded in foals, piglets, calves and lambs (Toth *et al.* 2012; Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). The animals reportedly shift into lateral recumbency, displaying motor inhibition, behavioural quiescence, and reduced responsiveness to external stimuli for as long as the squeeze is maintained (See Figure 1). Removal of the squeeze results in a rapid return of posture and responsiveness (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022).

The rope procedure was initially proposed as a therapy for low vigour neonates (Aleman *et al.* 2017). Low vigour is characterised by a delay in the onset of, or an inability to express, survival-related behaviours soon after birth. Such behaviours include uncoordinated and coordinated locomotion, teat seeking behaviours and sucking colostrum and milk (Muns *et al.* 2016). Instead, these low vigour neonates exhibit an inability to stand or walk, no affinity for the dam, and no sucking reflex (Aleman *et al.* 2017). Applying the squeeze to these 'dummy' neonates for twenty minutes reportedly facilitates the onset of normal behaviours that enable the neonate to thrive after birth (Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021).

The mechanisms underlying responses to the thoracic squeeze are currently unknown. However, various hypotheses have been proposed, including fainting, a birth-related theory and tonic immobility.

**Figure 1.** An example of a neonatal lamb's response to the thoracic squeeze applied with a soft fabric rope looped three times around the chest. Needle electrodes are attached to the scalp to record brain activity (electroencephalogram) and to the flank to record heart rate (electrocardiogram).

## 2.3 Potential explanations for the phenomenon

### 2.3.1 Fainting

Fainting was initially explored as a potential mechanism underlying responses to a thoracic squeeze, due to the compressive action applied from the squeeze. Chest compression caused by a thoracic squeeze may decrease the effectiveness of breathing and oxygenation due to ribcage restriction. This may affect blood flow from the heart to the brain and the rest of the body, resulting in fainting occurring after application of the squeeze. However, there is no evidence to suggest that the mechanism underlying the response to a thoracic squeeze is a fainting event. Fainting, or syncope, is a short-term loss of posture and consciousness caused by decreased cerebral vascular perfusion (van Dijk 2003). Typical signs of fainting are a sudden change in cardiovascular and respiratory function (increased breathing rate, decreased arterial blood pressure and blood oxygen saturation, increased heart rate) that lead to reduced oxygen delivery to the brain (Hainsworth 2003; Wieling *et al.* 2009). While presentation of fainting appears similar to the reported responses to a thoracic squeeze, the physiological responses are markedly different. No changes in arterial oxygen saturation or significant instability of cardiorespiratory parameters have been reported in the literature (See (Gilman and Marcuse 1949; Gallup Jr 1974; Jones 1986b; Ong 1993; Humphreys and Ruxton 2018) for reviews on physiological changes during immobility responses). Furthermore, in the case of fainting, loss of posture usually occurs after physiological parameters become unstable and oxygen deficiency occurs (Hainsworth 2003; Wieling *et al.* 2009). In contrast, during the thoracic squeeze, loss of posture often happens more rapidly than physiological instability.

### 2.3.2 Birth-related theory

Another theory that has proposed a mechanism underlying the observed responses of neonatal mammals to thoracic squeezing centres around the birth process. Based on the temporal proximity of the response to the birth process, it has been proposed that there may be specific birth-related neuroinhibitory factors involved, such as those believed to keep the fetus unconscious and behaviourally quiescent before birth (Aleman *et al.* 2017; Mellor 2017). This theory is specific to neonates of mammalian species which have the neural capacities to support consciousness before birth (i.e. relatively precocial mammals like sheep, horses, cattle, pigs) (Mellor and Diesch 2006; Mellor and Diesch 2007; Mellor and Lentle 2015).

Before and during the birth process, a variety of factors actively suppress the onset of consciousness in utero in order to maintain motor and respiratory quiescence and reduce the energy costs of increased cognitive functions associated with consciousness (Mellor and Diesch 2006; Mellor 2017). These factors originate in the placenta and fetal brain and include the combined actions of a sleep-inducing agent (Adenosine), two neurosteroidal sedatives (Allopregnanolone and Pregnanolone), and a sleep-inducing hormone (Prostaglandin $D_2$) (Lee and Walker 2002; Hunter *et al.* 2003; Nguyen *et al.* 2003). Unconsciousness is also enforced by a placental peptide (Alvaro *et al.* 1993), as well as the buoyancy, warmth, and cushioned tactile stimulation of the uterine environment (Mellor and Gregory 2003). Adenosine is the most powerful inhibitor of consciousness (Hunter *et al.* 2003).

These inhibitors of consciousness are present in high levels during the last third of gestation and throughout labour (Mellor and Diesch 2006). They work by inducing a deep state of unconsciousness during labour (Hunter *et al.* 2003). The fetus then enters the birth canal through the cervix, where its head and chest are compressed. This compression is proposed to trigger a brain reflex that effectively inhibits movement and reinforces fetal unconsciousness in partnership with the neurochemical inhibitors (Mellor 2017). Furthermore, it has been suggested that this reflex pathway simultaneously prepares the fetal brain for the postnatal onset of consciousness once the compression is discontinued (Mellor 2017).

The onset of consciousness and thus survival-related behaviours after birth requires the coordination of multiple events. As the mammal is born and separation from the placenta occurs, combined with the onset of continuous rhythmic breathing, the inhibitors of consciousness decline, so that the fetal brain is no longer actively kept unconscious (Mellor 2017). This decline is thought to be facilitated by the cessation of thoracic compression once the fetus has passed through the birth canal (Mellor 2017). Simultaneously, this compression, combined with transient episodes of hypoxaemia during the second stage of labour, results in the rapid increase of factors that activate consciousness. This occurs by the activation of the locus coeruleus (Berridge and Waterhouse 2003). Locus coeruleus activity is responsible for the release of noradrenaline, which exerts excitatory effects on the fetal brain (Berridge and Waterhouse 2003). Another powerful neuroactive steroid, 17β-oestradiol, has excitatory effects on the fetal and newborn brain (McEwen 2002). Concentrations of both activating factors become progressively elevated during labour and thoracic compression, facilitating the transition to consciousness by preparing the brain to support an increase in behavioural arousal and the onset of continuous rhythmic breathing (Mellor and Gregory 2003).

It has been proposed that the thoracic squeeze mimics the compressive actions of the birth canal during the birth process (Mellor 2017). By redoing this compression artificially with a soft, fabric rope looped around the thorax, it has been suggested that the transition between consciousness and unconsciousness can be repeated (Mellor 2017). There is currently no direct evidence to support this theory. For example, no studies have investigated whether responses to thoracic squeezing become extinct in older animals, which would provide support for a specific birth-related mechanism.

### 2.3.3 Tonic Immobility

The responses observed after application of a thoracic squeeze appear to most closely resemble characteristics of Tonic Immobility. Tonic Immobility (TI) refers to a temporary and reversible state of motor inhibition and behavioural quiescence with associated physiological changes and a reduction in responsiveness to external stimuli (Gilman *et al.* 1950). It is considered an innate response exhibited by animals in threatening or stressful situations, and is triggered by physical contact, often in the form of restraint. TI has been observed in a variety of vertebrate and invertebrate species (Gallup 1977; Marx *et al.* 2008).

TI has been widely studied for its role in animal survival, as it is just one component of a series of physiological and behavioral responses that occur in animals when presented with a perceived threat to survival (Marx *et al.* 2008). These responses make up the 'defence cascade' and also include orienting, freeze, flight, and fight. Each phase is considered to invoke a strong physiological stress response, commonly associated with negative emotional experiences like fear and anxiety (Kozlowska *et al.* 2015). Based on the observed similarities between responses to a thoracic squeeze and TI, the remainder of the literature review will explore TI in detail, including the stimuli required to induce TI, manifestations, and the neural mechanisms responsible for eliciting TI as part of the defence cascade.

## 2.4 History of TI

A brief exploration of the history of tonic immobility terminology and evolutionary significance is particularly important for understanding what TI is and how it manifests. It provides a contextual understanding of the conditions for characterising a response as TI.

### 2.4.1 Tonic immobility terminology

Tonic immobility is currently recognized as the prevailing term to refer to observed immobility. However, it is important to highlight that throughout history, there have been more than 50 different terms employed to describe similar responses (Gilman and Marcuse 1949). The expansive terminology has led to confusion within the scientific literature, making it challenging to establish clear criteria for what constitutes tonic immobility and what falls outside its boundaries. There has been a lack of a standardized and universally accepted terminology, and this has significantly limited development of a unified understanding of this phenomenon (Ong 1993). Popular terms that have been maintained throughout the history of TI research include animal hypnosis, feigning death, thanatosis, immobility response and reflex immobility (Braid 1855; Holmes 1908; Crozier 1923; Bleich 1928; Gilman and Marcuse 1949; Gravitz and Gerton 1984).

Tonic immobility emerged as the most neutral terminology to describe the observed decrease in responsiveness exhibited by animals when subjected to sustained physical contact (Gallup Jr 1974). This term is believed to encompass the behavioural and physiological changes associated with reduced responsiveness, without making explicit assertions about the underlying mechanisms of the response (Crozier 1923).

Further clarity on the terminology of TI comes from understanding the stimuli required to induce the response, as well as how TI manifests in animals. These features of TI are discussed in sections 2.5 and 2.6.

### 2.4.2 Evolutionary significance of tonic immobility

TI has remained a conserved defensive strategy throughout the animal kingdom (Peinkhofer *et al.* 2021) and is considered central to the handling of stressful situations and encounters (Tsoukalas 2018). The function of TI has always been of interest to researchers, due to the numerous species reported to induce into TI. From an ultimate evolutionary perspective, exploring the adaptive significance of tonic immobility helps further understanding of why this response has persisted in so many species.

The idea that tonic immobility is a terminal defensive response to a predator has remained the most widely accepted explanation for the phenomenon. It is considered a protective mechanism with the aim of reducing potential tissue damage (Moskowitz 2004). TI was proposed to serve two main functions in predator-prey models.

First, as predators were thought to be triggered by a prey's movements, prey that remained immobile were less appealing to a predator, and more likely to be abandoned than those that fought to escape (Moskowitz 2004; Marx *et al.* 2008). In studies evaluating behaviours of predators in response to moving or unmoving (where TI was simulated) prey, predators consistently attacked moving prey and ignored unmoving prey (Carli and Farabollini 2022a). One commonly observed example is the behavior between cats and mice, in which mice are reported to "feign death" when caught by a cat (Gallup 1998). This state is characterized by the suppression of mobility and reflexes, reduced responsiveness, and behavioral quiescence. As a result, the cat loses interest in the seemingly dead mouse and moves away to other activities. Eventually, the mouse regains its reflexes and mobility and scurries away (Gallup 1998).

Second, TI is thought to be an adaptive survival strategy. This is particularly relevant in situations where threat intensity or predator presence in an environment is variable (Carli and Farabollini 2022a). For example, damselflies that were placed into a high predation area were more likely to utilize TI and for longer durations than damselflies placed into a low predation area (Gyssels and Stoks 2005). Similarly, larvae faced with reduced locomotion from removed lamellae (propulsion appendage) in the presence of a predator were more likely to utilize TI than larvae with intact lamellae (Gyssels and Stoks 2005). These studies suggest flexibility in the TI response based on environmental factors, threat intensity, and the survival value of TI (Gyssels and Stoks 2005; Carli and Farabollini 2022b).

Human literature on TI has more recently proposed the idea that TI enables humans and other animals to dissociate from traumatic situations and enter a calm, relaxed state while under extreme stress (Marx *et al.* 2008). The accompanying literature on the analgesic effects of TI and a reduced responsiveness to external stimuli suggests that this may be the case (See Section 2.2.3.4 for details), though further research is needed to evaluate links between the emotional aspects of TI and TI responses observed to date. In the context of animal studies, information on an animal's emotional experiences during tonic immobility is severely limited.

While the ultimate evolutionary role of tonic immobility remains uncertain, it is believed to have adaptive significance in the context of survival and predator avoidance (Carli and Farabollini 2022a). However, the evolutionary role of tonic immobility is a complex and ongoing area of study, and further research is needed to fully understand the specific selective pressures and ecological circumstances that have shaped this behaviour.

## 2.5 Stimuli to TI

### 2.5.1 Phases of tonic immobility

Knowledge of the phases of TI is essential for understanding the manifestations of TI based on the sequence of events that occur to induce and maintain the response. TI occurs in multiple distinct phases: Induction, maintenance, and recovery. The induction phase helps identify the specific factors or stimuli that elicit the response. The maintenance phase informs the physiological and neurobiological changes that occur to sustain TI. The recovery phase is important for understanding the conditions that can arouse animals out of TI. It is important to note that there is currently no information on the time course of each phase of TI. Because of the variety in durations of induction and maintenance phases, it is not known when the switch from induction to maintenance occurs beyond what is reported for behavioural and physiological changes.

Prior to the onset of TI, animals reportedly struggle for a short time before TI is induced and they become unresponsive (Ong 1993). This struggling involves attempts to escape from restraint, righting reflexes to avoid inversion, and in some cases, animals attempt to bite or scratch as a means of defence (Ong 1993). It has been suggested that struggling may be necessary for the induction of TI (See section 2.7 Neural mechanisms of TI) (Lefebvre and Sabourin 1977b). As physical contact with the animal is sustained, induction occurs, and the animal enters the maintenance phase of TI.

In the maintenance phase, several behavioural and physiological changes occur that reflect the switch from a fighting/struggling state to the immobile state of TI. This transition is visualised by sudden onset of immobility and reduced responsiveness that is maintained from minutes to hours until the response is ended by arousal (Ong 1993).

Recovery from TI is commonly preceded by a return of spinal reflexes, an increase in the number of vocalisations, leg, ear or tail twitches, head and neck movements or blinking, and then finally a return of righting reflexes which results in the animal moving onto its feet (Hoagland 1928b; Gunter and McCaughan 1959; Gallup *et al.* 1971b; Rovee and Luciano 1973; Gallup Jr 1974; Rovee and Kleinman 1974). Recovery can occur either through self-arousal or externally-induced arousal. Self-arousal refers to the animal suddenly righting onto their feet and attempting to immediately escape (Gallup Jr 1974). Externally-induced arousal refers to an animal that is either stimulated by purposeful environmental stimuli or is placed onto its feet by a researcher (Ong 1993).

More details about behavioural and physiological characteristics that occur during the induction, maintenance and recovery phases are outlined below in section 2.6. It is not possible to integrate characteristics within each phase of TI because studies exploring behavioural and physiological responses did not indicate when measurements were taken during the TI response. However, inferences about the phase of TI at the time of measurement will be made where possible.

### 2.5.2 Methods of induction

Before understanding how TI manifests during each phase, it is important to review the conditions for inducing animals into TI. According to (Lalonde and Strazielle 2022), TI is characterised by specific stimuli criteria that provide a baseline for induction into the state . TI requires a brief handling period in order to keep the animal still to apply restraint. In this context, restraint refers to the application of sustained physical contact, either by a hand or facilitatory stimulus (objects such as rope, cloth, troughs, sandbags etc). Lalonde and Strazielle (2022) note that restraint can be either preceded or proceeded by inversion, whereby the animal is forcefully placed onto its side or back. Because animal handling is a well-recognised technique, it will not be discussed in detail below.

### 2.5.2.1 Restraint

Restraint is a fundamental feature of inducing TI, primarily achieved through physical contact with or without additional pressure (Lalonde and Strazielle 2022). Restraint has also been observed in natural conditions such as those seen in predator-prey interactions, whereby capturing prey triggers their induction into TI (Sargeant and Eberhardt 1975). Restraint can be achieved through the use of apparatuses to maintain physical contact. For example, restraining chicks on their side was found to rapidly cause induction into TI (Gallup 1977). Another approach involved restraining animals in specially designed troughs, a method that has been extensively documented as effective for inducing TI in various species (see reviews (Gallup Jr 1974; Ong 1993)). Each species exhibits similar responses during restraint, such as immobility, fewer vocalisations, and reduced responsiveness to environmental stimuli.

Pressure additional to that caused by restraint is a very effective method for facilitating the induction TI in animals and involves sustained, weighted contact on body parts such as the head, neck, distal limbs or thoracic area (Gallup *et al.* 1971b). Pressure can be applied using a hand, as has been demonstrated to result in faster induction times in rats that were placed on their backs and restrained with a hand applying pressure to the thorax than rats that were induced using only restraint (Meyer 1990). Similarly, the application of pressure using weighted sandbags on the thoraxes of animals after inversion into dorsal recumbency in a v-shaped trough resulted in a longer TI maintenance phase (Klemm 1966b; Ong 1993). In rabbits, pressure applied to the tips of the ears after being placed in a trough with their ears hanging over the edge was found to trigger a faster induction into TI than when pressure was not used (Oakley and Plotkin 1977). In crickets, TI can be induced rapidly by stretching out the hindlegs and applying pressure to the knees (Crawford 1977). Casting cattle using ropes looped around the abdomen and hindquarters has been an effective form of restraint and immobility for husbandry practices (Ewbank and Parker 2007). Pulling on the ropes causes cattle collapse on their sides and remain immobile until the ropes are removed (Ewbank and Parker 2007).

The use of pressure-based stimuli to maintain physical contact without the need for handling has been demonstrated to effectively and rapidly induce TI (Ong 1993). The use of localized pressure has become a recurring theme in TI literature and has demonstrated that induction into TI does not require pressure in one specific area. This has important implications for understanding the manifestations and influences of TI, as it suggests that the specific location of the pressure-based stimulus may not be the critical factor for inducing tonic immobility. Instead, it implies that the overall presence of pressure or the sensation of pressure on the body may be the key trigger for initiating the immobility response.

### 2.5.2.2 Inversion

Inversion refers to a sudden, forced loss of posture by placing the animal onto its back or its side (Gallup *et al.* 1971b; Webster *et al.* 1981; Meyer 1990). However, these differences in inversion methods may be limited by physical characteristics of different species. An animal with a pointed or sloped back will roll onto its side if placed in the dorsal position, thus potentially triggering recovery of TI (Ong 1993). Consequently, apparatuses such as a v-shaped trough or sling are used to overcome these morphological limitations (Klemm 1966b; Reese *et al.* 1985). This method was adopted for piglets and rabbits that self-aroused from TI due to rolling onto their sides (Klemm 1966b). Restraining piglets or rabbits on their backs in a v-shaped trough is documented to facilitate a rapid induction into TI (Ong 1993; Erhard *et al.* 1999) and to increase TI duration (Klemm 1966b), though it sometimes requires manual pressure to keep an animal in that inverted position until TI induction occurs (Klemm 1966b).

## 2.6 Factors influencing tonic immobility

Morphology is just one factor that can influence the induction and maintenance of TI in animals. This section provides insight into the relationships between methods for induction factors that influence induction and maintenance, such as the testing environment (physical and social surroundings), features of the animal (individual and species characteristics) and the type of stimulus applied (nature, duration and intensity).

### 2.6.1 Features of environment

Certain environmental factors are reported to influence TI induction, such as changes in ambient temperatures, light, noise, novelty and isolation (Ong 1993). All of these environmental factors can attenuate TI, though some have demonstrated a potentiating effect on TI induction and maintenance (Gallup Jr and Williamson 1972; Rakshit and Klemm 1980).

For example, lower ambient temperatures have been shown to decrease the duration of the maintenance phase in lizards (Hoagland 1928a) and similar changes have been observed in frogs (Dabrowska and Manikowski 1982) and rabbits (Whishaw *et al.* 1979). This suggests that ambient temperatures play a stimulatory role in TI, with lower ambient temperatures stimulating more arousals than higher temperatures.

Likewise, research exploring the effect of light on TI responses have demonstrated a faster induction time and longer maintenance phase duration in animals tested in darker light conditions (Sobotik *et al.* 2020). Manipulation of light resulted in a shorter maintenance phase of TI in animals tested in brighter conditions (Sobotik *et al.* 2020). In support of this, chickens with dark hoods placed over their head induced faster and had longer maintenance phases of TI (Gallup Jr 1974; Jones *et al.* 1998). The practice of covering an animal's head with a hood to induce reduced responsiveness is used to prevent injury during handling and clinical procedures in a wide range of species, including birds and small and large mammals and is demonstrated to significantly speed up induction, whilst increasing the duration of the maintenance phase (Gallup Jr 1974; Jones *et al.* 1998).

Loud noises also appear to have a stimulatory effect on TI, compared to quiet auditory environments (Blackshaw *et al.* 1998; Erhard *et al.* 1999). A TI study comparing silence to white noise, traffic and background noise in a poultry house showed that a quiet auditory environment is necessary to maintain the immobility response in fowl (Jones 1986a). In contrast, loud noises before TI induction in squirrel monkeys resulted in an increased duration of the maintenance phase (Hennig 1978), suggesting that loud noises have differing influences when presented before TI induction as opposed to during the maintenance phase (Hennig 1978).

A number of studies have also explored the effects of potential stressful conditions such as novelty and social isolation on TI. These factors have been shown to invoke a cortisol response in numerous species (see (Boissy *et al.* 2007; Coutellier *et al.* 2007; Forkman *et al.* 2007; Veissier and Boissy 2007; Hazard *et al.* 2008) for reviews). For example, isolation of chicks in an unfamiliar environment resulted in longer durations of the maintenance phase with shorter induction periods (Rovee-Collier *et al.* 1991). The novel environment was considered to have a significant impact on TI induction and maintenance due to the presentation of a number of novel objects in an unfamiliar environment (Rovee *et al.* 1973; Rovee-Collier *et al.* 1991). Similarly, a study in chickens reported increased maintenance phase durations after periods of social isolation in an unfamiliar environment (Eddy and Gallup Jr 1990). However, the maintenance of TI is attenuated by the sound or sight of conspecifics nearby (Gallup *et al.* 1971b; Rakshit and Klemm 1980). Overall, a number of studies evaluating the effects of both social isolation and novel environments found that social isolation has a larger impact on TI responses out of the two, causing shorter induction times and longer maintenance phases (Ong 1993).

### 2.6.2 Features of animal

The susceptibility to and duration of TI can also differ based on animal-specific influences. To date, there have been no significant differences in TI responses found between different sexes.

#### 2.6.2.1 Species

TI has been studied in many different species and responses have been demonstrably different across different taxa (See Table 1). Different species vary in their susceptibility to induction of TI, as well as the duration of the maintenance phase and specifics of the response (Ong 1993). For example, crickets are highly susceptible to TI and are rapidly induced, but their maintenance phase is very brief (Crawford 1977). Rabbits are reportedly highly susceptible to TI induction, while in contrast, cats, dogs, mice and rats are considered to be harder to induce (Svorad 1957; Carli 1977). The reasons why are unknown (Ong 1993).

Differences have also been reported in closely related species (Ong 1993). When subjected to the same conditions for TI induction, closely related species of snakes and rodents showed increased susceptibility to induction and varied maintenance phase durations (Crawford 1977; Webster *et al.* 1981). Likewise, coyotes and red foxes showed varying degrees of susceptibility, with coyotes demonstrating fast induction and long maintenance durations compared to red foxes, despite the same testing conditions (Klemm 1971).

**Table 1** Animal species reported to exhibit TI responses. Adapted from (Ong 1993) and the following reviews: (Hoagland 1928b, 1928a; Gilman and Marcuse 1949; Gilman *et al.* 1950; Gunter and McCaughan 1959; Fraser 1960; Gallup *et al.* 1971a; Gallup Jr 1974; Gallup 1977; Lefebvre and Sabourin 1977b; Maser and Gallup 1977; Prestrude 1977; Gallup and Rager 1996; Erhard *et al.* 1999; Humphreys and Ruxton 2018).

| **Invertebrates** | **Birds** | **Mammals** | **Reptiles, Amphibians, Fish** |
|---|---|---|---|
| Ants | Buzzard | Cat | Anole |
| Beetle | Canary | Crow | Boas |
| Caterpillar | Chicken | Cattle | Chameleon |
| Cockroach | Crow | Coyote | Crocodile |
| Crab | Cuckoo | Deer | Frog |
| Cricket | Duck | Dog | Fish |
| Crayfish | Falcon | Elephant | Gecko |
| Lobster | Finch | Fox | Iguana |
| Mantid | Goose | Goat | Lizard |
| Octopus | Guinea fowl | Guinea pig | Newt |
| Pill Bug | Gull | Horse | Salamander |
| Shrimp | Hawk | Human | Shark |
| Spider | Heron | Lion | Snake |
| Scorpion | Owl | Mice | Toad |
| Wood louse | Oyster catcher | Monkey | Tortoise |
|  | Parrot | Opossum |  |
|  | Partridge | Pig |  |
|  | Peacock | Rabbit |  |
|  | Pigeon | Rat |  |
|  | Quail | Sheep |  |
|  | Robin | Squirrel |  |
|  | sparrow | Tiger |  |
|  | Swan |  |  |
|  | Turkey |  |  |
|  | Vulture |  |  |

#### 2.6.2.2 Age

Age appears to influence the susceptibility of animals to be induced into TI. In some species, the success of TI induction and maintenance apparently decrease with age, with younger animals showing longer TI durations than older animals (Gilman *et al.* 1950; Rovee and Luciano 1973; Prestrude 1977). For example, in one study, newborn kittens induced into TI showed longer maintenance phases than weaning age kittens (6-8 weeks), and adult cats showed a shorter maintenance phase duration compared to the younger kittens (Rovee-Collier *et al.* 1991). In contrast, TI appears to be difficult to induce in some species of birds less than two weeks old, with older birds exhibiting longer TI durations than young ones (Ratner and Thompson 1960; Rovee and Luciano 1973; Rovee and Kleinman 1974; Gallup and Rager 1996). It is important to note that age-related effects reported in TI literature occur in animals that do not have mature brain development at birth (i.e. altricial species). As such, further research is required to determine if developmentally mature mammals such as sheep, cattle, horses and pigs demonstrate age-related effects in the same way that less developmentally mature animals do.

### 2.6.3 Features of stimulus

The induction of TI is dependent on some form of restraint. The differences in morphology of different species means that induction of TI is influenced by the stimulus used to induce it.

#### 2.6.3.1 Duration of stimulus

A common feature in TI literature is the idea that TI induction is faster, and maintenance of the response longer, when the stimulus applied to induce TI is high in both duration and intensity. The duration and intensity of stimuli reportedly vary depending on species (Ong 1993). For example, 15 seconds was considered the standard restraint time for inducing TI in chickens (Gallup *et al.* 1971b), whereas Simonov and Paikin (1969) found that increasing the duration of restraint to 45 seconds, and later 60 seconds, in rabbits resulted in significantly longer TI maintenance phases. A study assessing induction and maintenance phase duration in fish reported that only 10 seconds of restraint was required to induce TI with demonstrated longer maintenance phases (Lefebvre and Sabourin 1977a), suggesting that there is significant variation in stimulus duration required for TI induction and maintenance.

#### 2.6.3.2 Repeated testing/habituation

Habituation refers to a decrease in the strength of a response to a stimulus that occurs as a result of repeated application of the stimulus (Nash and Gallup 1976). Repeated testing of TI in animals causes a decrease in susceptibility to induction into the immobility response (Prestrude 1977). In addition, when an animal is repeatedly induced into TI, the duration of immobility is significantly reduced, and the animal becomes harder to induce into TI (Ong 1993). The attenuated TI response due to habituation was shown to last up to two and a half months in chickens (Nash and Gallup 1976). In addition, a more consistent occurrence of habituation to repeated TI inductions was observed when the induction tests were distributed over an extended duration, as opposed to multiple tests per day over a shorter time period (Crawford 1977). Interestingly, habituation occurred faster when animals were left to self-arouse from TI compared to animals for which recovery was triggered prematurely by researchers (Nash and Gallup 1976).

In some species, sensitization to induction has been demonstrated by leaving shorter intervals between repeated testing (Prestrude 1977). In chickens, a minimum interval of 15 seconds between recovery of the last TI event and the next induction was required to avoid sensitization to induction and potentiation of the maintenance phase (Nash and Gallup 1976).

## 2.7 Manifestations of tonic immobility

There is considerable variation in the reported responses during TI and this is likely to reflect the times at which the key features of this phenomenon are measured.

### 2.7.1 Behavioural characteristics

Behavioural changes during TI are most commonly reported to involve a change in posture and a cessation of movement. The most noticeable change associated with the onset and maintenance of TI is the motionless posture assumed by animals, even after restraint is removed. This posture apparently varies depending on the species of animal and method of induction (Ong 1993). For example, birds inverted onto their backs without arrangement of their wings reportedly splay their wings out to the sides and extend their head and neck in an unusual posture, whereas they keep their wings folded and head and neck in a neutral position when placed in lateral or ventral recumbency (Ong 1993). In contrast, manually folding the wings of hawks that are inverted onto their backs has been demonstrated to cause a relaxed posture with talons closed and legs relaxed against the body (Crawford

1977). In rodents induced into TI, either by scruffing the nape of the neck and lifting them off their feet or by being restrained on their backs in a v-shaped trough, immobility with extended forelegs has been observed (Webster *et al.* 1981). In comparison, crustaceans and spiders reportedly become immobile with their appendages folded into their body rather than extended (Gunter and McCaughan 1959). The position of limbs in different species is also dependent on their muscle tone during this period of immobility.

During immobility, muscle tone reportedly decreases in a range of species studied, including chickens, quail, and sharks (Klemm 1971; Carli 1977; Lefebvre and Sabourin 1977b; Ong 1993). However, TI has traditionally been described as rigid immobility or paralysis because of the characteristic rigid muscle tone observed in many species (Hoagland 1928b; Gilman and Marcuse 1949; Gallup Jr 1974). For example, muscle tone has been found to increase in some animals, such as in rats (Ong 1993), rabbits (Carli 1969), birds (Gilman and Marcuse 1949) and cats (Lefebvre and Sabourin 1977b). It is now understood that muscle tone is heightened during the induction of TI and steadily decreases over the period of immobility, followed by a sudden increase in tone preceding recovery (Gentle *et al.* 1989). This suggests that the time of testing of muscle tone during immobility is important and may explain variation in results.

A lack of overall movement is characteristic of TI, but a range of small movements have been reported to occur in some animals (Gallup and Rager 1996). These include slight head movements (Gentle *et al.* 1989), ear movements (Ong 1993) and muscle tremors (Gallup Jr 1974; Crawford 1977; Webster *et al.* 1981; Gentle *et al.* 1989; Gallup and Rager 1996), often in response to external stimuli. Periods of eye closure during TI have been observed in a range of animals (Gallup *et al.* 1971b; Rovee and Kleinman 1974; Campbell *et al.* 2019; Sakaguchi and Ishikawa 2019; Sobotik *et al.* 2020). However, eyes tend to remain open during the maintenance phase, with bouts of eye movements and blinking being reported (Hoagland 1928b; Rovee and Kleinman 1974; Webster *et al.* 1981; Gentle *et al.* 1989).

### 2.7.2 Physiological Characteristics

Several physiological changes reportedly occur during the onset and maintenance of TI, including cardiorespiratory activity, electroencephalographic activity, neural reflexes and hormone status. These changes appear to follow the transitions between phases of TI. The induction of TI is associated with an initial increase in heart rate, respiratory rate and blood pressure followed by a steady decline as the immobility response is maintained (Gentle *et al.* 1989; Overeem *et al.* 2002; Valance *et al.* 2008; Giannico *et al.* 2014). However, some studies found different responses, such as an initial decrease in respiratory rate in rabbits followed by a further decrease as TI continues (Klemm 1966a), while others found no change in respiration at induction or during TI in chickens (Gilman *et al.* 1950). Similarly, Gilman et al. (1950) also reported no changes for heart rate during TI (Gilman *et al.* 1950). In contrast, Reese et al. (1982) found a decrease in heart rate. As for behaviours, the changes reported may be due to cardiorespiratory variables being measured at a different time point during TI (Ong 1993).

Electroencephalographic (EEG) changes have been characterised in TI literature. Earlier reports described EEG patterns during TI being akin to brain activity during sleep (i.e. high voltage, slow wave activity representative of synchronous EEG patterns) (Svorad 1957). However, further investigation suggested that TI causes a desynchronisation (low voltage, fast activity) of the EEG (Ong 1993), a pattern that commonly occurs during awake states and is representative of alertness and hypervigilance. In support of this, more recent literature has characterised EEG obtained during the induction phase of TI in opossums and rabbits as low voltage fast activity (Barratt 1965; Vanderwolf *et al.* 1975). In contrast, in the maintenance phase, the occurrence of high voltage, slow wave patterns have been observed in a number of species (Schwarz and Bickford 1956; Kumazawa 1963; Lefebvre and Sabourin 1977b). This EEG pattern is thought to reflect a reduced state of awareness (Ong 1993). EEG activity then moves towards awake states characterised by low voltage fast activity in the seconds preceding recovery of TI (Ong 1993). Interestingly, during the maintenance phase, EEG characteristic of reduced states of awareness has been reportedly punctuated by periods of low voltage, fast activity consistent with awake states, and may reflect bouts of arousal in animals before TI is resumed (Ong 1993). It appears that, like other physiological parameters, the time of measurement plays a role in what presents on the EEG.

Reflexes and responsiveness to environmental stimuli are consistently reported to be reduced during TI. However, it is possible that some species variation exists, or that variation occurs that reflects a difference in the time of testing (Ong 1993). There appears to be consensus that during the maintenance phase, an absence of righting reflexes occurs alongside a decrease in overall responsiveness. In support of this, most studies have reported a decrease in spinal reflex responses during TI (Crawford 1977; Carli *et al.* 1984; Porro and Carli 1988; Gentle *et al.* 1989; Overeem *et al.* 2002). However, eye reflexes are often present if the animal has open eyes during TI (Ong 1993). The decreased reflexes are suggestive of a reduced responsiveness to environmental stimuli.

Marked increases in circulating concentrations of hormones linked to stress and emotional arousal, such as cortisol, corticosterone and adrenaline have been reported in TI literature. These increases above pre-restraint concentrations are reported to begin during the induction phase (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011), with concentrations peaking and then steadily declining throughout the maintenance phase (Braud and Ginsburg 1973; Campo and Davila 2002; Jones *et al.* 2005; Brooks *et al.* 2011; Baker *et al.* 2019). The time course of cortisol responses across the different phases of TI has not been studied, though the initial increase during induction is suggestive of a cortisol response related to the restraint and handling of animals prior to TI induction. As such, it is thought that hypothalamic-pituitary-adrenal (HPA) activation, resulting in cortisol release, is necessary for the induction but not the maintenance of TI (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011). However, further research is required to tease out the effect of the induction phase vs maintenance of TI on cortisol concentration, and whether these two phases can be differentiated by changes in cortisol concentration.

Likewise, one study demonstrated an activation of the sympathomedullary pathway (SAM) responsible for catecholamine-based stress responses during TI. Injection of adrenaline before the onset of TI was found to speed up induction and increase the maintenance phase of the immobility response, whereas injection during the maintenance phase was reported to increase the duration of immobility in chickens (Braud and Ginsburg 1973). This suggests that catecholamines like adrenaline play a facilitatory role in the induction and maintenance of TI and may provide insight into the neural mechanisms underlying the TI response (See section 2.3).

Reduced responsiveness is also evidenced by changes in nociceptive thresholds during the maintenance phase of TI. Antinociception is thought to occur at the onset of TI and continue throughout the maintenance phase, before behavioural and electrophysiological nociceptive responses return in the seconds preceding recovery (Baker *et al.* 2019). Repeated application of a noxious stimulus during the induction phase of TI resulted in a decrease in behavioural and electroencephalographic nociceptive responses in rabbits (Porro and Carli 1988), suggesting that both TI and pain transmission share similar mechanistic pathways (Baker *et al.* 2019). Nociceptive inhibition during TI has also been evidenced in chickens, rabbits, rats and guinea pigs, and other vertebrate species (Wallnau and Gallup Jr 1977; Porro and Carli 1988; de Oliveira *et al.* 1997; da Silva and Menescal-de-Oliveira 2006; Baker *et al.* 2019), suggesting that TI has an antinociceptive effect on animals that persists for the duration of the maintenance phase.

## 2.8 Neural mechanisms of TI

The behavioural and physiological changes that occur during TI are governed by specific neural pathways as part of a suite of responses called a ‘defence cascade’. This cascade represents a sequential progression of defensive responses by an animal to increasing proximity of a stressor (Marx *et al.* 2008). There are three levels to the cascade, beginning with an orienting response arising from sensory detection of a potential threat to the health of the animal. If the orienting response causes the threat to be considered serious rather than a false alarm, then flight, freeze and fight responses are elicited. TI is regarded as a terminal response within the defense cascade (Figure 2). This implies that when physical contact occurs, TI is elicited as a final attempt for survival (Marx et al., 2008).

It is important to mention that while responses in the defence cascade are considered to shift from orienting response, down to TI based on proximity of a threat, responses are mutually exclusive and can occur in no specific order. Rapid switches between defensive behaviours are done through active suppression of neural activity responsible for all other pathways while active stimulation of the target pathways occurs to elicit the desired response (Marx *et al.* 2008; Roelofs 2017; Terpou *et al.* 2019). Therefore, an animal can rapidly switch between any behaviour in the defence cascade depending on the circumstance. However, for the purpose of this review, behaviours have been labeled and described numerically (1, 2, 3) for ease of understanding.

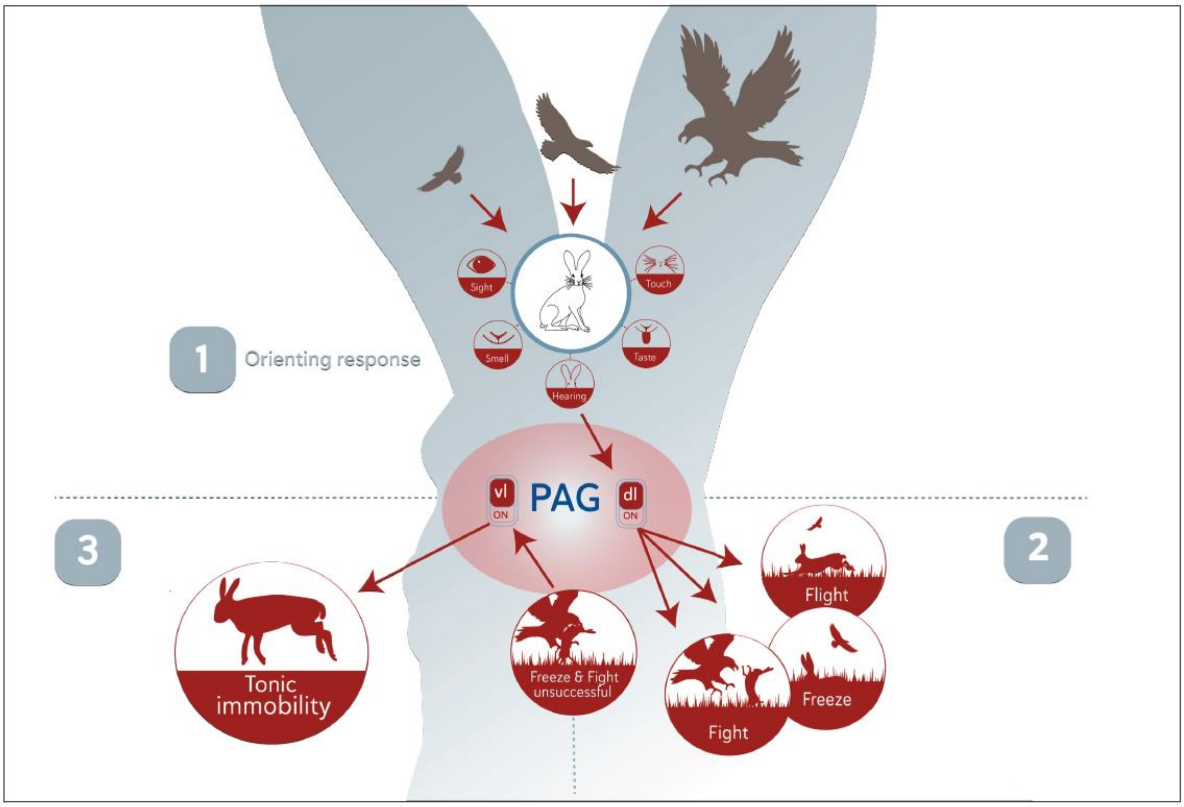


**Figure 2.** The defence cascade leading to the expression of tonic immobility. Based on the spatial proximity of a threat, different areas of the periaqueductal grey (PAG) are activated or suppressed. Flight, Freeze and Fight behaviours are facilitated by dlPAG (dorsolateral periaqueductal grey) activation, while vlPAG (ventrolateral periaqueductal grey) activation triggers a shift into tonic immobility.

The complete neural pathway that governs the defence cascade and leads to TI combines autonomic processes (sympathetic and parasympathetic nervous system activity), stress pathway responses (Hypothalamic-pituitary-adrenal axis), modulation of nociceptive information, and behavioural changes to provide the best outcome to the animal when facing a threat (Brandão *et al.* 2008). The main points of control by which each of these responses occur are the brainstem and spinal cord (See Figure 3) (Roelofs 2017).

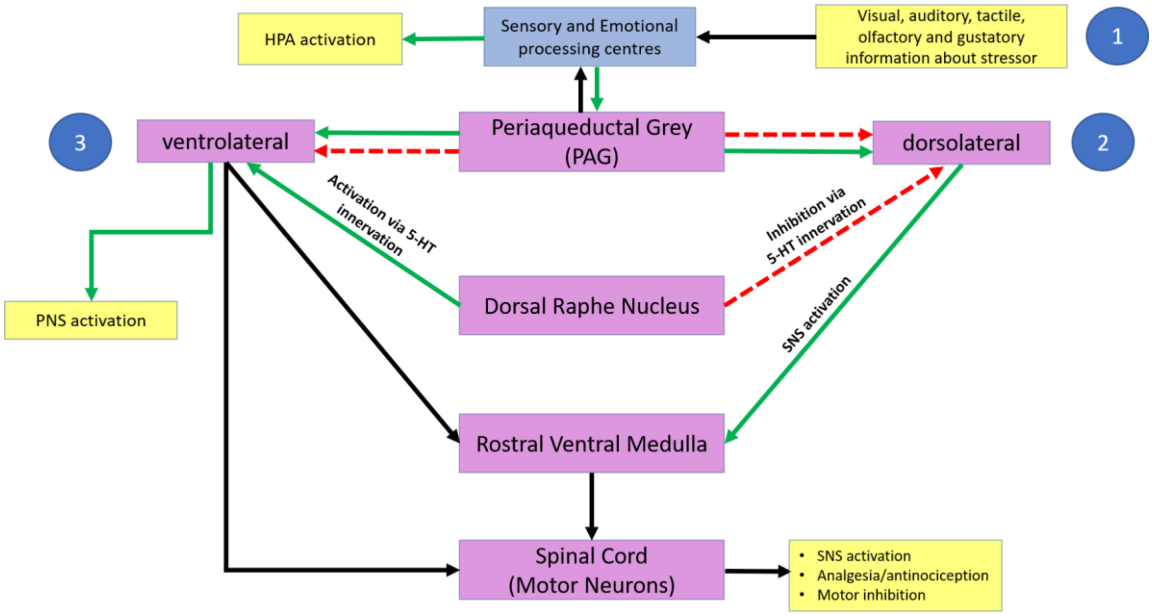


**Figure 3.** Neural pathways involved in all three responses of the defence cascade after detection of a stressor, beginning with the orienting response (1). Activation of the primary structures (purple) result in the sequential progression of flight, freeze and fight activation (2). If these behaviours are unsuccessful at removing the animal from the stressor, then tonic immobility occurs (3). Sensory input pathways (yellow) connect to brain systems (blue), with behavioural and/or physiological outputs shown in yellow. Direct projections between brain structures are shown in black. HPA = hypothalamic-pituitary-adrenal axis; SNS = Sympathetic nervous system; PNS = Parasympathetic nervous system.

### 2.8.1 Orienting response

The orienting response is activated following detection of a potential threat in the environment. It enables animals to rapidly gather information about the surroundings and prioritise responses that are most beneficial to survival (Figure 4) (Terpou *et al.* 2019). This is achieved via sensory integration of visual, auditory, olfactory, tactile or gustatory cues. If assessment of the stressor finds it to be a serious threat to the animal, then immediate progression into the next level of the defence cascade occurs (Terpou *et al.* 2019).

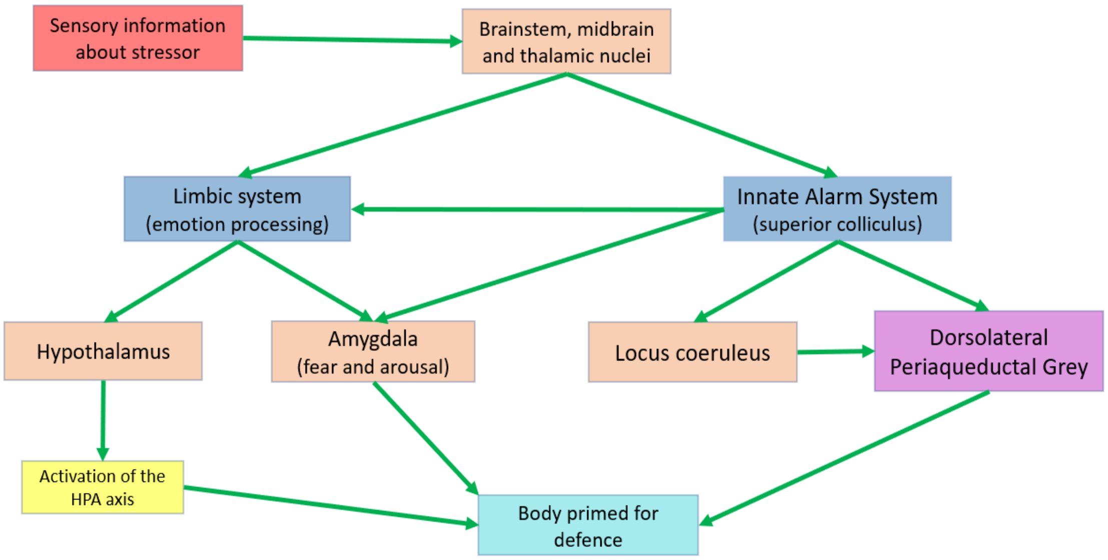


**Figure 4** Neural pathways involved in the orienting response part of the defence cascade after detection of a stressor. Sensory input pathways (red) connect to brain areas (orange), or brain systems (blue) involved in the orienting response. While the hypothalamus and amygdala are part of the limbic system, their roles in the defence cascade are specific compared to the role of the whole limbic system, and as such have been presented separately. Primary structures involved in the defence cascade are shown in purple, with behavioural and/or physiological outputs shown in yellow. Projections between brain structures play either an activatory (green arrows) or inhibitory (red arrows) role to result in the behavioural and physiological priming of the body for defence (turquoise). HPA = hypothalamic-pituitary-adrenal axis.

During the orienting phase, information about the environment is collected and transmitted to the emotion and arousal processing centres of the brain via an array of interconnected brainstem, midbrain and thalamic nuclei (Brandão *et al.* 2008) (Figure 4). This process doesn't require involvement of the cerebral cortex which allows information to be processed reflexively and almost instantaneously (Terpou *et al.* 2019).

Simultaneously with information processing, activation of an innate alarm system occurs. This system involves the superior colliculus of the brainstem which has direct projections to the locus coeruleus, amygdala and dorsolateral area of the periaqueductal grey (PAG) (Terpou *et al.* 2019). The locus coeruleus contains noradrenergic fibres that also stimulate the dorsolateral PAG, while projections to the amygdala help specify whether the stressor should be taken seriously (Brandão *et al.* 2008). Stimulation of this system indirectly triggers activation of the hypothalamic-pituitary-adrenal axis (HPA) via the limbic system, which causes a release of stress hormones (e.g glucocorticoids such as cortisol) that act on organs and muscles to effect a cascade of physiological changes (Terpou *et al.* 2019). Depending on the type of threat and its proximity to the animal, this orientation response manifests into a flight, freeze or fight response.

### 2.8.2 Flight, Freeze, Fight responses

Flight, freeze and fight responses are considered the second stage of the defence cascade, and are characterised by a heightened state of arousal with associated physiological and motor responses aimed at enabling the animal to escape predation (Terpou *et al.* 2019).

Based on proximity to the threat, one or all of flight, freeze and fight responses occur sequentially. When the threat is far away, the animal is more likely to initiate a flight/escape response (Marx *et al.* 2008). As distance from the threat decreases, the animal initiates a freeze response. Freezing refers to a brief absence of coordinated movement that is rapidly reversible (Marx *et al.* 2008). Breathing, blinking, head movements, vocalisations and tense body posture reportedly occur throughout the duration of a freeze response with an associated increase in responsiveness to environmental stimuli (Roelofs 2017; Terpou *et al.* 2019). When escape or freeze behaviours are not possible due to the proximity of the threat, a rapid shift to fighting or struggling with the threat occurs, with physical contact activating this shift in defensive behaviour (Marx *et al.* 2008). If the fight or struggle is successful, the animal will shift backwards in the defence cascade towards flight responses.

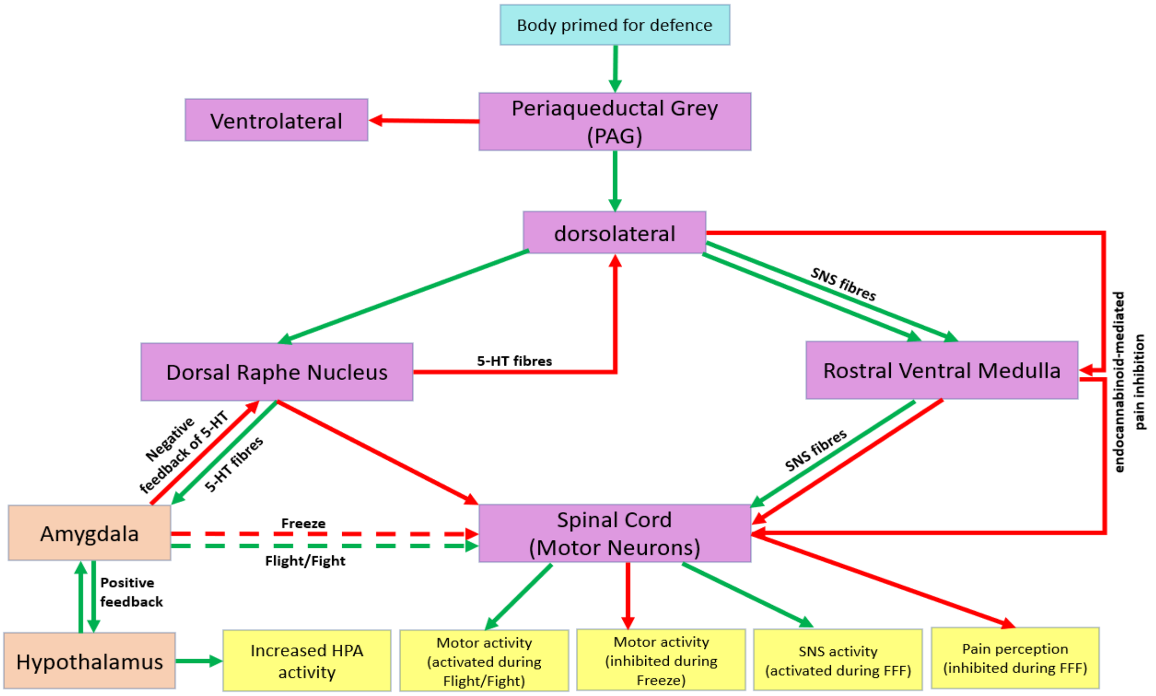


**Figure 5.** Neural pathway of the flight, freeze or fight responses that occur after the orienting response. Activation of the periaqueductal grey (purple) results in either activation (green arrows) or inhibition (red arrows) of other primary brain structures and brain areas (orange). The two branches of the periaqueductal grey (ventrolateral and dorsolateral) are mutually opposing. Behavioural and physiological outputs are shown in yellow. Some pathways denote both activatory and inhibitory actions, based on whether the defensive response is Flight/Fight (green arrows) or Freeze (red arrows). Dashed arrows refer to connections that are known, but the mode of the connection is currently unknown. HPA = hypothalamic-pituitary-adrenal axis; 5-HT = serotonin; SNS = sympathetic nervous system.

However, if this is not possible and the physical contact is sustained, then the animal will shift into the terminal level of the defence cascade, TI.

The neural pathways governing this suite of responses begins in the PAG (See Figure 5). The PAG is responsible for coordinating multiple processes (Lovick 1993). Most notably, activation of different branches of the autonomic system in conjunction with analgesia/antinociception, vocalisations and defensive responses is achieved via the opposing actions of the PAG's subunits, the ventrolateral (vl) and dorsolateral (dl) areas (Behbehani 1995; Brandão *et al.* 2008). The dlPAG is thought to coordinate active defence responses like flight, freeze and fight, while the vlPAG coordinates passive defence responses like TI (Carrive 1993; Behbehani 1995; Brandão *et al.* 2008; Roelofs 2017).

Neural activity during the orienting response triggers an increase of activity in the dlPAG. This simultaneously inhibits activity in the vlPAG, consequently inhibiting the onset of TI (Vieira *et al.* 2011; Vieira-Rasteli *et al.* 2018). The dlPAG projects to the rostral ventral medulla in the midbrain, where projections to the preganglionic sympathetic neurons in the spinal cord activate the sympathetic nervous system (Lovick 1993). Activation of the sympathetic nervous system initiates the flight, freeze, fight response and is involved in redirecting blood flow to organs responsible for fighting or escaping (Brandão *et al.* 2008). This activation is reflected by increased heart rate, blood pressure and breathing rate and muscle tone (Brandão *et al.* 2008). Alongside autonomic changes, activity of the HPA axis is maintained and cortisol is continuously released to promote stress responses (i.e. energy provision and immune system modulation) (Roelofs 2017). Hormones of the HPA pathway also interact with the central nucleus of the amygdala to coordinate fear responses and potentiate HPA activity via direct projections to the hypothalamus (Spinieli and Leite-Panissi 2018). Corticotropin-releasing factor (CRF) from the hypothalamus has been implicated in the experience of the freeze response, with CRF acting on the amygdala to inhibit the motor pathway and potentiate emotional arousal associated with the freeze response, resulting in longer immobility durations and increased anxiety and fear (Donatti and Leite-Panissi 2011; Spinieli and Leite-Panissi 2018).

In order to induce the freeze response instead flight or fight, the dlPAG must transmit information to the motor neurons of the spinal cord to inhibit activity and induce immobility. However, the dlPAG does not have direct projections to the motor neurons of the spinal cord (Roelofs 2017). Instead, it inhibits motor output indirectly, via two other areas that have direct projections to the spinal cord. The dorsal raphe nucleus sits in front of the PAG in the midbrain and projects to the intermediolateral column of the spinal cord, while the rostral ventral medulla is located caudally in the brainstem and projects directly to the motor neurons of the spinal cord (Roelofs 2017). Combined, projections from both areas inhibit motor neurons to induce a freeze response that is rapidly reversible (Roelofs 2017). Furthermore, the dorsal raphe nucleus projects to the amygdala with serotonin mediated fibres, which act on the emotion processing centres to increase anxiety and fear based responses (Leite-Panissi *et al.* 2006). In particular, increased serotonin concentration in the basolateral part of the amygdala promotes flight and fight responses and inhibits immobility associated with the freeze response (Leite-Panissi *et al.* 2006). While serotonin concentrations in the basolateral nucleus are reaching certain levels, a negative feedback pathway projects back to the dorsal raphe nucleus to slow serotonin release (Hicks *et al.* 1975). Interestingly, serotonin release from the dorsal raphe nucleus also acts as a threshold for the switch between flight, freeze and flight responses and TI (Figure 6) (Wallnau and Gallup Jr 1977; Farkas *et al.* 1997).

The neural pathway active during the freeze response is also responsible for activation of a non-opioid, endocannabinoid-mediated analgesic pathway (Terpou *et al.* 2019). Endocannabinoids play an important role in modulating biological stress, by inhibiting neurotransmitter release associated with stress and pain (Baker *et al.* 2019). This pathway is involved in pain modulation and works by inhibiting ascending nociceptive transmission from the spinal cord to the brain (Terpou *et al.* 2019). It does this via projections from the dlPAG to the rostral ventral medulla, where these projections then descend to nociceptive relay neurons in the spinal cord to induce a pain-relieving state (Baker *et al.* 2019).

While freezing and TI appear to have similar behavioural characteristics, the neural mechanisms underlying both support that the two phenomena are quantitatively different and activate different neural pathways.

### 2.8.3 Tonic immobility

The shift from fighting/struggling to TI is triggered by sustained physical contact (Marx *et al.* 2008). It is thought that intense emotional arousal associated with the sustained physical contact causes a neural threshold to be reached that physiologically shifts the animal from a freeze or fight response into TI, an event that occurs almost instantaneously and can be reversed just as rapidly (Terpou *et al.* 2019). This transition into TI is supported by a physiological shift that brings the animal from an alert, tense and active state into a quiet, less responsive state (Terpou *et al.* 2019).

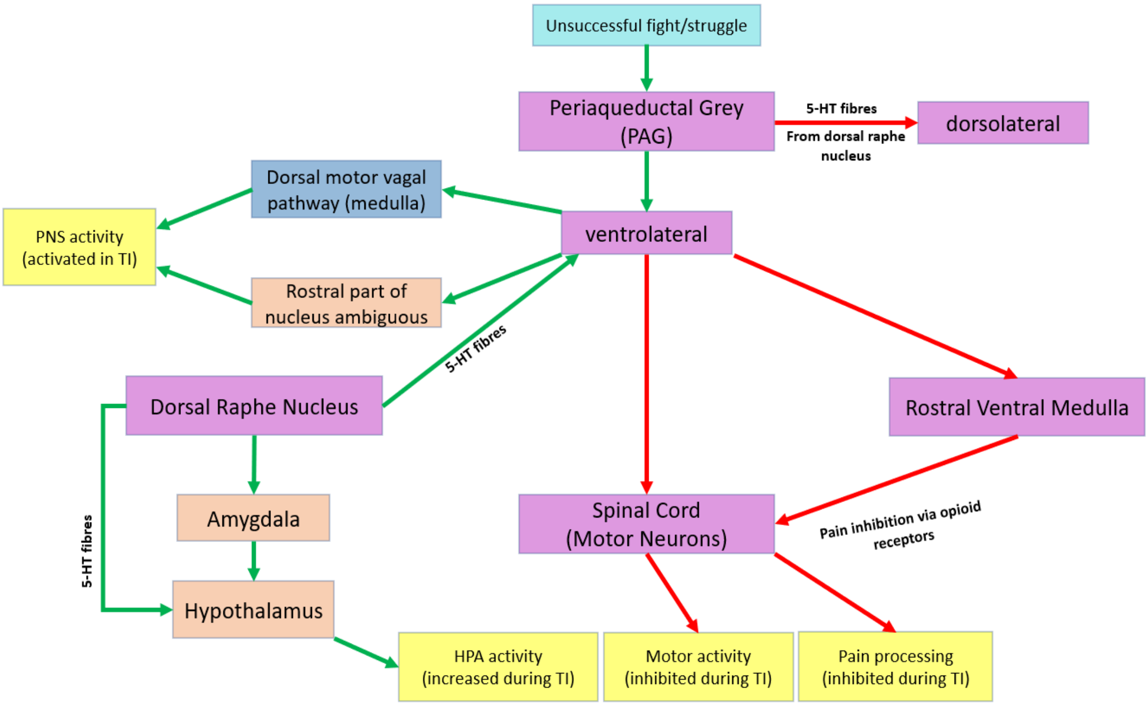


**Figure 6.** Sustained physical contact resulting from an unsuccessful flight, freeze or fight response causes activity in the periaqueductal grey to switch from dorsolateral activity (red arrow) to ventrolateral activity (green arrow). The link between sustained physical contact and serotonin threshold activity from the dorsal raphe nucleus is not yet known. The result is direct inhibition (red arrows) from the periaqueductal grey to primary brain structures (purple) that trigger induction into tonic immobility. Activation (green arrows) of other primary structures (purple), brain areas (orange) and brain systems (blue) are responsible for behavioural and physiological outputs (yellow) that are responsible for the maintenance phase of tonic immobility. HPA = Hypothalamic-pituitary-adrenal axis; PNS = parasympathetic nervous system; 5-HT = serotonin.

As serotonin levels in the dorsal raphe nucleus increase, serotonin-mediated projections from the dorsal raphe nucleus act on the dlPAG to inhibit its activity and to the vlPAG to stimulate activity (Ferreira and Menescal-de-Oliveira 2009). The vlPAG has direct projections to the motor neurons of the spinal cord that, when activated, potently inhibit motor output and righting reflexes to bring about the induction phase of TI (Vieira *et al.* 2011; Vieira-Rasteli *et al.* 2018). Simultaneously, the vlPAG acts on a range of brainstem structures to activate the parasympathetic nervous system (Terpou *et al.* 2019). It does this via direct projections to the rostral part of the nucleus ambiguous and the dorsal motor vagal pathway in the medulla (Farkas *et al.* 1997). The nucleus ambiguous is a collection of motor neurons that contain preganglionic parasympathetic neurons (Farkas *et al.* 1997). Preganglionic parasympathetic neurons are also found in the vagal pathway of the dorsal motor nucleus (Roelofs 2017). Activation of these areas stimulates the parasympathetic nervous system to cause a reduction in heart rate, blood pressure and breathing rate, as well as relaxation of muscle tone (Roelofs 2017), changes that are characteristic of the maintenance phase of TI.

Serotonin has been demonstrated to directly affect HPA activity and circulating cortisol levels during TI. Serotonin release from the dorsal raphe nucleus can increase HPA activity via projections to the hypothalamus and thus potentiate the stress response activated during the orienting response (Yehuda and Meyer 1984; Weidenfeld and Ovadia 2017). Furthermore, serotonin acts on the amygdala to further potentiate HPA activity via direct projections to the hypothalamus (Yehuda and Meyer 1984; Weidenfeld and Ovadia 2017). Thus, it appears that serotonin plays an activatory role in TI whilst also potentiating the stress response caused by activation of the HPA axis.

The vlPAG also plays an important role in nociceptive modulation (Baker *et al.* 2019). It does this via projections to the rostral ventral medulla where opioid-mediated fibres innervate the spinal cord and inhibit transmission of ascending nociceptive signals (Terpou *et al.* 2019). Endogenous opioids acts as pain relievers and are also associated with emotional regulation by reducing emotional arousal caused by activation of the defence cascade (Ong 1993). This also acts to ensure the animal remains immobile during TI despite noxious inputs due to potential tissue damage from the sustained physical contact (Terpou *et al.* 2019).

A thorough understanding of the neural mechanisms underlying TI enables the identification of specific criteria responsible for the classification of TI. From there, these criteria can be used as a framework for evaluating whether a technique such as the thoracic squeeze can be characterised as a stimulus of TI.

## 2.9 Discussion

### 2.9.1 Exploring the similarities between responses to the thoracic squeeze and tonic immobility

TI is a phenomenon that is elicited in a broad range of species, with similarly presenting characteristics. In addition, the induction and maintenance phases of TI are strongly influenced by a range of factors, with particular emphasis on the stimuli used to induce TI. The use of pressure to maintain physical contact and therefore induce animals into TI has been a popular method for restraint in animals. There is a likelihood that such techniques may inadvertently induce TI, and it raises the possibility that there may be instances of immobility described in the literature, that utilise TI as an underlying mechanism. In particular, the behavioural and physiological similarities reported between responses of neonates to thoracic squeeze and TI suggest that thoracic squeezing may be a manifestation of TI.

Building upon the existing knowledge of tonic immobility, it is worth exploring the potential link between the well-known manifestations of TI and the observed behavioural and physiological responses to a thoracic squeeze. The thoracic squeeze, as a proposed stimulus of TI, involves the application of pressure to the thorax to maintain physical contact. To date, characterisation of the thoracic squeeze has focused on neonatal mammals from several precocial species, namely horses (Toth *et al.* 2012; Aleman *et al.* 2017), cattle (Stilwell *et al.* 2019; Holdsworth *et al.* 2022), sheep (Flora *et al.* 2021) and pigs (Holdsworth *et al.* 2021), all of which demonstrated a generalised reduced responsiveness. By examining responses to a thoracic squeeze, we can assess whether it elicits similar behavioural and physiological changes to those observed during tonic immobility. The current, known responses to a thoracic squeeze are summarised below.

#### 2.9.1.1 Features of the thoracic squeeze technique

##### *2.9.1.1.1 Behavioural*

In previous research by the author (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022), the most prominent behavioural change after the squeeze was applied was an almost immediate loss of posture (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). All piglets and calves studied thus far collapsed onto their sides (lateral recumbency) with posture similar to animals in a ‘sleep-like state’ during the squeeze (Toth *et al.* 2012). Furthermore, induction appeared to follow the same sequential pattern as phases of TI, where a period of fighting and struggling occurred before the reduced responsive state commenced

(Holdsworth *et al.* 2021). During application of the thoracic squeeze and for a short period thereafter, all piglets and calves exhibited struggle behaviours like kicking, jumping, vocalisations and vigorous head and limb movements (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022), before a loss of posture suddenly occurred and the reduced responsiveness commenced.

Muscle tone was reduced in animals after application of the thoracic squeeze, though it followed a similar pattern to TI whereby muscle tone was reduced for the duration of immobility but increased before recovery from the squeeze (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Furthermore, transient changes in muscle tone during the squeeze appeared to coincide with arousals (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). These arousals were characterised by bouts of kicking, head movements, vocalisations, and righting reflexes in which animals attempted to move into sternal recumbency or attempted to stand. In both piglets and calves, self-arousal from the thoracic squeeze occurred in about half of all animals while the squeeze was still applied, with the other half having the squeeze ended by the experimenter after 10 minutes (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022).

In all foals, piglets and calves squeezed, a lack of head, neck and limb movements were maintained for the entire squeeze duration, punctuated by bouts of arousals (Toth *et al.* 2012; Aleman *et al.* 2017; Stilwell *et al.* 2019; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). In some studies, eyes reportedly remained closed for the entire squeeze duration in all animals (Toth *et al.* 2012; Aleman *et al.* 2017). However, Holdsworth *et al.* (2022 reported minimal or no eye closure in all calves squeezed. Although it is unknown why eye closure did not occur in these one-day old calves, open eye behaviour did appear to follow the typical behavioural patterns consistent with TI.

#### *2.9.1.1.2 Physiological*

A range of physiological changes have been reported to occur in all animals subjected to a thoracic squeeze. In most of the piglets and calves studied, cardiorespiratory levels such as heart rate, breathing rate and blood oxygen levels did not change during the 10 minute squeeze application (Holdsworth *et al.* 2021). However, these parameters were only measured intermittently throughout the squeeze duration. In healthy foals, cardiorespiratory rates reportedly decreased steadily throughout the maintenance period (Toth *et al.* 2012), following a similar pattern to parameters measured during TI.

EEG activity during the thoracic squeeze has only been explored previously in two healthy foals (Toth *et al.* 2012). Toth et al. found distinctive pattern changes from a desynchronised wave pattern to a synchronisation of the EEG throughout the maintenance phase. The presence of K-complexes and spindles, characteristic of sleep, were also briefly observed during the maintenance phase, before the EEG transformed into high voltage, slow-wave electrical activity characteristic of reduced responsive states for the rest of the maintenance period (Toth *et al.* 2012). However, further research is needed to explore changes in EEG activity at different phases of the thoracic squeeze, with more detailed characterisation needed to determine the brain state animals go into during the squeeze.

Reflexes tested during the thoracic squeeze reportedly vary considerably between species. Reflexes are commonly used to assess the depth of unconsciousness in animals under general anaesthesia and have more recently been explored in animals after stunning for slaughter (Verhoeven *et al.* 2015). A pedal reflex assesses the depth of unconsciousness at the spinal cord level, whereas a palpebral reflex assesses the depth of unconsciousness at the brainstem level (Verhoeven *et al.* 2015). The absence of pedal and palpebral reflexes in animals under general anaesthesia have been reported as indicative of unconsciousness (Verhoeven *et al.* 2015; Bradbury and Clutton 2016). In most piglets, both spinal and cranial reflexes remained reduced or absent for the duration of the squeeze (Holdsworth *et al.* 2021). However, in calves, cranial reflexes remained present in nearly all calves tested, while spinal reflexes were reduced but rarely absent for the duration of the squeeze (Holdsworth *et al.* 2022). Interestingly, spinal reflexes in both species returned before self-arousal in individuals that did so, and some animals that had the squeeze ended by the experimenter also showed reflexes before arousal (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). These results suggest that animals with absent spinal and cranial reflexes were potentially less responsive to external stimuli than those with reflexes present reflexes.

A single study reported significant increases in HPA activity, reflected in circulating adrenocorticotropic hormone (ACTH) levels, in healthy foals before application of the thoracic squeeze and during maintenance of a reduced responsive state (Toth *et al.* 2012). This suggests that stress may be necessary for induction during thoracic squeezing in a similar manner to induction of TI. However, this HPA activity was not well characterised in this paper due to the small sample size (8 foals) and the frequency of assessment during and after the squeeze (Toth *et al.* 2012). Further research is required to determine whether a cortisol response is a prerequisite for the induction phase of the thoracic squeeze, as well as to characterise how cortisol concentrations change throughout each phase (induction, maintenance, recovery) of the squeeze.

Behavioural and electrophysiological responses to nociceptive input during the squeeze have not been well studied. The responsiveness of healthy foals to an electric shock applied to parts of the tail was measured during the maintenance phase (Toth *et al.* 2012). They found that nociceptive thresholds during the squeeze increased compared to thresholds before the squeeze, suggesting that the thoracic squeeze may inhibit nociception. However, this is another area of thoracic squeeze research that needs further investigation, in order to characterise pain responses during the squeeze in a more detailed way. In particular, there is a need to explore how pain responses change during each phase of the thoracic squeeze.

## 2.10 Future research

The numerous similarities in behavioural and physiological responses between the thoracic squeeze technique and TI suggest that the two may be connected. However, further research is needed to investigate the similarities between the thoracic squeeze technique and TI. By utilizing our understanding of the neural mechanisms underlying TI, we can establish key factors to evaluate the thoracic squeeze as a potential stimulus of TI. These factors specifically involve HPA activity, and antinociception. However, based on current theories about the mechanisms underlying the thoracic squeeze technique being related to birth (See Section 2.1.2), it is first necessary to find evidence to support or refute this theory.

There have currently been no studies exploring the effects of age on responses to the thoracic squeeze. The thoracic squeeze has only been applied to neonatal mammals in the range of 2 hours to 4 postnatal days of age (Toth *et al.* 2012; Stilwell *et al.* 2019; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). In contrast, TI has been explored in a wide range of ages, and responses vary with age in different taxa. Furthermore, one of the proposed mechanisms of responses to the thoracic squeeze is related to the proximity of birth and birth-related mechanisms (Aleman *et al.* 2017; Mellor 2017). Thus, exploring the age-related effects of a thoracic squeeze could determine whether TI is a feasible explanation for the response if the age-related responses follow the same pattern as those reported for TI. Alternatively, further research into the proposed birth-related theory may be warranted if the response becomes extinct completely in mammals older than 4 postnatal days.

If TI can be considered the most feasible explanation for the observed responses to a thoracic squeeze, further research is needed to determine the effects of application of a thoracic squeeze on cortisol responses in neonates. Activation of the HPA axis and release of cortisol have been explored in TI literature and are considered necessary for the induction of TI based on the neural mechanisms described for TI (Brandão *et al.* 2008). If animals show a cortisol response to application of a thoracic squeeze, this would be consistent with the TI mechanism, particularly as it would also highlight a disparity in the other mechanisms considered, such as the birth-related mechanism that proposes an inhibition of cortisol responses. This has important implications for the use of the thoracic squeeze as a therapy for low vigour animals or a ‘low-stress’ restraint. A cortisol response to the squeeze would suggest that it is a negative experience for the animals in a similar way to TI, thus raising concerns about the welfare of animals during the squeeze and TI.

Lastly, more detailed characterisations of nociceptive responses during the squeeze are required to determine if they are diminished by the thoracic squeeze, in the same way that they may be during TI. Very little research has been done to characterise pain responses during the thoracic squeeze (Toth *et al.* 2012). Considering the significant overlap of neural pathways for both TI and nociceptive inhibition (Baker *et al.* 2019), a finding of decreased nociceptive responses during the squeeze would provide further support that the thoracic squeeze is equivalent to TI. This has further implications for the use of both the thoracic squeeze and TI in veterinary and agricultural settings. There is a potential for the thoracic squeeze to be used as a rapid, reversible, and non-chemical analgesic in mammals for the purpose of applying painful husbandry procedures.

## 2.11 Conclusions

This literature review has provided a comprehensive overview of the thoracic squeeze technique and its implications for neonatal farm mammals. By examining the literature, insights were gained into the mechanisms underlying the thoracic squeeze response and its potential connection to TI.

The potential mechanisms underlying the thoracic squeeze response remain an area of active investigation. In recent years, TI has emerged as the most feasible explanation for the responses observed after application of a thoracic squeeze. While a main feature for eliciting TI is physical contact, various stimuli can induce TI, including sustained physical pressure on body parts. TI manifests in a range of behavioural and physiological changes, governed by specific neural pathways as part of the defence cascade. Considering the knowledge presented in this literature review, it is reasonable to propose that the thoracic squeeze may serve as a stimulus for inducing TI in farm mammals. There are evident similarities between the responses observed in animals subjected to the thoracic squeeze and those displaying TI, supporting the hypothesis that the thoracic squeeze can elicit TI-like responses.

Future research should focus on investigating the specific aims outlined in this thesis. This includes further exploration of the behavioural responses of lambs to a thoracic squeeze, with particular focus on the age-related responses of lambs to a thoracic squeeze (Chapter 3); and using the known neural mechanisms underlying TI to inform comparisons about HPA responses (Chapter 4), antinociceptive effects (Chapter 5) of a thoracic squeeze on healthy neonatal male piglets. By advancing our understanding of the thoracic squeeze technique, and its underlying mechanisms in relation to TI, we can contribute to the refinement of animal handling practices and inform the appropriate use of the thoracic squeeze as it is currently being used in the veterinary industry.

## 2.12 Research objectives

The objectives of this research were:

- Characterise the behavioural and electroencephalographic responses of healthy neonatal lambs to application of a thoracic squeeze.
- Evaluate whether postnatal age influences the behavioural responses of healthy lambs to the squeeze.
- Compare the HPA responses of healthy neonatal piglets to a thoracic squeeze and sham squeeze.
- Provide further evidence of the similarities between responses to the thoracic squeeze and TI using comparisons between cortisol responses to the thoracic squeeze and TI.
- Evaluate the effects of a thoracic squeeze on the electroencephalogram in anaesthetised, healthy neonatal piglets.
- Evaluate the effects of a thoracic squeeze on electroencephalographic responses to noxious stimuli in anaesthetised, healthy neonatal piglets.

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# CHAPTER 3

## 3 Characterising the responses of neonatal lambs to a thoracic squeeze, and a preliminary exploration of age-related extinction of the response

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## 3.1 Abstract

Compressing the chests of healthy neonatal farmed mammals causes them to enter a state of reduced responsiveness, characterised by a loss of posture and cessation of movement. Upon removal of this ‘thoracic squeeze’ these newborns rapidly resume healthy, vigorous behaviours. However, this response has not yet been characterised in healthy lambs. In addition, the mechanism underlying this response is unknown, though it has been speculated that there may be a link to the compressive actions of the birth canal on the fetus. If this is correct, the neonatal response may become extinct in older animals, but this has not yet been evaluated. Our aim was to characterise the behavioural and electroencephalographic (EEG) responses of healthy neonatal lambs to two standardised methods of applying a thoracic squeeze. We also evaluated whether lambs in two older age ranges respond to a thoracic squeeze. A thoracic squeeze was applied to neonatal lambs (12-36 hours) for up to 10 minutes using either a rope (n = 10) or inflation cuff (n = 10) and the latencies to lose posture and to subsequently cease movement were measured, along with EEG. Six lambs approximately 3-weeks old (n=3 rope, n=3 cuff) and three lambs approximately 3-months old (n=3 cuff) were evaluated in the same way. Nineteen of the 20 neonatal lambs were induced into a state of reduced responsiveness, though reflexes remained mostly present amongst individuals. For 13 (68%) of these, the squeeze was discontinued before 10 min, either due to spontaneous arousal or physiological instability (i.e. gasping and blue oral mucosa colour). There was no difference in the efficacy of induction between the two squeeze methods, though the rate of physiological instability and thus discontinuation was higher in lambs squeezed with the cuff. This may be due to the cuff design which requires further development. Five of the six 3-week old lambs were induced, though the squeeze was discontinued for four lambs due to spontaneous arousal or physiological instability. Moreover, two of the three 3 month old lambs were induced with the cuff, but were later discontinued due to physiological instability (gasping). These findings suggest a generalised response to thoracic compression across mammalian species. Moreover, the success of induction in older lambs provides support for the interpretation of the response to the squeeze as a form of Tonic Immobility (TI), and further research should explore TI as a potential mechanism underlying responses to a thoracic squeeze.

**Keywords:** Thoracic Squeeze, Compression, Lambs, Sheep, Neonates, Tonic Immobility

## 3.2 Introduction

'Thoracic squeeze' is a term used to describe the purposeful compression of an animal's thorax, which is usually achieved using a rope or an inflatable cuff. This technique was first described as a potential therapeutic intervention for 'low vigour' foals, i.e., neonatal foals born apparently healthy which do not exhibit normal behaviours such as sucking from the dam after birth and are at risk of death without intervention (Aleman *et al.* 2017). Compression of the thorax has been shown to cause low vigour neonatal foals, calves, and lambs to enter a temporary state of immobility and behavioural quiescence with reduced responsiveness to external stimuli and associated physiological changes (Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021). Upon removal of the squeeze, such animals reportedly recovered, and some began to express more normal vigour.

In addition to the effect observed in low vigour neonates, healthy (normal vigour) newborn foals, piglets and calves have been shown to respond similarly to the application of a thoracic squeeze (Toth *et al.* 2012; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). In addition, brain electrical activity using electroencephalography (EEG) was observed to change in two healthy foals during the squeeze, reflective of a state of reduced responsiveness (Toth *et al.* 2012). However, due to the small sample size, EEG responses to the squeeze need further investigation. EEG is a useful tool for evaluating changes in cortical electrical activity relating to conscious awareness. Complete suppression of cortical activity, as seen during states of unconsciousness and deep anaesthesia, results in an isoelectric EEG trace (Verhoeven *et al.* 2016). Sleep-like states and changes in vigilance can be determined through alterations in the amplitude and frequency of the EEG (Ternman *et al.* 2012; Verhoeven *et al.* 2015).

The mechanisms underlying this response have not been elucidated, although it has been speculated that it may be linked to processes occurring during parturition whereby the fetus is compressed as it passes through the birth canal (Mellor 2017). If the mechanism does in some way relate to the birth process and peri-natal hormonal environment, then it might be expected that the response would become extinct at a certain point after birth. To date, the thoracic squeeze has only been applied to neonatal farm mammals up to 4 postnatal days of age. Evaluating the responses of older farm mammals would provide insight into the mechanisms underlying responses to the thoracic squeeze. In particular, should older lambs respond to a thoracic squeeze, this would provide support for the alternate theory that such responses are a form of Tonic Immobility (TI) (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022).

The effects of a thoracic squeeze have never been characterised in healthy lambs. Likewise, the effects of postnatal age on the response have not been explored in any species. Due to the very limited previous research on responses to the thoracic squeeze and the total absence of research on age effects, this study was designed to generate hypotheses, rather than to test them. Thus, the aims of this hypothesis-generating study were 1) to systematically characterise the behavioural and electroencephalographic responses of healthy neonatal lambs to application of a thoracic squeeze, 2) to compare the method of application of a thoracic squeeze (rope or cuff) on responses, 3) to explore whether postnatal age influences the behavioural responses of healthy lambs to the squeeze, and 4) to explore the effect of sex on responses to a thoracic squeeze.

## 3.3 Methods

This study was undertaken at Ngapouri Research Farm (Liggins Institute, University of Auckland) near Reporoa, New Zealand. All of the procedures were approved by the University of Auckland Animal Ethics Committee (ref: 2846).

### 3.3.1 Selection of the animals

Twenty nine Romney-Dorset crossbreed lambs (*Ovis aries*) were included in the study from a synchronised breeding programme. Making statistical inferences was not an aim of this study, and therefore, no power calculation was conducted to determine sample sizes. Instead, twin lambs were used in both the neonatal and 3-6 week age groups, based on availability of healthy twins within the sampling period. Twins were selected for use in the study and individual lambs within a pair of twins were assigned to  treatment group to control for any potential litter effect. Sex across the groups was also balanced (Rope: Females: n= 6, Males: n=7; Cuff: Females: n=8, Males: n=8). These comprised three age groups: Group 1 (n=20) were aged 12 to 36 hours, Group 2 (n=6) were aged 3 to 6 weeks, and Group 3 (n=3) were approximately 3 months of age (±7 days). All lambs were selected based on the following criteria establishing them as healthy (described in detail by Holdsworth *et al.* (2021): Normal locomotion for age, vigour, body posture, respiratory rate, heart rate, and rectal temperature. To assess locomotion, lambs were observed moving around the indoor pens with the ewe, with and without the presence of humans. Normal locomotion was considered as coordinated movements. Vigour was assessed as alertness and responsiveness to the presence of humans, including moving away and vocalising (Squires 1975; Hergenhan *et al.* 2012). Normal body posture required the head to be slightly raised or level with the body and the ears level and relaxed. Normal rectal temperature for lambs was considered between 38-40°C (Piccione *et al.* 2007; Elnageeb and Abdelatif 2013). The lower age limit of 12 hours postpartum for Group 1 lambs was to ensure that the lamb had bonded properly with the ewe and consumed sufficient amounts of colostrum.

### 3.3.2 Procedures

Data were collected for 7 days over a 3-month period between October and December 2020. The number of lambs tested per day depended on the availability within the desired age ranges; up to five lambs were tested in a single day. Lambs in each age range were alternately assigned to either the cuff (Holdsworth *et al.* 2021) or rope method (Toth *et al.* 2012). For the pairs of twin lambs in Group 1 (neonates), one twin was assigned to the cuff group, and the other twin assigned to the rope group, in order to control for litter effects across treatment groups. For Group 3, the squeeze was only applied using the cuff because the lambs were too large to apply enough force to the rope to create a successful squeeze. Overall, 16 lambs were squeezed using the cuff treatment across the three age groups (Group 1 n=10; Group 2 n=3; Group 3 n=3), and 13 lambs were squeezed using the rope (Group 1 n=10; Group 2 n=3). Each animal was squeezed only once to prevent a cumulative response.

Ewes and lambs were farmed on pasture outside of the experimental phases. Ewes were 5-6 years old and were all assured successful mothers and so assistance was rarely required. Lambs were docked at 3 weeks of age and weaned at 3 months after birth. Each day during the study, three ewes and their lambs were drawn from the mob were quietly herded into large pens inside the research facility with their dams (Groups 1 and 2), or with their flock mates (Group 3), for assessment. Once deemed suitable for inclusion, the test lamb was moved alone from the indoor group pen into an adjacent room for testing. This ensured that temperature, lighting, and noise could be controlled during the treatment. Following treatment and monitoring for complete recovery, the lamb was returned to the group pen. Each group of ewes and lambs were held inside for a total of 4 hours, with access to water and a total replacement concentrate pellet diet (Uni-C, Dunstan, Hamilton, NZ) *ad libitum*.

### 3.3.3 EEG and ECG recording

Prior to application of the squeeze, lambs were instrumented for EEG and electrocardiogram (ECG) recording using surface electrodes. Individual lambs were positioned in a custom sling and five subcutaneous 27-gauge stainless steel needle electrodes were positioned to record EEG activity and monitor heart rate, as follows: A three electrode montage was used to record EEG from the right cerebral hemisphere, with the inverting electrode placed parallel to the midline over the left frontal bone zygomatic processes, the non-inverting electrode over the right mastoid processes and a common ground electrode placed caudal to the occipital process (see Murrell and Johnson (2006). Electrodes were secured into the skin with superglue to prevent them falling out during animal movements. ECG was recorded using a base-apex configuration. Signals were amplified with a gain of 1000 and a band-pass of 1.0 –500 Hz (Iso-Dam isolated biological amplifier, World Precision Instruments Inc.) and digitised at a rate of 1 kHz (Powerlab 4/20, ADInstruments Ltd, Colorado Springs, Co). The digitised signals were recorded on an Apple Macintosh computer. ECG recordings were monitored in real time, whilst EEG was analysed offline at the conclusion of the experiment.

Following instrumentation, the lamb was held in a standing position with one of the researcher's arms supporting the hind end and the other around the front of the chest until the squeeze was applied. Once the lamb was gently restrained in a standing position, the squeeze device (rope or cuff) was firmly secured around the thorax, and the squeeze was applied as described below and in Holdsworth et al. (Holdsworth *et al.* 2021). Following application, the lamb was allowed to relax into sternal or lateral recumbency as that occurred naturally. For 3-month-old lambs, two people were required to restrain the lamb for application of the squeeze, and to ensure the lamb was safely supported into recumbency.

A timer was started upon the application of the squeeze and the latency to induction into a state of reduced responsiveness was recorded, based on loss of standing posture (lateral recumbency) and cessation of limb movements. The squeeze was applied for 10 minutes unless the lamb met the discontinuation criteria (see Section 2.5 below) in which case the squeeze was immediately discontinued. Prior research on neonatal piglets demonstrated that 10 minutes is sufficient to demonstrate induction into a state of reduced responsiveness and maintenance of the state (Holdsworth *et al.* 2021) while ensuring that any risks associated with prolonged squeezing were minimized. After the squeeze was completed or discontinued, each lamb was monitored for up to 5 minutes to ensure that it had recovered and was displaying the behaviours described in the inclusion criteria. The lambs were encouraged into sternal recumbency and were allowed to remain there for two minutes before being encouraged to stand to assess their recovery.

### 3.3.4 Treatments

The rope squeeze was applied using a soft polyester fabric rope 3 cm in diameter and 3 m in length. A small loop was created on one end of the rope using a bowline knot and the squeeze was applied as described previously (Holdsworth *et al.* 2021) (Figure 7). The timer was started once all rope loops had been tightened.

**Figure 7.** Thoracic squeeze being applied to a neonatal lamb using a soft fabric rope. Leads from the electroencephalogram and electrocardiogram electrodes can be seen attached to the scalp and chest/abdomen regions, respectively.

The inflation cuff, consisting of a custom-designed inflatable band, was wrapped around the thorax, and secured snugly using Velcro (Figure 8). The dimensions of the nylon band were based on thoracic length and girth of lambs within the three age ranges tested, as follows: Group 1 (12-36 hours old), 12.5 cm wide × 65 cm long; Group 2 (3-6 weeks old), 19.5 cm wide x 90 cm long; Group 3 (3 months old), 23.5 cm wide x 105 cm long. The band housed an inflatable rubber bladder, and a nylon strap was placed around the chest to prevent slippage of the cuff on to the abdomen. Once the cuff was in place, the bladder was inflated to a pressure of 140 ± 20 mmHg using regulated compressed air from the facility's central gas supply system. This pressure was determined by steadily inflating the cuff until the lamb went into lateral recumbency, as was done previously in piglets and calves (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Based on this, a "minimum effective pressure" of 140 mmHg was established. The timer was started when the minimum effective pressure was achieved.

**Figure 8.** Inflation cuff applied to a 3-week-old lamb, secured around the thorax with Velcro and fastened to a strap around the neck to prevent slippage onto the abdomen. Electroencephalogram and electrocardiogram electrodes can be seen attached to the scalp region.

### 3.3.5 Criteria for the discontinuation of the squeeze

To ensure lambs remained physiologically stable throughout the squeeze, the following variables were monitored alongside the data collected before and 2 and 8 minutes after application of the squeeze: heart rate, respiratory rate, oxygen saturation (where possible), oral mucosa colour, rectal temperature, and muscle tone (for more details, see Holdsworth *et al.* (2021). If any parameter approached the bounds of the normal range at the 2-minute check (Piccione *et al.* 2007; Silva *et al.* 2016), lambs were subsequently monitored every 30 seconds. If any parameter was outside of the reported healthy range, the squeeze was immediately discontinued.

The lambs were also monitored for persistent struggling once the squeeze was applied and during the initial increase in pressure/tightening. If struggling persisted for 2 minutes after the start of application, the treatment was discontinued. Likewise, if the lamb was calm during the application of the treatment but there was a failure of induction by 2 minutes—that is, posture was not lost, or movement did not cease following loss of posture—then the squeeze was immediately discontinued. The squeeze was also discontinued if an induced lamb exhibited spontaneous arousal e.g. struggling or thrashing, vocalization, attempting to stand (Holdsworth *et al.* 2021) (Table 3) before the end of the 10-minute period.

### 3.3.6 Data collection

Behavioural parameters, electrical brain activity (EEG) and neural reflexes (tested at 2 and 8 minutes) were assessed before and during application of the squeeze (Table 3). Behaviours were assessed both in real time and by reference to video recordings captured using two Sony Handycam DCR-SR85 Cameras (Sony Corporation, Tokyo, Japan).

**Table 2.** Behavioural and neural reflex responses recorded during the 10-min thoracic squeeze, adapted from Holdsworth *et al.* (2021).

| Variable | Description |
|---|---|
| **Latency to loss of posture** | Time from the start of cuff inflation or rope tightening to the lamb losing posture and relaxing into lateral recumbency from a standing position. |
| **Latency to Induction** | Time from the start of cuff inflation or rope tightening to the cessation of all limb and head movements for a minimum of 3 seconds following loss of posture. |
| **Pedal reflex** | Level of reflex determined by pinching the interdigital cleft between the claws of a front foot to elicit withdrawal of the limb. Present-D was assigned to lambs for which the squeeze was discontinued due to spontaneous arousal or physiological instability, and were therefore categorised as having a full limb withdrawal response; Present-I was assigned to lambs for which the squeeze was maintained during reflex testing and exhibited full limb withdrawal. Reduced for a slight limb withdrawal or a limb twitch response; and Absent for no muscle twitch or limb movement. |
| **Palpebral reflex** | Level of reflex determined by lightly brushing or touching the eyelashes or skin in the lateral corner of the eye, to elicit a blink or eye twitch response. Present-D was assigned to lambs for which the squeeze was discontinued due to spontaneous arousal or physiological instability, and were therefore categorised as having a full twitch or blink response; Present-I was assigned to lambs for which the squeeze was maintained during reflex testing and exhibited full twitch or blink response; Reduced for a small twitch response; and Absent for no movement. |
| **Spontaneous arousals** | High intensity movements, lasting longer than 5 seconds. These movements are considered a 'spontaneous arousal', characterised by opening of the eyes and vigorous movements with righting onto all four feet, and triggering immediate discontinuation of the squeeze. |

### 3.3.7 Data analysis

A four-category scale was used to characterize the responses of individual lambs to the squeeze (Holdsworth *et al.* 2021). The categories were based on successful induction into a state of reduced responsiveness— characterised by loss of posture and cessation of movement—and for how long this state was maintained:

A: No induction into a state of reduced responsiveness within 120 seconds of the start of application of the squeeze.

B: Induction into a state of reduced responsiveness occurred within 120 s of the start of the application of the squeeze but this state was not maintained for the full 10-minutes observation period due to spontaneous arousals.

C: Induction into a state of reduced responsiveness occurred within 120 s of the start of the application of the squeeze and this state was maintained for the 10-min squeeze duration.

D: Induction into a state of reduced responsiveness occurred within 120 s of the start of the application of the squeeze, but the squeeze was discontinued due to concerns based on physiological monitoring.

While previous research in piglets included eye closure as a criterion for induction into a state of reduced responsiveness (Holdsworth *et al.* 2021), here we chose to follow the induction criteria used for healthy calves, none of which closed their eyes after application of the squeeze (Holdsworth *et al.* 2022).

### 3.3.8 Statistical analysis

Due to the the fact that the main objective was hypothesis-generating and not statistical inference and the small sample sizes in this preliminary study, data analysis focused on descriptive statistics to characterise responses. Data were presented as number and percentage of animals in each age group based on method of application and induction category (with 95% CI, calculated using the exact binomial method). Kaplan-Meir graphs were used to visualize the effects of induction category, method of application, lamb age and sex on the latency to loss of posture and latency to induction. The relationships between the method of squeeze application and presence of pedal and palpebral reflexes at 2 and 8 minutes into the squeeze were depicted using plot graphs. All statistical analyses were conducted using R Studio version 1.2.1335 (R Version 4.0.4) (RStudio Team 2018).

## 3.4 Results

### 3.4.1 Characterisation of responses in neonatal lambs

#### 3.4.1.1 Success and maintenance of induction

Nineteen of the 20 neonatal lambs squeezed were successfully induced (Table 4). Five of these (25% of total squeezed) completed the full 10 minute squeeze (Category C) while 14 did not (Categories B and D). Eleven lambs spontaneously aroused (Category B: Med = 261 s, Range = 62-459 s) after induction, whereas the squeeze was discontinued for three due to physiological instability (Category D: Med = 115 s, Range = 113-126 s).

**Table 3.** Number and proportion (with 95% confidence intervals) of lambs of three different age ranges in each induction category, according to the method of squeeze application. Category A lambs had the squeeze discontinued as they failed to induce, Category B lambs were induced but had the squeeze discontinued due to spontaneous arousals, Category C lambs were induced and maintained in a state of reduced responsiveness for the full 10-minute duration, and Category D lambs had the squeeze discontinued due to physiological instability.

| | **12-36 hours old** | | **3-6 weeks-old** | | **3-month-old** | | | |
|---|---|---|---|---|---|---|---|---|
| **Category** | **Rope** | **Cuff** | **Rope** | **Cuff** | **Cuff** | **Total** | **%** | **95% CI** |
| A (not induced) | 1 | 0 | 1 | 0 | 1 | 3 | 10 | (2–27) |
| B (induced, discontinued) | 4 | 7 | 1 | 3 | 0 | 15 | 52 | (33–71) |
| C (induced, maintained) | 5 | 0 | 1 | 0 | 0 | 6 | 21 | (8–40) |
| D (Induced, unstable) | 0 | 3 | 0 | 0 | 2 | 5 | 17 | (6–36) |
| Total | 10 | 10 | 3 | 3 | 3 | 29 | - | |
| % | 35 | 35 | 10 | 10 | 10 | - | 100 | |

In the cuff group, the squeeze was discontinued in all lambs, three due to a rapid change in physiological parameters (Category D: Med = 115 seconds, Range = 113-126 s), and the other seven due to spontaneous arousals (Category B: Med = 136 s, Range = 78-321 s). In the rope group, half of the neonates squeezed remained in a state of reduced responsiveness for the full 10-minute duration and of the nine neonates successfully induced in the rope group, the squeeze was discontinued in four lambs due to spontaneous arousals (Category B: Med = 434 s, Range = 62-459 s).

#### 3.4.1.2 Effect of induction category and method of application on time to loss of posture and induction

Due to the small number of Category D (induced but discontinued due to physiological instability) lambs, this category was excluded from the analysis. The lambs for which the squeeze was discontinued due to spontaneous arousals (Category B) appeared to lose posture faster than the lambs that maintained reduced responsiveness for the full 10 minutes (Category C) (Cat B: Med = 33 s, Range = 10-50; Cat C: Med = 44 s, Range = 18-78; Figure 9a). Time to induction did not differ between Category B and C lambs (Cat B: Med = 47 s, Range = 16-94; Cat C: Med = 50 s, Range = 25-92; Figure 9b).

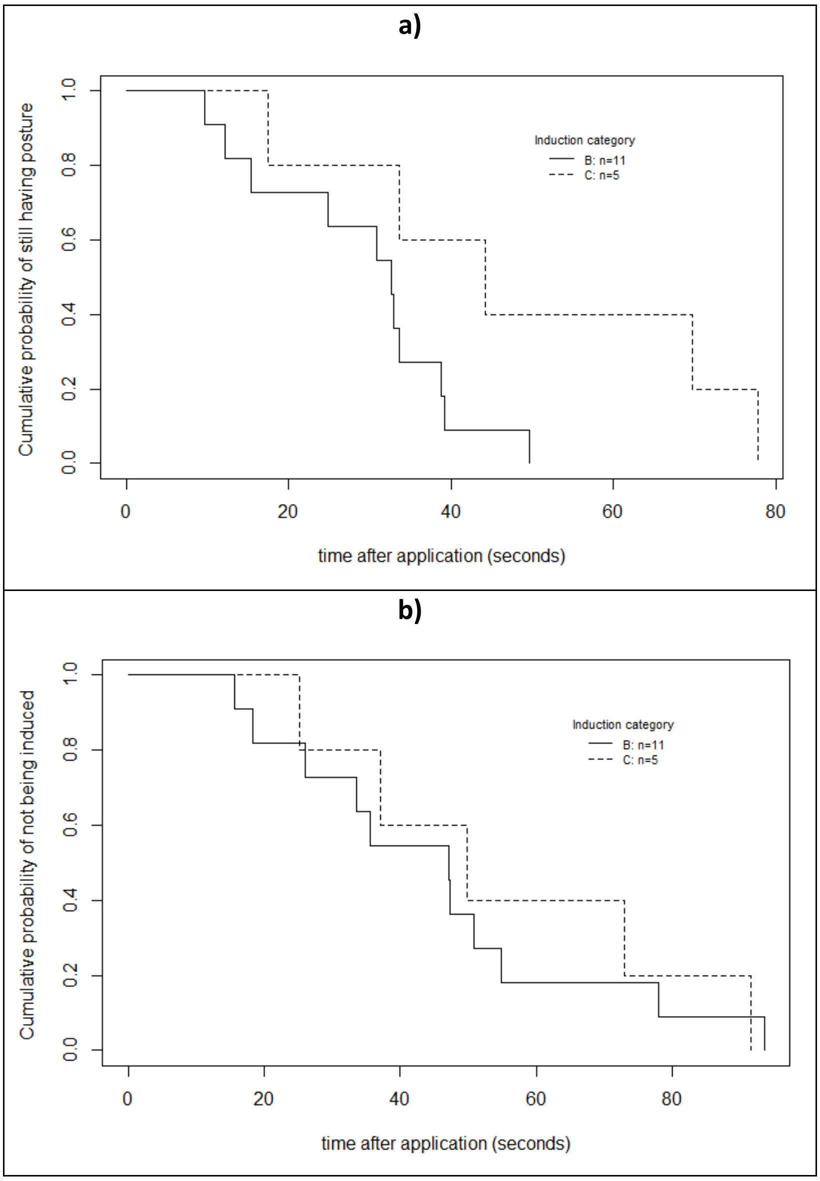

**Figure 9.** Kaplan-Meier graphs of (a) Latency to lose posture, (b) Latency to Induction in 19 neonatal lambs that were successfully induced into a state of reduced responsiveness with either the cuff or the rope, and that did not have the squeeze discontinued after physiological instability was observed. Category B refers to lambs that were induced but had the squeeze discontinued due to spontaneous arousals, and Category C refers to lambs that were induced and maintained in a state of reduced responsiveness for the full 10-minute duration.

Neonatal lambs that were squeezed with the Cuff appeared to lose posture as quickly as those that were squeezed with the Rope (Cuff: Med = 33 s, Range = 10-39 s; Rope: Med = 34 s, Range = 15-78 s; Figure 10a). There was also no apparent difference in the time to induction between the Cuff and Rope treatments in this age group (Cuff: Med = 47 s, Range = 16-94 s; Rope: Med = 50 s, Range = 18-92 s; Figure 10b).

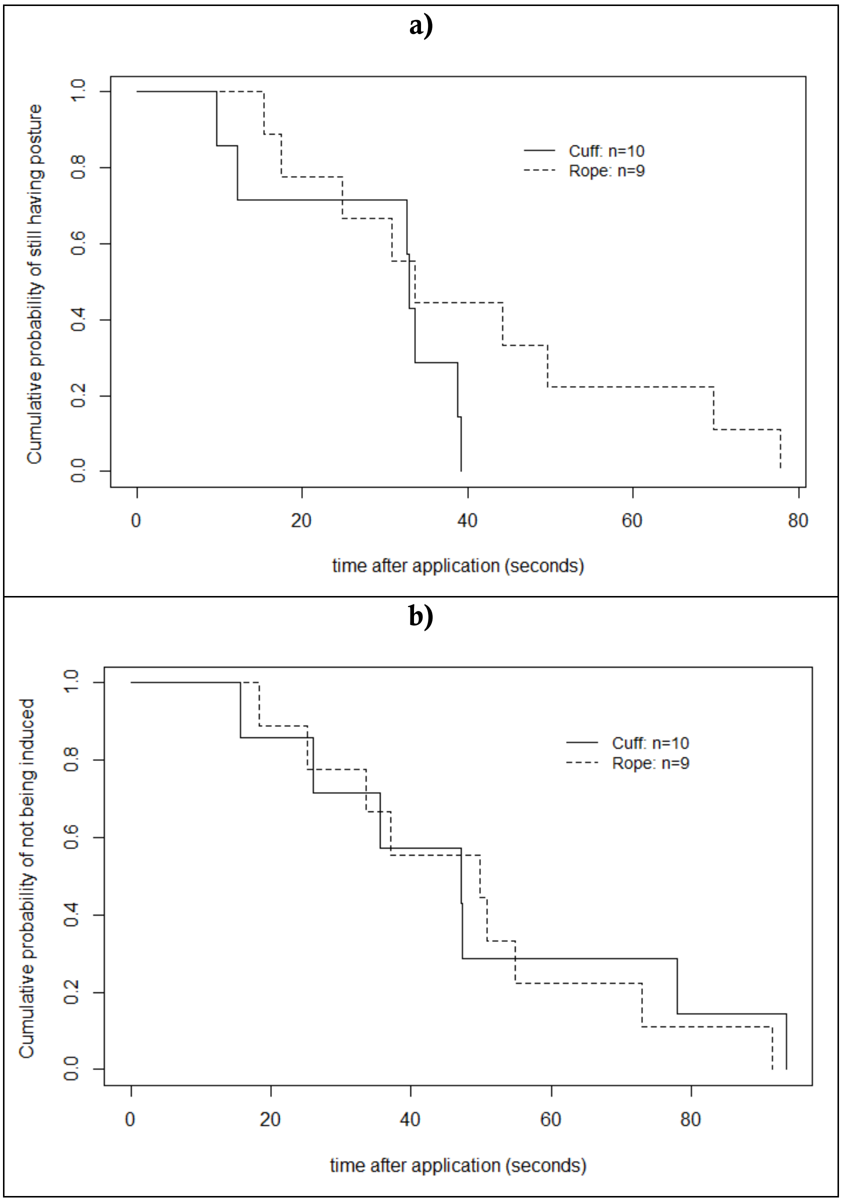

**Figure 10.** Kaplan-Meier graphs of (a) Latency to lose posture, (b) Latency to induction in 19 neonatal lambs that were successfully induced into a state of reduced responsiveness with either the cuff (n=10) or the rope (n=9).

### 3.4.1.3 Neurophysiological measures

Lambs that did not induce (Category A: n=1) or had the squeeze discontinued due to spontaneous arousal (Category B: n=11) or physiological instability (Category D: n=3) were categorized as 'Present-Discontinued' following discontinuation of the squeeze, as they retained reflex responses and were thus included in the analysis.

At the 2-minute reflex testing, eight lambs squeezed with the cuff, and two lambs squeezed with the rope could not be tested as the squeeze had already been discontinued due to spontaneous arousals or physiological instability. These animals were assigned present reflexes (Present-D). Of the two cuff lambs tested, one had a reduced, and one had a present pedal reflex. In the rope group, two of the eight lambs tested had reduced pedal reflexes, while the remaining six all retained pedal reflexes. In contrast, the two cuff lambs retained palpebral reflexes at the 2-minute testing while one lamb in the rope group showed reduced palpebral reflexes. The remaining seven rope lambs retained palpebral reflexes.

At the 8-min reflex testing, the squeeze was discontinued for all 10 lambs in the cuff group, and as such could not be tested and were categorized as retaining pedal and palpebral reflexes. In the rope group, two lambs had the squeeze discontinued and were categorized as retaining pedal and palpebral reflexes. Of the remaining six lambs, two had absent and one had reduced pedal reflexes, while three lambs had present pedal reflexes. In contrast, all six lambs in the rope group retained palpebral reflexes (Figure 11a, b).

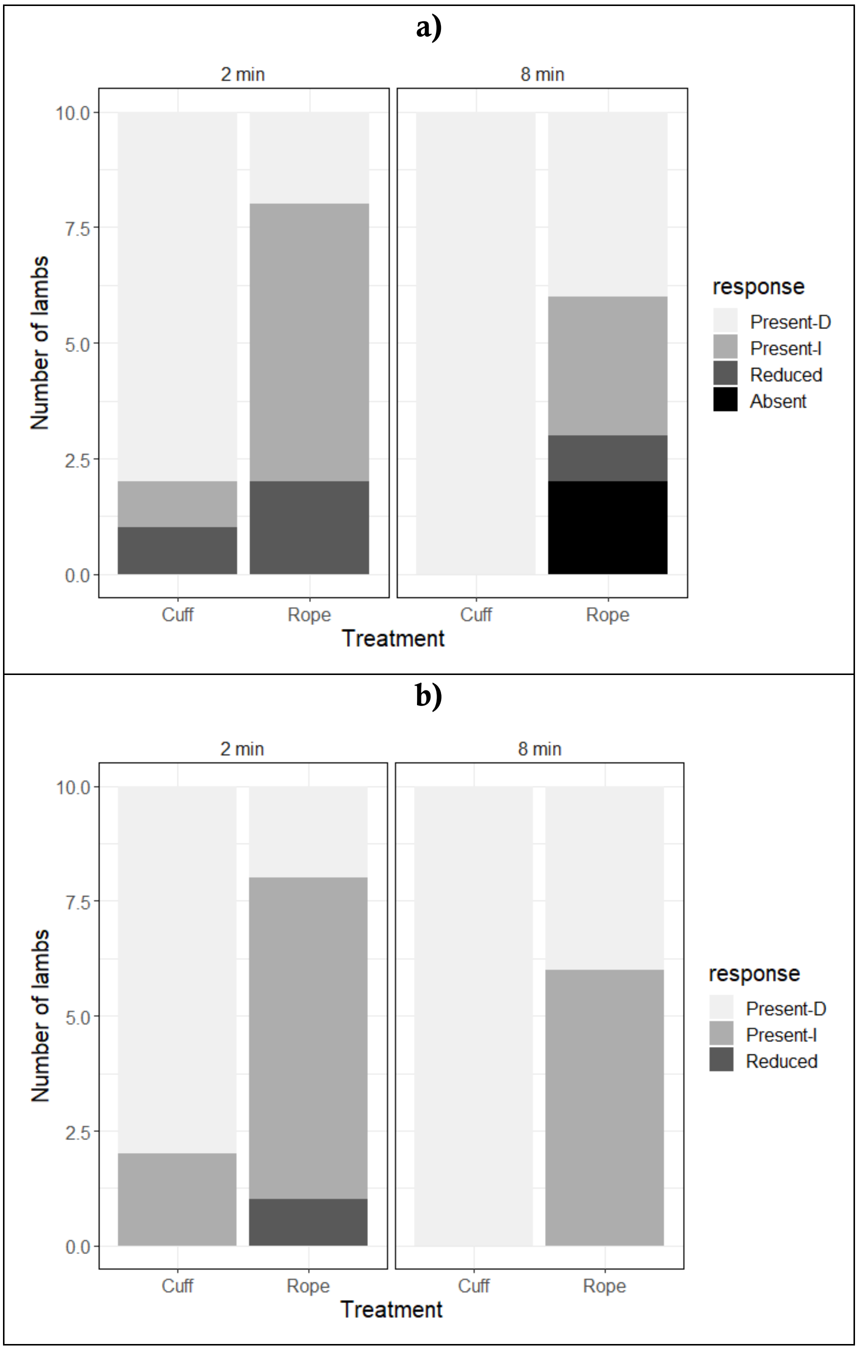

**Figure 11.** Number of neonatal lambs exhibiting each level of a) Pedal reflex responses and b) Palpebral reflex responses at 2 and 8 minutes after squeeze application for each method. "Present - D" refers to lambs for which the squeeze was discontinued due to no induction (Category A), spontaneous arousal (Category B) or physiological instability (Category D) and were all marked as retaining present reflexes. "Present-I" refers to lambs for which the squeeze was maintained during the reflex testing, and exhibited full limb withdrawal or complete blink of the eyelid. "Reduced" refers to slight limb withdrawal and twitch of the eyelid; "Absent" refers to no responses to reflex testing.

EEG traces were heavily contaminated with artefacts from skeletal muscle activity due to movements of the head and/or body of the lambs during application of the squeeze or due to researchers inadvertently disturbing the recording electrodes or leads during support and monitoring activity. Although small sections of clear records were obtained from some individuals, there were insufficient data for subsequent analyses.

## 3.4.2 Responses of older lambs

### 3.4.2.1 Effect of age on induction after application of the squeeze

Five of the six 3-6 week old lambs were successfully induced (Table 4). Four of the five lambs induced did not complete the 10-minute squeeze period due to spontaneous arousals (Category B: Med = 323 s, Range =182-349 s), one with the rope and three with the cuff. The one lamb that completed the full 10 minute squeeze (Category C) was squeezed with the rope.

Of the 3-month old lambs (n=3), two were induced into a state of reduced responsiveness using the cuff but neither was maintained for the full 10 minutes. The squeeze was discontinued for both due to physiological instability (Category D: Med = 138 s, Range = 136-141 s).

### 3.4.2.2 Effect of sex on latency to loss of posture and Induction

The time to the loss of posture appeared to be shorter and less variable in male lambs than it was in female lambs (Females: n = 13, Med = 34 s, Range = 12–78 s; Males: n = 13, Med = 25 s, Range = 10–44 s; Figure 12a). In contrast, there was no apparent difference in the time to induction between female and male lambs (Females: Med = 47 s, Range = 19–91 s; Males: Med = 34 s, Range = 16-93 s; Figure 12b).

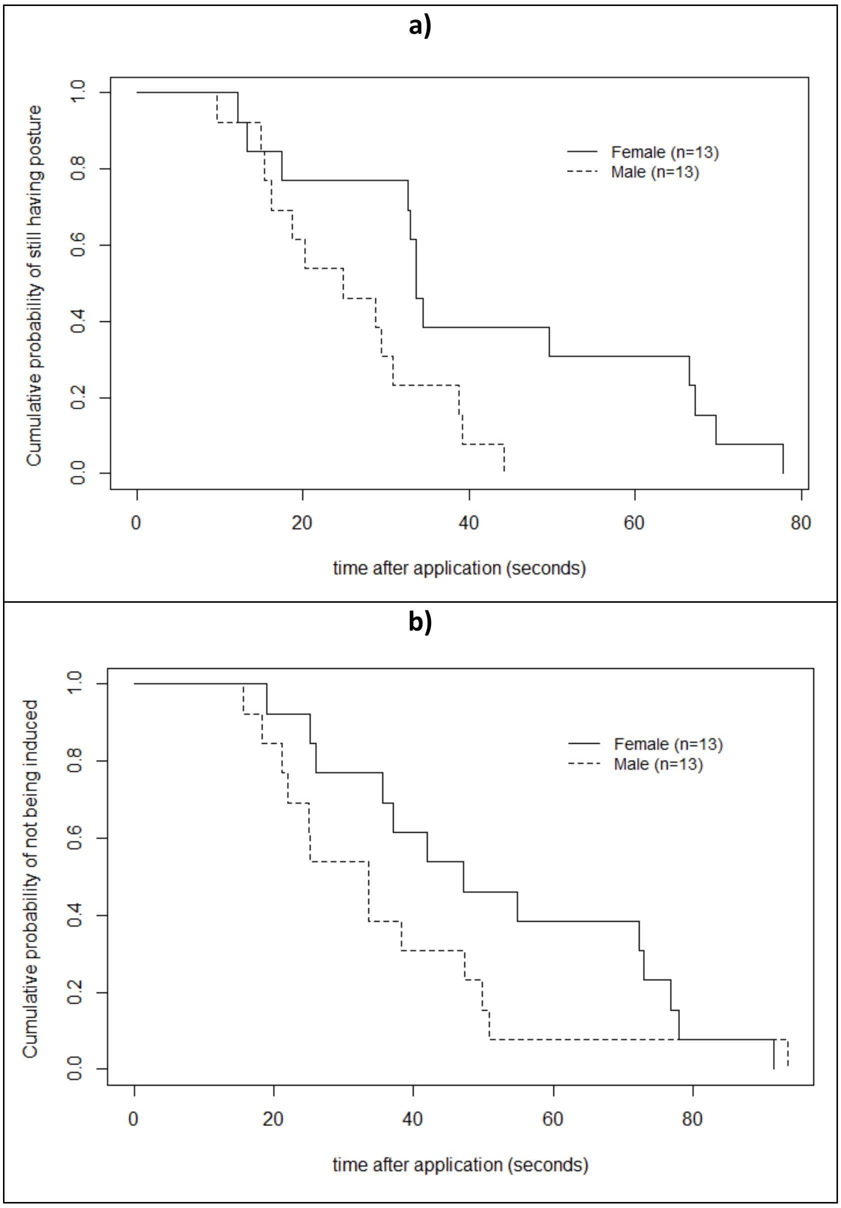

**Figure 12.** Kaplan-Meier graphs of (a) Latency to lose posture, (b) Latency to induction in Female and Male lambs that were successfully induced into a state of reduced responsiveness.

## 3.5 Discussion

The primary aim of this study was to characterise the responses of healthy newborn lambs to a standardised application of the thoracic squeeze. Additional aims were to evaluate the effect of the method of application on those responses and to explore whether older lambs responded to application of the squeeze. Due to the very limited amount of previous research, this was an exploratory, hypothesis generating study and therefore, descriptive analysis of the data was considered the most appropriate method.

### 3.5.1 Behavioural responses in neonatal lambs

Nineteen out of the 20 neonatal lambs (95%) entered a state of reduced responsiveness, characterized by loss of posture and cessation of movement, following application of the squeeze. This is consistent with induction rates reported in low-vigour foals, calves and lambs (Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021), and healthy neonatal calves and piglets (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Only around one quarter (26%, n=5/19) of the neonatal lambs that were induced remained in a state of reduced responsiveness for the full 10-minutes, a number that is lower than the proportion of piglets and calves that maintained a state of reduced responsiveness for the full squeeze duration. Of 17 piglets squeezed, nine (53%) piglets maintained a state of reduced responsiveness (Holdsworth *et al.* 2021), while in 13 out of 16 calves successfully induced, seven (54%) maintained a state of reduced responsiveness for the full 10 minute squeeze (Holdsworth *et al.* 2022). In Category B neonates, discontinuation due to spontaneous arousals occurred with large variability, with some individuals spontaneously arousing as far as 8 minutes after start of squeeze application. While the median time to induction for Category B neonates was 47.2 s, the median for discontinuation was just over 4.5 minutes after the start of the squeeze. Thus, the time spent in a state of reduced responsiveness was just over 3.5 minutes. It is important to note that the 10 minute squeeze duration is arbitrary, thus there is no known cause for why some of the lambs that spontaneously aroused did so at nearly 8 minutes into the squeeze, and whether there are any biological differences between those that stayed in a state of reduced responsiveness for 3 minutes and those that stayed for the full 10 minutes. In the present study, the lower rate of maintained low responsiveness may be attributed to the method of squeeze application; when considering the rope data alone, 50% of lambs remained in a reduced state of responsiveness for the full 10 minutes. This is explored in more detail below.

### 3.5.2 Neurophysiological responses

Neural reflexes remained present in most neonates for the duration of the squeeze, with more neonates displaying reduced or absent pedal reflexes compared to one lamb that showed reduced palpebral reflexes. This is consistent with previous observations in healthy newborn calves , whereby only one calf showed consistently reduced pedal and palpebral reflexes during the thoracic squeeze (Holdsworth *et al.* 2022). In contrast, pedal and palpebral reflexes were reduced or absent in most healthy piglets subjected to a thoracic squeeze. Similarly, whilst lambs and calves' eyes remained open following induction, piglets maintained eye closure for the duration of the squeeze. Neural reflexes are generally considered a reliable indicator of the level of consciousness or awareness in animals under general anaesthesia (Guntheroth and Kawabori 1975; Spyer 2009). When reflexes are present, it is suggested that the animal is not in a deep stage of anaesthesia (Guntheroth and Kawabori 1975; Spyer 2009). Collectively, these observations suggest that healthy newborn lambs and calves do not reach the same level of reduced responsiveness as neonatal piglets following application of the squeeze. The reason for these differences between species is unknown.

In addition to testing neural reflexes, we also attempted to collect EEG data to quantitatively evaluate changes in brain state (e.g. consciousness, reduced responsiveness, unconsciousness) when lambs responded behaviourally to the squeeze. We also aimed to evaluate differences in brain state between lambs for which the squeeze was discontinued (Category B and D) and those that maintained a state of reduced responsiveness for the full 10 minutes. Despite the use of cyanoacrylate to secure these in place, the electrodes became dislodged in several lambs due to their vigorous activity. In those lambs where the electrodes remained in place, EEG traces were contaminated to varying degrees by skeletal muscle artifacts and/or physical disturbance of the leads by head and body movements. Although the use of surgically implanted electrodes would have overcome some of these issues, this was not deemed feasible, given that surgery could not be performed within the first 12 hours of birth (so as not to interfere with mother-young bonding), and the requirement for 24–48 hours recovery after surgery would have effectively prohibited data collection from the desired age range (12–36 hours).

### 3.5.3 Comparison of rope and cuff methods in neonates

Loss of posture seemed to occur more quickly in lambs squeezed with the cuff than those squeezed with the rope, although there were no differences in latency to induction (cessation of movement following loss of posture) between methods. These findings are similar to those observed for piglets and calves. Piglets squeezed with a cuff initially lost posture faster than those squeezed with a rope, although again there were no differences in the time to induction between the two methods (Holdsworth *et al.* 2021). In contrast, healthy calves squeezed with a cuff appeared to lose posture and stop moving (become induced into a state of reduced responsiveness) faster than calves squeezed with a rope (Holdsworth *et al.* 2022).

Overall, maintenance of a state of reduced responsiveness in the neonatal lambs was relatively rare, observed in only 26% lambs, and only in lambs squeezed with the rope. The squeeze appeared to be successful in maintaining behavioural quiescence when applied using the rope compared to the cuff, with 50% of lambs squeezed with the rope maintaining reduced responsiveness for the full 10 minutes, compared to no lambs squeezed with the cuff. This contrasts with previously reported responses of piglets and calves in which more animals maintained a state of reduced responsiveness when squeezed with the cuff than those squeezed with the rope. These findings, along with the greater incidence of physiological instability and spontaneous arousals in lambs squeezed using the cuff, suggest that poor maintenance in the lambs was more likely due to features of the cuff design than species differences.

While the three neonates that became physiologically unstable (Category D) induced relatively quickly, all had the squeeze discontinued within approximately 2 minutes after application of the squeeze. These lambs exhibited evidence of hypoxaemia - rapid onset of hyperventilation (gasping) characterised by an open mouth and forceful inspiration, and some lambs exhibited a rapid change from a pink to blue oral mucosa colour (Guntheroth and Kawabori 1975; Spyer 2009). It may be that these lambs did not switch from thoracic to abdominal breathing after application of the squeeze, resulting in hyperventilation and oral mucosa colour changes within the first 126 s. In previous research, proportionally fewer piglets had the squeeze discontinued due to physiological instability, with only one piglet out of 17 being discontinued due to gasping significantly later than the lambs in this study (265 s after start of squeeze application) (Holdsworth *et al.* 2021). Similarly, only two of 16 healthy calves were discontinued due to physiological instability, one due to a marked increase in breathing rate at 322 s, and the other due to a rapid decrease in heart rate at 332 s (Holdsworth *et al.* 2022). The change in physiological status of both the piglets and calves occurred much later than in the lambs.

In all cases of physiological instability in these three species, the animals were squeezed with an inflation cuff. While the reasons for this effect are unknown, a possible explanation is the cuff dimensions. The dimensions were based on estimates of the thoracic girth and thoracic length in each lamb age range. However, it appeared that the cuff was too long for some lambs, compressing both the thorax and parts of the abdomen and impeding breathing. Piglets and calves squeezed using a cuff appeared to rapidly switch from thoracic to abdominal breathing, thus maintaining oxygen saturation, while the ones that became physiologically unstable all showed signs of impaired breathing (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). The apparent failure of lambs to successfully adopt abdominal breathing may explain the large proportion of spontaneously arousing and physiologically unstable neonates in the cuff group compared to the rope group. Due to the remote location and age specific time constraints of the cohort studied, alterations to the cuff design were not possible after the commencement of the data collection.

From a practical application point of view, the cuff was easier and faster to apply than the rope and enabled rapid discontinuation of the squeeze if a lamb became physiologically unstable. In contrast, application of the rope was difficult, due to the inability to standardise the squeeze across lambs of different sizes and ages. Looping the rope three times around the thorax as is required to perform a squeeze caused friction on the rope, which meant that each loop had to be tightened separately, and the greatest force applied to the thorax was uneven across each loop. These findings are similar to that of healthy piglets, with the inflation cuff being applied faster and eliciting induction more quickly than the rope (Holdsworth *et al.* 2021). Further work is needed to evaluate the safety of the cuff in neonatal lambs with a cuff design that is better tailored to the anatomy of lambs at different ages.

### 3.5.4 Effect of lamb age on induction

Of the nine older lambs squeezed with the cuff, seven were induced into a state of reduced responsiveness within 80 seconds of the squeeze application. One (3-month-old) cuff lamb failed to induce within 2 minutes of application of the squeeze. None of the seven older lambs squeezed with the cuff that induced were maintained for the full 10-minutes. However, as discussed previously, potential issues with the cuff design may have impaired the maintenance of reduced responsiveness.

Of the three older lambs squeezed with the rope (3-6 weeks old), one did not induce into reduced responsiveness within 2 minutes and two were successfully induced. Of the two that were induced, one lamb maintained this state for the full 10-minutes, while the remaining lamb later had the squeeze discontinued due to spontaneous arousal.

Although the small sample sizes preclude drawing robust conclusions, this preliminary exploration suggests that responses to the squeeze, characterised by an induction into a state of reduced responsiveness, persist beyond the early postnatal period. Thus, it provides an interesting reference point for further exploring responses in older animals.

### 3.5.5 Effect of sex on behavioural responses

Although males initially lost posture faster than females, there were no apparent differences in the latency to induction into a state of reduced responsiveness between sexes. These limited data suggest that sex has no effect on responses to the squeeze. While sex differences have not been reported in healthy mammals for the thoracic squeeze, these findings are consistent with a lack of reported differences in sexes when placed into TI

(Lefebvre and Sabourin 1977b; Jones and Faure 1981; Mills and Faure 1986; Erhard *et al.* 1999). In 219 pigs (106 females, 113 males) tested for TI induction and duration, no sex differences were observed in the latency to induce and the duration of TI behaviours (Erhard *et al.* 1999). In 28 domestic fowl (14 females, 14 males) placed into TI, there were no sex differences found in the duration of TI behaviours (Jones and Faure 1981). The findings in this study suggest that sex has no effect on responses to the squeeze, and also supports the hypothesis that responses to the squeeze are similar to those of animals induced into TI.

### 3.5.6 Potential mechanisms underlying the thoracic squeeze

The present study has demonstrated that lambs up to 3 months of age can be induced into a state of reduced responsiveness by the squeeze, in the same way as neonates. These findings indicate that responses to the squeeze are retained beyond the post-natal period and suggest that proposed mechanisms related to the birth process are unlikely to explain the observed responses to a squeeze. The apparent success of the squeeze at subsequently invoking normal behaviours in neonatal foals born with low vigour (Aleman *et al.* 2017) led researchers to propose that the mechanisms might relate to birth processes. It has been posited that the thoracic squeeze mimics the compressive actions of the birth canal on the fetus during parturition (Aleman *et al.* 2017). This compression, along with a range of neurohormonal factors that can suppress and activate consciousness, facilitates the transition from unconsciousness *in utero* to consciousness after birth. It is suggested that applying the squeeze to neonates that are not exhibiting critical survival behaviours at birth 'resets' the animal, leading to the expression of normal survival behaviour (Mellor 2017). To date, no evidence has been generated to support or refute this theory. However, a crucial element of the theory is its function in neonates due to proximity to birth. The current findings in this study do not support the birth process hypothesis, and therefore suggest an alternative hypothesis be explored.

The responses of older lambs to a thoracic squeeze, as well as the generalised behaviours observed during induction and maintenance after application of a thoracic squeeze bear strong similarities to reported changes associated with TI. TI is a temporary and reversible phenomenon seen in animals of numerous species and ages (Moore and Amstey 1962; Rovee *et al.* 1973; Rovee and Kleinman 1974; Prestrude 1977; Rovee-Collier *et al.* 1991; Erhard *et al.* 1999; Sobotik *et al.* 2020). Animals induced into TI become rapidly immobile, display reduced responsiveness to environmental stimuli and exhibit accompanying physiological changes, including diminished responses to painful stimuli (Verhoeven *et al.*

2015). The reduced responsiveness observed during TI can be reflected in changes to brain electrical activity. During induction into TI, EEG patterns are characterised as low voltage, fast activity – a pattern that represents hypervigilance and alertness. In contrast, as the TI response is maintained, EEG patterns reflect high voltage, slow wave activity – a pattern that represents a state of reduced responsiveness (Schwarz and Bickford 1956; Kumazawa 1963; Barratt 1965; Vanderwolf *et al.* 1975; Lefebvre and Sabourin 1977b). TI is considered a stress-induced state triggered by sustained physical contact. Sustained physical contact elicits activation of the hypothalamic-pituitary-adrenal (HPA) axis or equivalent and results in by increased secretion of stress hormones (glucocorticoids) (Ong 1993). The TI response is regulated by specific brainstem and forebrain structures that enact these behavioural and physiological changes that result in complete immobility (Brandão *et al.* 2008; Terpou *et al.* 2019).

The results of this study, along with previous research, have demonstrated a generalised behavioural quiescence in response to thoracic compression, characterised by a loss of posture, motor quiescence, reduced responsiveness to external stimuli and, sometimes, attenuation of neural reflexes (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). TI is characterised by the same features, along with physiological changes such as decreased heart rate, breathing rate, and blood pressure, changes in brain electrical activity, and onset of analgesia (Davie *et al.* 1993; Monassi *et al.* 1997; Vieira *et al.* 2011). We attempted to collect EEG in this study in order to evaluate whether brain electrical activity changes observed after application of a thoracic squeeze could provide more information about the type of brain state animals enter, and whether EEG patterns reflected those reported for animals induced into TI. The data contamination that occurred during EEG collection meant that brain activity could not be evaluated. Further research could explore the effects of a thoracic squeeze on EEG patterns to determine whether the similarities between responses to a squeeze and reported responses during TI are consistent.

Further similarities between the two phenomena are reflected in the rapidity of onset of the state of reduced responsiveness. In the present study, induction almost always occurred within 2-minutes of squeeze application if indeed it happened. The same was found in calves (Holdsworth *et al.* 2022), piglets (Holdsworth *et al.* 2022), and foals (Toth *et al.* 2012). Similarly, induction into TI is considered to be neurally mediated with inputs from reflexes, making induction rapid (Webster *et al.* 1981; Gentle *et al.* 1989; Terpou *et al.* 2019). In addition, the high proportion of lambs that were induced and had the squeeze discontinued appears to follow a similar pattern to animals induced into TI. Individual variability within and across species is observed when restrained animals become unresponsive, i.e., TI (Moore and Amstey 1962; Rovee *et al.* 1973; Rovee and Kleinman 1974; Prestrude 1977;

Rovee-Collier *et al.* 1991; Erhard *et al.* 1999; Sobotik *et al.* 2020). Specifically, environmental factors and personality differences affect the success of induction into TI, and maintenance of reduced responsiveness in a wide range of species (Moore and Amstey 1962; Rovee *et al.* 1973; Rovee and Kleinman 1974; Prestrude 1977; Rovee-Collier *et al.* 1991; Erhard *et al.* 1999; Sobotik *et al.* 2020). Regardless, it can be concluded that the variability in responses to a thoracic squeeze follow a similar pattern to those observed in TI.

Based on these similarities, TI appears to be a potentially valid explanation for the mechanism underlying responses to the thoracic squeeze. However, further research is required, such as evaluating alterations in HPA activity during the squeeze, and determining whether there are any analgesic effects of the squeeze in mammals.

## 3.6 Conclusions

Application of a thoracic squeeze resulted in induction into a temporary state of reduced responsiveness in 90% of the lambs in this study, characterised by a loss of posture and cessation of movement. These findings are consistent with previous research characterising the responses of healthy neonatal piglets and calves to a thoracic squeeze and suggest that such responses may be a generalised phenomenon across multiple species of farm mammals. Furthermore, both the cuff and rope were effective at inducing reduced responsiveness. However, of the 26 lambs induced, only six (21%) of these lambs maintained reduced responsiveness for the full 10-minute squeeze duration, all of which were squeezed using a rope. There were issues with the cuff design that affected both its efficacy at maintaining a state of reduced responsiveness and the physiological stability of the lambs during the squeeze. Further research is needed to evaluate the safety of the cuff following design modifications.

Additionally, we found that lambs up to 3 months of age respond to a thoracic squeeze. Of the nine older lambs squeezed, seven (78%) were induced into a state of reduced responsiveness. While the sample sizes were low, these results suggest that responses to a thoracic squeeze persist beyond the early postnatal period. However, further research is needed to characterise the responses of older mammals to a thoracic squeeze and explore the efficacy of this technique in adult mammals.

## 3.7 Acknowledgments

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# CHAPTER 4

# 4 Evaluating the Hypothalamic-Pituitary-Adrenal responses of healthy neonatal piglets to a thoracic squeeze

## 4.1 Abstract

Tonic Immobility (TI) is a phenomenon whereby application of sustained physical contact causes animals to enter a state of reduced responsiveness with immobility and behavioural quiescence. A technique called the ‘thoracic squeeze’, which involves applying sustained pressure to the thorax of mammals, has been observed to induce similar behavioural changes, suggesting a connection to TI. Induction into TI has been reported to require a significant increase in Hypothalamic-Pituitary-Adrenal (HPA) activity reflected by increased circulating cortisol concentrations. If the thoracic squeeze is a stimulus to TI, we would expect it to elicit a measurable increase in circulating cortisol concentration following induction. The aim of this study was to evaluate the HPA responses of neonatal piglets to a thoracic squeeze. Healthy male piglets aged between 12 and 48 hours old were subjected to no handling (n=8), thoracic squeeze applied using an inflated cuff (n=16) or sham squeeze (n=16). A single blood sample was taken from each piglet at -4 (control), 15 or 30 minutes after treatment and plasma samples were analysed for cortisol concentration. All 16 thoracic squeeze piglets induced (became laterally recumbent, closed eyes and ceased moving), while 10 out of 16 sham squeeze piglets also displayed induction behaviours. Cortisol concentration was significantly higher at 15 minutes for both the thoracic squeeze and sham-squeezed piglets than for control piglets at time -4. There were no significant differences in cortisol concentrations between -4 and 30 minutes or between thoracic-squeezed and sham-squeezed groups at 15 minutes. The significantly higher plasma cortisol levels in piglets that were induced by thoracic squeezing follow similar patterns to those observed during induction into TI, and thus provide support for the notion that this technique is a stimulus of TI. Additionally, the lack of difference in cortisol levels between the sham squeezed and thoracic squeezed piglets suggests that handling before application of the cuff is stressful, and the sustained physical contact elicited by the cuff results in immobility. This is supported by previous literature of animals induced into TI and provides further support that the thoracic squeeze is a stimulus for inducing TI.

## 4.2 Introduction

Tonic Immobility (TI) is a behavioural phenomenon whereby physical contact with an animal induces a state of immobility accompanied by behavioural quiescence and reduced responsiveness to external stimuli (Gallup 1977; Marx *et al.* 2008). This response has been observed to last anywhere from seconds to hours, with reversal of the behaviours occurring either spontaneously, or on removal of the physical contact. TI has been documented in numerous vertebrate and invertebrate species at a range of ages (Hoagland 1928b; Gallup 1977; Maser and Gallup 1977; Erhard *et al.* 1999). While the behavioural responses of TI are similar across numerous animal taxa, the stimuli used to induce the response vary significantly. Lalonde and Strazielle (2022) define TI by three stimuli required for induction: handling, restraint, and forced inversion. In this context, restraint refers to sustained physical contact, sometimes with additional application of pressure (Lalonde and Strazielle 2022). Inversion involves rapidly and forcefully placing an animal on its back or side from a standing position (Gallup *et al.* 1971b; Webster *et al.* 1981; Meyer 1990). Adding extra pressure to that already caused by restraint is documented to facilitate faster induction into the state of TI and sustaining that pressure leads to longer maintenance of the immobility response (Klemm 1966b; Gallup *et al.* 1971b; Meyer 1990; Ong 1993). Overall, there seems to be some variation to this TI stimulus definition, in that some studies have achieved TI induction without forced inversion (Rovee *et al.* 1973; Williamson *et al.* 2018).

A technique called the 'thoracic squeeze' has recently been demonstrated to induce an immobility response in neonates of several species of farmed mammals: foals, calves, piglets and lambs (Toth *et al.* 2012; Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). The squeeze induces behavioural changes similar to those observed during TI (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). The technique involves applying sustained pressure to the thorax of the animal using an inflatable cuff or a rope (Toth *et al.* 2012; Aleman *et al.* 2017; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). In the majority of cases, this causes the animal to enter lateral recumbency and become immobile and behaviourally quiescent. In terms of a stimulus to TI, the thoracic squeeze involves restraint with the application of additional pressure. However, inversion of the animal is not forcibly done as part of the induction stimulus. Instead, when the squeeze is applied, the animal loses posture spontaneously. The response is then maintained until either the squeeze is removed, or the animal spontaneously arouses (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022).

Originally, it was postulated that the squeeze re-activates a specific neonatal mechanism that operates during parturition, whereby compression of the fetus during its passage through the birth canal induces behavioural quiescence (Aleman *et al.* 2017). However, recent research evaluating the responses of lambs to a thoracic squeeze found that the response persists beyond the neonatal period, suggesting this is a generalised phenomenon rather than a mechanism that is specific to neonates (Holdsworth et al., under review). Similarities between the behavioural changes observed after application of a thoracic squeeze and during TI suggest that the squeeze may act as a pressure-based stimulus to induce TI in neonates.

To date, only behavioural similarities between responses to a thoracic squeeze and TI have been evaluated. In addition to immobility and behavioural quiescence, TI is characterised by activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011; Baker *et al.* 2019). Physical restraint stimulates activity in the HPA axis that triggers a switch from escape behaviours to complete motor inhibition and behavioural quiescence and stimulates glucocorticoid secretion (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011). Studies have demonstrated a significant increase in circulating glucocorticoid levels associated with restraint and handling of animals prior to the onset of TI. As such, glucocorticoid concentrations are highest at the time of induction, before plateauing and eventually returning to baseline as TI persists (Braud and Ginsburg 1973; Campo and Davila 2002; Jones *et al.* 2005; Brooks *et al.* 2011; Baker *et al.* 2019). Because of this, it is thought that HPA activation, accompanied by a significant increase in glucocorticoid concentration, is necessary for the induction of TI, but not in the maintenance of the response (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011).

While the behavioural effects of the squeeze are well documented, little is known about the accompanying physiological responses of neonatal farmed mammals to the squeeze, particularly cortisol responses. Considering the significant role of HPA activity in TI, exploring the cortisol responses of mammals to the application of a thoracic squeeze can provide more information about whether the squeeze can be classified as a stimulus to TI. In addition, whether the features of the of the physical contact influence the cortisol response to the thoracic squeeze has not been explored. In particular, it is not known whether the application of sustained pressure around the thorax (i.e. the actual squeeze) enhances the cortisol response compared to handling and physical restraint alone. As such, the aim of this study was to compare the HPA responses of healthy neonatal piglets to a thoracic squeeze and sham squeeze, with a view toward establishing whether or not the thoracic squeeze may be considered a stimulus for inducing TI.

We hypothesised that piglets in the thoracic squeeze group would mount a larger HPA response than piglets in the control group, and that piglets in the sham squeeze group would also have higher cortisol concentration than control piglets. However, HPA responses of sham squeeze piglets would be smaller than the thoracic squeeze piglets.

## 4.3 Methods

The study was undertaken on a commercial pig farm in the Manawatū region, New Zealand. All procedures were approved by the Massey University Animal Ethics Committee (MUAEC Protocol 21/40).

Forty healthy, male, Large White X Landrace piglets (*Sus scrofa domesticus*) aged between 12 and 48 hours old were selected for the study based on specific inclusion criteria: normal locomotion, social behaviour and body posture (Holdsworth *et al.* 2021). Piglets had to be able to stand, walk, vocalise, or move their head or limbs when undisturbed or approached by researchers. Normal social behaviour was demonstrated by seeking contact with, or reciprocating social interactions made by, the dam or littermates. Normal body posture required a curled tail and relaxed, forward-facing ears. Piglets that were small, apparently weak or had abnormal conformation (e.g. protruding forehead, shorter snout, leg deformities) were rejected to avoid any confounding impacts of underlying health problems (Alonso-Spilsbury *et al.* 2007; Baxter *et al.* 2008). Only piglets from litters of multiparous sows were selected.

Using a power calculation, an estimated sample size of 8 piglets per group was needed to detect a difference of 14 ng/ml plasma cortisol concentration, assuming a variance of 87.1 ng/ml (derived from a pooled standard error of the mean) (Morrison and Hemsworth 2020a), with 95% confidence and a power of 80%.

In order to get a sample size of 40 animals in total (n = 8 per treatment x timepoint), ethics approval was granted for up to 80 animals. Any animals for which a blood sample could not be obtained within the 2 minute time frame or animals not induced by the thoracic squeeze were not included in the dataset.

### 4.3.1 Procedures

The study took place in a farrowing house over a period of two months (October and November 2021). To reduce the effects of circadian changes in cortisol concentrations (Munsterhjelm *et al.* 2010; Martínez-Miró *et al.* 2016), piglets were tested between 9 am and 12 pm each day. As a result, a maximum of 4-5 piglets were tested in a single study day.

Forty piglets were assigned to three treatment groups using a random number generator:

1. Thoracic squeeze (TS) treatment - an inflatable cuff was secured around the thorax and inflated for 4 min before being deflated and removed.
2. Sham squeeze (SS) treatment - an inflatable cuff was secured around the thorax but not inflated and left for 4 min before being removed.
3. Control (C) - piglets were unhandled prior to blood sampling.

Within these treatment groups, piglets were randomly allocated into subsets for blood sampling based on three timepoints relative to removal of the inflated cuff or sham squeeze (Table 4). A randomised controlled trial design was used, in which each piglet had only one sample taken to prevent the cumulative effects of blood sampling on cortisol concentrations. The study included unhandled controls to separate the effects of handling from treatment. The sham squeeze group was included in order to separate the effects of handling and sustained physical contact from the squeeze itself.

**Table 4.** Treatment groups and sample sizes. Sampling times are relative to the end of the 4-minute treatment (removal of the cuff).

| | Sampling Time | | | Total |
|---|---|---|---|---|
| Group | -4 min | 15 min | 30 min | |
| Control | 8 | | | 8 |
| Sham Squeeze (SS) | | 8 | | 16 |
| | | | 8 | |
| Thoracic Squeeze (TS) | | 8 | | 16 |
| | | | 8 | |

The duration of the squeeze was based on previous research that showed that piglets which spontaneously aroused from a state of reduced responsiveness did so during the first 4 minutes following thoracic squeezing (Holdsworth *et al.* 2021). As the aim of this study was to evaluate cortisol responses specifically to the application of a squeeze (induction), 4 minutes from the start of cuff inflation to the start of cuff deflation was considered sufficient. Any piglets that did not induce (enter a state of immobility and reduced responsiveness) following thoracic squeeze application were excluded from the study.

### 4.3.2 Treatments

For the control group, piglets were removed from the pen for immediate blood sampling, without prior handling.

In the thoracic squeeze group, a selected piglet was removed from the pen and lifted on to a foam lined wooden board (Figure 1) located under a heat lamp at the caudal end of the sow's farrowing crate. An inflatable cuff, created by Holdsworth et al. (2021), was placed around the chest of the piglet and secured with velcro around the chest and a neck strap. The squeeze was applied according to Holdsworth et al. (Holdsworth *et al.* 2021) up to a maximum pressure of 180 mmHg, and maintained for 4 minutes and then the cuff was deflated and removed. Following this, the piglet was identified on its back with its treatment number using an animal-safe marker and placed back into the pen. The piglet was removed from the pen a second time for blood sampling at either 15 or 30 minutes after cuff removal, depending on sampling group.

**Figure 13.** An inflated cuff applied to the thorax of a piglet, secured with velcro and prevented from slipping onto the abdomen by a soft fabric strap.

In the sham-handling group, piglets were picked up and gently restrained on the table in the same way as the thoracic squeeze group. The inflation cuff was placed around the thorax and secured with Velcro and the neck strap, but not inflated (Figure 2). The piglet was then placed back into the farrowing pen for the 4-minute treatment duration (to avoid the need for restraint by the experimenter). After 4 minutes, the cuff was removed while the piglet remained in the farrowing pen. The piglet was removed a second time from the pen for blood sampling at 15 or 30 minutes depending on the sampling group.

**Figure 14.** A sham-handled piglet, with inflation cuff applied and secured but not inflated, after being returned to the farrowing pen for the 4-minute treatment.

Video footage was captured for the duration of each thoracic squeeze and sham squeeze treatment to keep a record of behaviours.

### 4.3.3 Discontinuation criteria

Physiological parameters such as respiratory rate and colour of the snout (shifts from pink to blue that are indicative of oxygen deficiency) were visually assessed in real time with no contact, once before the treatment and again 1 minute after the start of the treatment.

During the thoracic squeeze treatment, each piglet was assessed visually for physiological instability, characterised by increased respiratory rate or gasping (forced inspiration of air with open mouth) (Guntheroth and Kawabori 1975; Spyer 2009). Discontinuation of the squeeze occurred immediately if the piglet showed signs of physiological instability and the animal was excluded from the study.

Thoracic squeezed piglets that did not enter lateral recumbency and stop moving by 2 minutes after cuff inflation were not considered induced, and the squeeze was immediately discontinued. Piglets that did induce but spontaneously aroused before 4 minutes had the squeeze immediately discontinued and were excluded from the study.

Any piglets excluded from the study were not included in the dataset, and therefore not subjected to statistical analysis.

### 4.3.4 Data collection

Each piglet was blood sampled only once. Samples were collected at one of three time points: before treatments began on the sampling day (C), ensuring as little disturbance as possible prior to sample collection, or at 15 or 30 minutes after the end of treatment (TS or SS). These time points were selected based on previous research that demonstrated a cortisol response within 15 minutes of applying a stressful (and painful) stimulus, with concentrations beginning to return towards baseline levels by 30 min (Prunier *et al.* 2005a; Morrison and Hemsworth 2020a, 2020b). By sampling at 15 and 30 min after treatment, the aim was to capture the time course of cortisol responses to TS or SS.

Piglets were picked up and restrained in dorsal recumbency with the front legs held down against the body for blood sampling. Samples (2-4 mL) were collected from the jugular vein using a 1.5 inch 21 gauge needle and 4mL EDTA vacutainer within two minutes of the piglet being restrained. Blood samples were centrifuged immediately or after having been on ice for a maximum of 15 minutes, and up to 1 mL of plasma was extracted from the supernatant using a transfer pipette. Samples were suspended in phosphate buffered solution (PBS) for enzyme-linked immunosorbent assay (ELISA) analysis using Invitrogen Cortisol Competitive Human ELISA Kits (ThermoFisher Scientific). Although a human ELISA kit was used, the assay is commonly used for other mammals, including pigs (Thomsson *et al.* 2014; Gholib *et al.* 2019).

All piglets were weighed following blood sampling, using a luggage scale attached to a bucket. Weights were recorded and included in the statistical analysis.

Behavioural observations were assessed post hoc via video footage. Induction behaviours for the thoracic squeeze and sham squeeze groups were characterised using Behavioural Observation Research Interactive Software (BORIS) version 8.13 (Friard and Gamba 2016). Induction was characterised as follows:

Piglets collapsed into lateral recumbency and stopped moving their limbs for at least 3 seconds. In order to generalise induction behaviours between sham squeeze and thoracic squeeze piglets, eye closure, included in previous studies (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022), was excluded from the induction criteria.

### 4.3.5 Data Analysis

Differences in plasma cortisol concentrations between treatments and time points were evaluated using a linear regression in R Studio (version 4.3.0.), with an interaction of treatment and time as fixed effects, as well as the weight of the piglets after centring on the minimum weight (Posit Team 2023). A Pearson correlation coefficient  was determined for piglet weight compared to cortisol concentration. Assumptions of homoscedasticity, linearity and normal distributions were assessed visually using plots of standardised residuals against fitted values, normal quantile-quantile plot of the standardised residuals, and standardised residuals against weight (centred), and no evidence of violation of the assumptions was observed.

## 4.4 Results

A total of 64 piglets were used to obtain a sample size of 40 piglets. Of these 64 piglets, 21 were not included due to inability to obtain blood samples within the 2 minute timeframe. The remaining three piglets were excluded from the study due to discontinuation: one due to physiological instability at 113 seconds after cuff inflation, another due to no induction, and one due to spontaneous arousal at 127 seconds.

Within the 40 cortisol samples, two piglets (one SS30 and one TS30) had plasma cortisol concentrations that were considered biologically implausible (981.3 and 1653.2 ng/mL, respectively) (Parrott *et al.* 1989; Rodarte *et al.* 2004; Munsterhjelm *et al.* 2010) and were therefore excluded from the dataset prior to statistical analysis.

**Table 5:** Results of linear regression analysis comparing cortisol concentrations between treatment groups against Control as the intercept, adjusting for piglet weight.

| Coefficient Variable | Estimate Coefficient | Standard Error of the coefficient | T-value | P-value |
|---|---|---|---|---|
| **Intercept (Control)** | 83.98 | 12.62 | 6.654 | <0.001 |
| **Treatment SS 15** | 83.94 | 17.90 | 4.689 | <0.001 |
| **Treatment SS 30** | 22.93 | 18.76 | 1.222 | 0.230 |
| **Treatment TS 15** | 90.41 | 17.69 | 5.111 | <0.001 |
| **Treatment TS 30** | 14.55 | 18.24 | 0.798 | 0.431 |
| **Weight centered** | 24.66 | 17.46 | -1.412 | 0.167 |

At 15 minutes following the end of treatment, both the SS, and TS piglets had significantly greater cortisol concentrations than control piglets sampled at time -4 (C: average cort conc = 83.98 ng/mL, p = < 0.001; SS 15: average cort conc = 167.92 ng/mL (83.98 + 83.94), p = < 0.001; TS 15: average cort conc = 174.39 ng/mL, p = < 0.001; Table 5, Figure 15). Within the TS and SS treatments, cortisol concentration was significantly higher at 15 minutes than at 30 minutes post-treatment. There were no statistically significant differences between the control piglets (time -4) and the other treatment groups at 30 minutes.

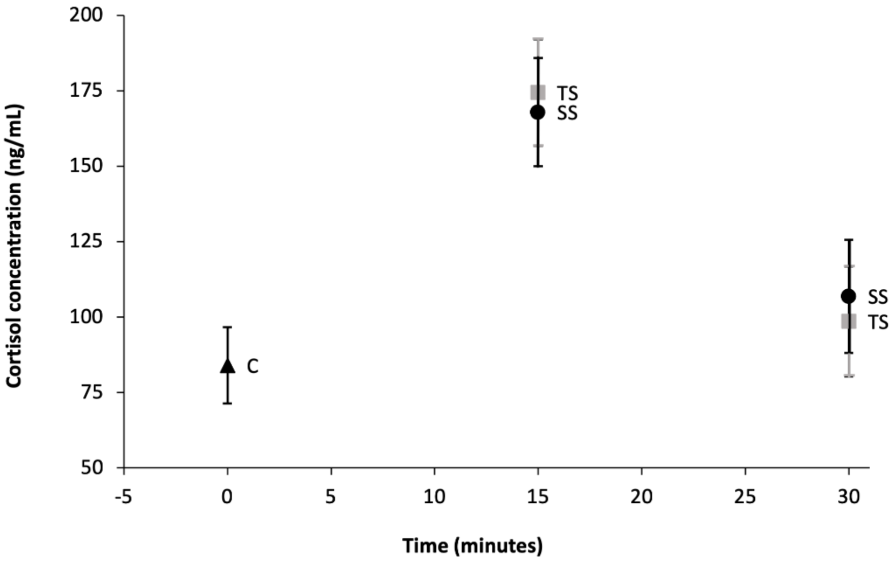

**Figure 15.** Average (± SD) cortisol concentration at each timepoint following the end of the 4-minute treatment in 38 healthy male piglets. C refers to Control piglets (n = 8); TS (grey) refers to thoracic squeeze piglets (15 min: n = 8; 30 min: n = 7) and SS (black) refers to sham-squeeze piglets (15 min: n = 8; 30 min: n = 7).

There was a very weak correlation between piglet weight and cortisol concentration (R = 0.60, p = 0.17).

### 4.4.1 Behavioural observations

All 16 piglets subjected to a thoracic squeeze were induced into a state of immobility, characterised by a loss of posture, eye closure and cessation of movement. Of the piglets that induced, all stayed immobile for the full 4 minutes before being aroused by removal of the cuff.

Unexpectedly, the behaviours displayed by some sham-squeezed piglets were similar to those shown by the squeezed piglets. Of the 16 piglets in the sham squeezed group, 10 (62.5%) also displayed loss of posture and cessation of movement after having the cuff attached and being placed back in the pen. However, none of these piglets showed eye closure following loss of posture. After loss of posture and cessation of movement, all 10 piglets vocalised continuously until they spontaneously aroused, or the cuff was removed. They responded to physical contact from littermates with 2-3 second bouts of uncoordinated kicking. Six were aroused out of immobility by removal of the cuff at the end of the 4-minute period. The remaining four spontaneously aroused before 4 minutes and displayed rapid locomotion around the pen. Two of the 10 'induced' sham squeeze piglets showed bouts of high intensity struggling lasting 5 seconds or more, consisting of coordinated, vigorous limb movements, including directed kicking towards the uninflated cuff with both hindlegs. The six sham squeezed piglets that did not 'induce' displayed loud vocalisations and bouts of fast locomotion (walking or running) for the 4-minute period after being placed back into the pen.

## 4.5 Discussion

### 4.5.1 HPA responses to application of a thoracic squeeze

The main aim of this study was to evaluate the HPA responses of healthy neonatal piglets to a thoracic squeeze to progress understanding of whether this technique is a stimulus for inducing TI rather than a low-stress therapy with a special birth-related mechanism. The increase in plasma cortisol concentrations observed in response to the thoracic squeeze appears consistent with changes demonstrated during the induction of TI. Furthermore, cortisol concentration at 30 minutes after cuff removal was not significantly different from the control piglets. Research on TI to date has demonstrated a clear increase of cortisol caused by induction into the state (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011). The observed switch from struggling to complete motor inhibition and behavioural quiescence (i.e. TI), in response to sustained physical contact in the form of restraint or sustained pressure, is accompanied by a significant rise of HPA activity in a variety of vertebrate animal species (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011). For example, plasma corticosterone concentrations were found to be significantly higher in quail sampled 8.5 minutes following induction into a state of TI compared to unhandled quail (Jones *et al.* 2005). The increase in corticosterone in quail occurred between 2 and 10 minutes after induction into TI (cessation of movement), with peak concentrations occurring within 10 minutes of TI induction (Hazard *et al.* 2008). Thus, increased HPA activity is considered to relate specifically to the induction phase as cortisol concentrations are considered to be highest at induction, before plateauing and eventually returning to baseline if TI is maintained (Braud and Ginsburg 1973; Campo and Davila 2002; Jones *et al.* 2005; Brooks *et al.* 2011; Baker *et al.* 2019).

Only one previous study has evaluated HPA responses specifically to application of a 20 minute thoracic squeeze. That study was focussed on healthy neonatal foals and applied the squeeze using loops of rope rather than a cuff (Toth *et al.* 2012). They found no difference in cortisol concentration between the pre-squeeze period and 15 minutes into the 20 minute squeeze. These findings contrast with this study, where thoracic squeeze piglets mounted a significant cortisol concentration at 15 minutes compared to control piglets sampled at -4 minutes. However, several features of the methodology may limit comparison of the results to the present study. First, the foals were acclimated to the presence of a researcher for 15 minutes prior to application of the squeeze, through gentle handling. Thus, the 'pre-squeeze' concentration may have been higher than a true baseline because of the prior handling, and as such an increase in cortisol concentration would be smaller. Given that certain forms of novel handling have been reported to elicit HPA responses in animals (Mears *et al.* 1999; Prunier *et al.* 2005a), the nonsignificant rise in cortisol concentration from 'baseline' (i.e. pre-squeeze following handling) to during and post-squeeze concentration therefore might be the result of pre-squeeze handling rather than indicating that the squeeze did not elicit an HPA response. The inclusion of an unhandled control group in the present study allowed a true baseline cortisol concentration to be determined, leading to more accurate information about the effect of a thoracic squeeze on HPA activity.

In this study, blood samples were taken within a two minute timeframe from when the piglet was picked up to prevent handling effects on cortisol concentrations. However, limited conclusions can be made about the temporal effects of a thoracic squeeze and sham squeeze on cortisol concentrations due to the limited sampling time frames. Previous research on the time-course of cortisol after the application of a physiological stressor found that a detectable increase in plasma cortisol concentration was often observed from 2 to 15 minutes, with concentrations highest at 30 minutes (Moe and Bakken 1996; Hopster *et al.* 1999; Sutherland *et al.* 2002; Prunier *et al.* 2005b; Numberger *et al.* 2016). Thus, more frequent sampling may have been useful for capturing the time course of cortisol responses. However, a limited number of sampling points were selected in order to minimise the number of animals used. Additionally, cortisol peak has been reported in the 15–30 minute period in pigs undergoing tail docking (Morrison and Hemsworth 2020a, 2020b), informing the time points used in the current study. Furthermore, this experimental design limits information about the individual variability in cortisol concentrations over time, since only one blood sample was taken from each animal. Blood sampling itself has been shown to elicit a cortisol response (Moe and Bakken 1996; Hopster *et al.* 1999; Stilwell *et al.* 2008), which may mask responses to a subsequent stressor. In sheep that were handled, and then subjected to a secondary stressor of either shearing or sham-shearing (Mears *et al.* 1999), there were no significant differences in cortisol concentrations between the two treatment groups, and the cortisol responses to shearing or sham shearing after handling did not elicit a further significant increase in cortisol (Mears *et al.* 1999). This suggests that there may have been a cumulative effect caused by the sampling. The current study overcame this limitation by adopting a randomised controlled trial study design with single sampling.

### 4.5.2 HPA responses and behaviours to a sham squeeze

The specific effects of applying pressure to the thorax, in addition to the effects of brief handling to apply the cuff, does not appear to cause a greater cortisol response than brief handling alone. Handling is known to be stressful to piglets, with handled or sham handled piglets often exhibiting higher cortisol responses than unhandled piglets (Hemsworth *et al.* 1981; Herskin and Jensen 2002; Torrey *et al.* 2009). Based on the literature, the sham squeezed piglets were expected to mount larger HPA responses than the control piglets, but smaller responses than piglets in the thoracic squeeze group.

However, this study found that cortisol responses did not differ between squeezed and sham squeezed piglets at either 15 or 30 minutes after removal of the cuff and both were higher than non-handled controls. These findings suggest that HPA responses to a successful thoracic squeeze are not different to responses from non-squeezing methods of handling and restraint. In other words, application of squeezing pressure around the thorax did not increase the stressfulness of treatment compared to the sham squeeze. In support of this, piglets in both the thoracic squeeze and sham squeeze showed similar induction behaviours. As expected, all squeezed piglets induced, characterised by a loss of posture, eye closure and cessation of movement for at least 3 seconds (Holdsworth *et al.* 2021) and maintained reduced responsiveness for the 4 minute duration. Unexpectedly, nearly two thirds of the sham-squeezed piglets also displayed some features of induction: loss of posture and cessation of movement despite sustained pressure around the thorax not being applied. There are currently no reports of sham-handled animals displaying behaviours similar to those observed in this study (loss of posture and cessation of movement) which supports the notion that these responses are unusual, and suggests that something about the application of the cuff following handling causes a type of induction in sham piglets. In contrast to the squeezed piglets, eye closure did not occur in the sham-squeezed piglets and induction was not maintained for the full 4 minutes. This suggests that the treatments applied caused qualitatively different types of induction and it is possible that the sham squeeze piglets were in a lighter plane of reduced responsiveness that did not include a maintenance period in the same way as thoracic squeeze piglets.

However, there is not enough information to compare post induction behaviours of the two groups, since the thoracic squeeze piglets remained outside the pen and undisturbed by littermates, while the sham squeezed piglets were often stimulated by activity from littermates. Returning sham squeezed piglets to the pen for the 4-minute following handling was a potential confound. However, this was done in order to eliminate the need for the piglet to be physically restrained by a researcher and to mitigate the stress associated with isolation had the piglets been kept away from the littermates and sow.

In contrast to these findings, there were no significant differences in cortisol responses of one week old piglets that received no handling and piglets that were sham castrated (restrained for 45 seconds before being returned to the pen), but castrated piglets mounted significantly higher responses than sham castrated pigs when sampled at 15 and 30 minutes (Prunier *et al.* 2005a). The findings in this study suggest that the sham squeeze was more stressful than sham castration in the study by Prunier *et al.* (2005a).This may explain why no significant differences were observed in cortisol concentration between the squeezed and sham squeezed piglets. However, piglets in the castration study were acclimated to handling prior to treatment to facilitate repeat blood sampling, which may have affected their response to sham castration (which consisted of 45 seconds of restraint).

An alternative interpretation of the lack of differences observed between sham squeeze and thoracic squeeze piglets could be that the thoracic squeeze was in fact more stressful than sham squeezing, but the sustained maintenance period for the squeezed piglets initiated recovery. In a review by Carli and Farabollini (2022a), it was suggested that the maintenance phase of TI is responsible for preparing animals physiologically for recovery out of the state. The initial HPA responses mounted by induction into TI begins to return towards baseline during the maintenance period, readying the animal for recovery out of TI (Carli and Farabollini 2022a). Therefore, it is possible that by the 15 minute sampling time point, cortisol concentration for the squeezed piglets had begun returning towards baseline, and appeared at the same concentration as sham squeeze piglets. In support of this, the sham squeezed piglets exhibited a different quality of behaviours compared to the thoracic squeeze piglets. While the squeezed piglets maintained immobility and behavioural quiescence, the sham squeezed piglets were easily stimulated by the sound and presence of littermates, and the maintenance period of the 4 minute treatment was not sustained for any sham piglets. It is possible that the sham squeezed piglets never entered a maintenance period that would have physiologically prepared them for recovery, such as with a decline of cortisol concentration towards baseline that may have occurred with the thoracic squeeze piglets.

This interpretation is further supported by previous research, whereby immature piglets subjected to tooth clipping mounted a significant HPA response as early as 5 minutes following treatment, before a return of cortisol concentration towards baseline was captured at the 15 minute sampling timepoint (Prunier *et al.* 2005a). Thus, it is possible that the same HPA responses occurred in the thoracic squeeze piglets of this study, and the sampling timepoints of 15 and 30 minutes were not frequent enough to capture the peak cortisol response to application of a thoracic squeeze. As such, the thoracic squeeze being more stressful than sham squeezing is a viable explanation. However, further research is required to provide support for this interpretation, with sampling done more frequently in order to properly evaluate the time course of HPA responses in piglets to application of a thoracic squeeze.

It should be noted that the lack of difference observed in cortisol concentration between the thoracic squeeze and sham squeezed piglets was not due to a ceiling effect, whereby all piglets reached a maximum value of cortisol concentration following treatment (Llamas Moya *et al.* 2006). In a previous study measuring HPA responses of one day old piglets to insults such as ear notching, tooth clipping, and tail docking, cortisol concentration reached as high as 250 ng/mL following treatments (Llamas Moya *et al.* 2006). Given that the highest cortisol concentration exhibited by the thoracic squeeze piglets averaged 174 ng/mL, it is unlikely that a ceiling effect occurred in this study.

Additionally, the results suggest that application of the squeeze for 4 minutes is no more stressful to piglets of this age than typical handling. Thus, the squeeze may have applications as a non-chemical form of restraint, with the purpose of immobilising animals for painful husbandry procedures. However, further research is needed to explore this potential application of the thoracic squeeze in industry settings. In particular, there is a need to explore the time course of HPA activity during the thoracic squeeze using a quick secreting hormone such as adrenocorticotrophin hormone (ACTH), and the effects of induction vs maintenance of a thoracic squeeze on ACTH concentrations. To date, no studies have explored whether HPA activity changes between the induction and maintenance period after application of a thoracic squeeze. Understanding the time course of HPA activity during each phase would further inform whether responses to a thoracic squeeze follow the pattern of HPA activity reported for TI.

## 4.6 Conclusion

Piglets subjected to a thoracic squeeze demonstrated an increase in plasma cortisol consistent with HPA activation observed with the induction of TI. The resolution of responses at 30 minutes is consistent with the reduction in circulating cortisol observed during the maintenance of TI. Of note, both the thoracic squeeze and sham-squeeze piglets exhibited increased plasma cortisol relative to unhandled controls at 15 minutes. This suggests that the thoracic squeeze was no more stressful than handling alone. Alternatively, the limited sampling time points may not have captured the peak cortisol response, meaning it is possible that the thoracic squeeze elicited a larger cortisol increase that was beginning to resolve by 15 minutes. However, the finding that almost 2/3 of sham squeezed piglets exhibited some form of induction (loss of posture and cessation of movement) may also explain the lack of treatment differences. Further research is required to define the time course of HPA activity more clearly in response to a thoracic squeeze. Should it be confirmed that a thoracic squeeze is no more stressful than sham squeezing, then the potential use of a thoracic squeeze as a non-chemical form of restraint for routine husbandry procedures could be explored.

Overall, the findings of this study appear consistent with changes in cortisol concentrations demonstrated during the induction of TI, and thus provide evidence that the thoracic squeeze may be a stimulus to TI. In order to support or refute these conclusions, future research should investigate whether or not the squeeze shares other features with tonic immobility, such as changes in brain state consistent with reduced awareness.

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# CHAPTER 5

# 5 Exploring the electroencephalographic (EEG) responses of anaesthetised neonatal piglets to a thoracic squeeze and to noxious stimuli after application of a thoracic squeeze.

## 5.1 Abstract

A technique called a ‘thoracic squeeze’, applied using sustained pressure to the thorax of animals, has been observed to cause immobility and behavioural quiescence with associated physiological changes. Similar behavioural and physiological changes have been documented during Tonic Immobility (TI) suggesting a connection between the two phenomena. TI is a temporary and reversible fear-induced state, whereby a stressor such as handling and restraint causes animals to become immobile and behaviourally quiescent. TI has been reported to cause changes in neural activity characteristic of reduced states of awareness and has been observed to have antinociception and analgesic effects on animals. The aims of this study were to evaluate the effects of a thoracic squeeze on the EEG, and on EEG responses to noxious stimuli in anaesthetised neonatal piglets. Twenty healthy male piglets aged between 12 and 48 hours old were subjected to no handling (control), or thoracic squeeze (squeeze) applied under light halothane anaesthesia. Following induction of anaesthesia, animals were instrumented with EEG electrodes and EEG was continuously recorded. After 5 minutes of either no handling or thoracic squeeze, animals in both groups were subjected to a noxious stimulus (toe clamp) for 3 seconds. EEG spectral analyses were conducted at the conclusion of the experiment. No immediate changes in the EEG were observed in response to the squeeze. Four minutes after squeeze application, an increase in F95 was observed, along with decreases in F50 and Ptot. This pattern of response was not consistent with transition to a sleep-like state or deepening anaesthesia, suggesting that either the squeeze had no effect on the state of conscious awareness, or that the model used was not suitable for detecting changes in brain activity associated with a reduced state of awareness. Likewise, application of a toe clamp in control piglets failed to induce changes in any of the EEG summary variables (F50, F95, Ptot). This unexpected finding meant that no inferences could be made about possible antinociceptive or analgesic effects of the thoracic squeeze. The lack of EEG response to a toe clamp may have been due to the stimulus itself, or a function of piglet age.

Future research exploring brain responses of mammals to a thoracic squeeze, and to noxious stimuli during the squeeze, should consider adopting a modified approach. For example, using older animals to ensure that EEG responses to noxious stimulation are fully mature, employing a more potent noxious stimulus, or utilising surgically implanted electrodes to record from conscious animals to overcome the potential confounds of general anaesthesia.

## 5.2 Introduction

The application of a "thoracic squeeze", using either a rope or inflation cuff to apply pressure around the thorax, causes animals to collapse on their side, close their eyes and stop moving (Toth *et al.* 2012). Removal of the squeeze results in animals regaining their posture and displaying normal behaviours (Toth *et al.* 2012). To date, the thoracic squeeze has been observed to cause a state of reduced responsiveness in foals, piglets, calves, and lambs of neonatal ages (Toth *et al.* 2012; Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). The mechanism underlying responses to the thoracic squeeze in mammals is not known. It was originally thought that the squeeze evokes a neonatal-specific mechanism whereby fetal compression during parturition induces behavioural quiescence (Mellor 2017). However, a recent study demonstrated that responses to the squeeze persist beyond the postnatal period, suggesting a generalised age response rather than a phenomenon specific to birth.

It has recently been speculated that responses to the thoracic squeeze may be equivalent to those observed in Tonic Immobility (TI) (Ong 1993). TI is a temporary and reversible state of immobility and behavioural quiescence that can be induced by sustained physical contact. Reversal of TI occurs through spontaneous arousal of the animal or by removal of physical contact (Gallup 1977; Marx *et al.* 2008). Numerous invertebrate and vertebrate species have been documented to be induced into TI, and the response is reported to persist with age (Hoagland 1928b; Gallup 1977; Maser and Gallup 1977; Erhard *et al.* 1999). In addition to immobility and behavioural quiescence, TI is accompanied by physiological changes, including activation of the HPA axis (Braud and Ginsburg 1973; Campo and Davila 2002; Jones *et al.* 2005; Brooks *et al.* 2011; Baker *et al.* 2019), changes in brain activity consistent with a reduced state of awareness (Schwarz and Bickford 1956; Kumazawa 1963; Lefebvre and Sabourin 1977b), and reduced responsiveness to nociceptive stimuli (Wallnau and Gallup Jr 1977; Porro and Carli 1988; de Oliveira *et al.* 1997; da Silva and Menescal-de-Oliveira 2006; Baker *et al.* 2019).

Changes in brain activity during TI are reflected by changes in electroencephalogram (EEG) activity occurring at different phases during the period of reduced responsiveness (Ong 1993). At induction, EEG patterns reportedly show desynchronisation (or low voltage, fast activity) that is characteristic of awake or alert brain states (Barratt 1965; Vanderwolf *et al.* 1975). As TI is maintained, EEG patterns show more synchronisation (high voltage, slow wave activity) (Schwarz and Bickford 1956; Kumazawa 1963; Lefebvre and Sabourin 1977b), a pattern reflective of reduced awareness. Additionally, TI is known to induce antinociception in numerous species (Baker *et al.* 2019; Terpou *et al.* 2019). It is thought that induction into TI triggers activation of antinociceptive pathways at the brainstem and spinal cord levels that modulate the transmission of noxious stimuli to the brain (Baker *et al.* 2019). The result of this is decreased behavioural and physiological responsiveness to noxious stimuli during TI (Baker *et al.* 2019; Terpou *et al.* 2019).

In addition to similarities between behavioural responses to a thoracic squeeze and TI, recent research has demonstrated similarities in the pattern of HPA activation between the squeeze and TI (Holdsworth et al., In prep). To date, no studies have characterised the brain responses of neonatal mammals to the application of a thoracic squeeze or to the application of a noxious stimulus during the squeeze. To establish a conclusive connection between the thoracic squeeze and TI, as proposed by Holdsworth et al. (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022), it is necessary to investigate changes in brain states and responses to noxious stimuli during application of the squeeze. To investigate these aspects, neurophysiological techniques, such as EEG recording, can be employed. The EEG provides a representation of neural activity in the cerebral cortex (Murrell and Johnson 2006), enabling the detection of changes in brain responses to external stimuli in mammals. Changes in the frequency and amplitude of the EEG have been used to characterise sleep-like states (Saito *et al.* 2005). Sleep is typically associated with an increase in amplitude and decrease in frequency of the EEG, with the presence of sleep spindles and k-complexes during non-rapid eye movement (REM) sleep (Saito *et al.* 2005). As such, changes in the EEG may be useful for inferring changes in the state of awareness. Additionally, changes in the frequency spectrum of the EEG recorded under light anaesthesia have been used to quantify responses to noxious stimuli and to evaluate anti-nociceptive strategies (Murrell and Johnson 2006). Light anaesthesia prevents conscious perception of an applied noxious stimulus without affecting neural transmission to the cortex, thus providing an ethical model for pain-related studies in animals. Anaesthesia provides the additional advantage of immobilising animals, thus minimising contamination of EEG traces with movement artefacts (Murrell and Johnson 2006).

The aims of this study were to evaluate the effects of a thoracic squeeze on the electroencephalogram, and to evaluate the effects of a thoracic squeeze on electroencephalographic responses to noxious stimuli in lightly anaesthetised, healthy neonatal piglets.

## 5.3 Methods

This study was approved by the Massey University Animal Ethics Committee (Approval number: 22/30). Twenty healthy Large White X Landrace piglets (*Sus scrofa domesticus*) aged between 24-48 hours old were obtained from a commercial premises (Ratanui Piggery, Halcombe) and transported to the Massey Neurosciences Lab in Palmerston North, Manawatu, New Zealand. The piglets were housed in a temperature-controlled, ventilated room until testing later the same day. Piglets were chosen for this study based on their previously demonstrated consistent responses to the thoracic squeeze (Holdsworth *et al.* 2021). Furthermore, piglets are the easiest of production animals to source in large numbers due to being non-seasonal breeders, and as such were the practical choice for the study.

A power calculation was performed using data obtained from tail docking in healthy young pigs with or without prior analgesia (Kells *et al.* 2017a). Using the average median frequency values (F50) for control vs analgesia, and based on a power of 80% and type 1 error rate of 5%, the required sample size per treatment was calculated as 8. To allow for any unforeseen issues during data collection, such as equipment failure or data contamination, a sample size of 10 animals per treatment was used.

Piglets were selected from litters based on sow parity (number of previous litters), with litters from gilts being excluded in order to prioritise the largest and healthiest piglets. Litters of 12 or fewer piglets were used to reduce the likelihood of enrolling individuals with underlying short-term or long-term pathophysiology (Mellor 1988; Svendsen 1992; Alonso-Spilsbury *et al.* 2007). Within the litter, piglets that appeared most vigorous (e.g. showing behaviours such as walking, teat seeking and sucking milk) were selected. Piglets that were small, apparently weak or had abnormal conformation (e.g. protruding forehead, shorter snout, leg deformities) were rejected to avoid any confounding impacts of underlying health problems (Mellor 1988; Svendsen 1992; Alonso-Spilsbury *et al.* 2007).

### 5.3.1 Procedures

Piglets were randomly assigned to one of two groups: thoracic squeeze (TS) or control (C). Piglets in both groups were anaesthetised using the minimal anaesthesia model developed by Murrell and Johnson (2006).

Piglets were anaesthetised with 3–4% halothane vaporised in oxygen, delivered via facemask. Once adequate anaesthesia was achieved (assessed by loss of posture, eye reflexes and limb withdrawal reflexes), sub-dermal stainless steel needle electrodes (Ambu Neuroline 24K, Ambu, Ballerup, Denmark) were applied. Following placement of electrodes, halothane concentration was reduced to achieve an end tidal concentration of 0.95–1.05 %, and baseline EEG activity was recorded for 5 minutes.

In the thoracic squeeze piglets, a custom inflation cuff device was applied according to the methods used by Holdsworth *et al.* (2021) and inflated for 10 minutes (Figure 16). The cuff was fitted around the chest and secured with Velcro. A nylon strap attached to the cuff and secured around the neck prevented the cuff from slipping down on to the abdomen. The cuff was inflated using a hand pump with an attached sphygmomanometer (pressure gauge), to a maximum of 180mm Hg (the effective pressure found to cause a state of reduced responsiveness in piglets) (Holdsworth *et al.* 2021). EEG data were collected continuously prior to application of the squeeze, and for the 10 minutes duration of the squeeze (or equivalent in control pigs).

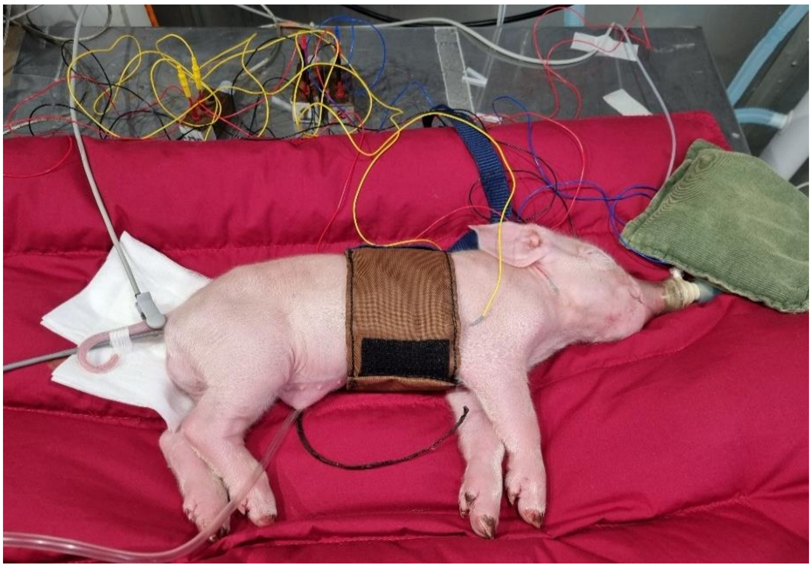

**Figure 16.** An Uninflated cuff applied to piglet. An inhalation mask administering halothane covers the nose and mouth. A temperature probe is inserted into the rectum, and pulse oximeter clipped on to the tail. EEG electrodes (red and blue leads) are attached to the scalp, and ECG electrodes (yellow leads) attached to points of the thorax.

Control piglets had no inflation cuff applied and remained untouched until application of a noxious stimulus (Figure 17). Piglets in both groups then had a known noxious stimulus (toe clamp) applied once between the toes of the hind foot at 5 min after application of the cuff or 5 min after the conclusion of baseline EEG recording (Diesch et al., 2010). To avoid attenuation of EEG responses caused by multiple applications of a noxious stimulus, the toe clamp was applied only once. EEG was then recorded for a minimum of 5 min after application of the noxious stimulus to assess responses. Following this, the thoracic squeeze was removed.

**Figure 17.** A control piglet anaesthetised with halothane, administered via face mask. A pulse oximeter is attached to the ear and a rectal probe inserted into the rectum. EEG electrodes (white, black and red leads) are attached to points in the scalp and ECG electrodes (green) are placed under the skin at points on the thorax.

At the conclusion of data collection, pigs in both groups were euthanised, as they could not be returned to the farm for biosecurity reasons and were too young to be successfully hand reared. This was carried out via an injected overdose of sodium pentobarbital whilst still under anaesthesia.

### 5.3.2 Monitoring

End tidal halothane and $CO_2$ levels, blood oxygen, heart rate, respiratory rate and rectal temperature were monitored via an anaesthetic monitoring machine, for the duration of the piglet’s time under anaesthesia. A Bair Hugger was used to maintain piglet body temperature between 38–40°C.

### 5.3.3 Statistical Analysis

Raw EEG data were manually inspected, and any artefacts, defined as periods of the recording where the voltage was outside of the recording range (out-of-range data) and therefore not feasibly generated by cortical neuronal activity, were excluded from subsequent analyses. Data were only excluded for the out-of-range period before the three summary variables were extracted (typically a few seconds at most), thus excluding the data for all three variables for the specified time (epochs) for a given pig, with all other data from the animal's recording retained for further analyses. Such out-of-range data likely represented EEG contamination due to skeletal muscle activity or manual disturbance of the recording leads. The raw data was then subjected to Fast Fourier transformation using purpose-written software (Spectral Analyser; CB Johnson, Massey University, Palmerston North, NZ, 2002), yielding the summary variables total power (Ptot), median frequency (F50) and 95% spectral edge frequency (F95) for consecutive 1-second epochs. Mean F50, F95 and Ptot were calculated for 60 consecutive epochs (60 seconds; 1 minute) at the following time points: 60 seconds prior to cuff inflation and 60 seconds after cuff inflation (squeeze only); 60 seconds prior to toe clamp and 60 seconds after toe clamp (control and squeeze).

Mixed-effect linear regressions were built for each of the three outcomes of interest with an interaction between treatment and timepoint as a fixed effect and pig as a random effect. The analyses were conducted in R Studio (version 4.3.0.), using packages nlme (version 3.1-162), multicomp (version 1.4-23) and emmeans (version 1.8.6) (Posit Team, 2023). Animals acted as their own controls in order to reduce the number of piglets needed for the study. Assumptions of homoscedasticity, linearity and normal distributions were assessed visually using plots of standardised residuals against fitted values and normal quantile-quantile plot of the standardised residuals, and no evidence of violation of the assumptions was observed.

## 5.4 Results

EEG data were successfully collected from all 20 piglets. Application of a toe clamp in control piglets (no squeeze) failed to induce a statistically significant change in any of the EEG summary variables (F50, F95, Ptot) (Figure 18). Additionally, application and inflation of the cuff caused no changes in the EEG over the first 60 seconds after inflation. The proportion of variance that was due to variation between pigs was 95.3% for F50, 87.4% for F95 and 87.9% for Ptot.

**Median Frequency**

There were no signficant differences found between groups in the mixed effects linear regression (Table 6).

**Table 6:** Mixed-effects linear regression with an interaction between treatment and timepoint for Median Frequency (F50), with pig as a random intercept.

| Coefficient Variable | Estimate Coefficient | Standard Error of the coefficient | T-value | p-value |
|---|---|---|---|---|
| Intercept | 13.7 | 0.66 | 20.8 | <0.001 |
| Control post-clamp | 0.06 | 0.20 | 0.32 | 0.75 |
| TS baseline | 0.82 | 0.93 | 0.88 | 0.39 |
| TS cuff | 0.57 | 0.93 | 0.61 | 0.55 |
| TS pre-clamp | 0.33 | 0.93 | 0.35 | 0.73 |
| TS post-clamp | 0.33 | 0.93 | 0.35 | 0.73 |

### Spectral Edge Frequency

There were no signficant differences found between groups in the mixed effects linear regression (Table 7).

**Table 7:** Mixed-effects linear regression with an interaction between treatment and timepoint for Spectral Edge Frequency (F95), with pig as a random intercept.

| Coefficient Variable | Estimate Coefficient | Standard Error of the coefficient | T-value | p-value |
|---|---|---|---|---|
| Intercept | 27.22 | 0.20 | 136.59 | <0.001 |
| Control post-clamp | 0.03 | 0.10 | 0.29 | 0.77 |
| TS baseline | 0.09 | 0.28 | 0.31 | 0.76 |
| TS cuff | 0.16 | 0.28 | 0.58 | 0.56 |
| TS pre-clamp | 0.38 | 0.28 | 1.35 | 0.19 |
| TS post-clamp | 0.57 | 0.28 | 2.02 | <0.06 |

### Total Power

There were no signficant differences found between groups in the mixed effects linear regression (Table 8).

**Table 8:** Mixed-effects linear regression with an interaction between treatment and timepoint for Total Power (Ptot), with pig as a random intercept.

| Coefficient Variable | Estimate Coefficient | Standard Error of the coefficient | T-value | p-value |
|---|---|---|---|---|
| Intercept | 15.42 | 1.51 | 10.19 | <0.001 |
| Control post-clamp | 0.56 | 0.74 | 0.75 | 0.46 |
| TS baseline | 0.17 | 2.14 | 0.08 | 0.94 |
| TS cuff | -0.07 | 2.14 | -0.03 | 0.97 |
| TS pre-clamp | -1.48 | 2.14 | -0.69 | 0.49 |
| TS post-clamp | -1.84 | 2.14 | -0.86 | 0.39 |

Four minutes after cuff inflation, F50 decreased (relative to pre-cuff baseline; Table 6, Figure 18a); F95 increased (relative to pre-cuff baseline AND 1-minute after cuff inflation; Table 7, Figure 18b); and Ptot decreased (relative to pre-cuff baseline AND 1-minute after cuff inflation; Table 8, Figure 18c). Applying a toe clamp during the squeeze (at 5 minutes) resulted in a small increase in F95 relative to pre-clamp (Table 7, Figure 18b), whereas F50 remained lower than the pre-cuff baseline. Similarly, Ptot remained lower than baseline and decreased relative to the first 60 seconds following cuff inflation (Table 8, Figure 18c).

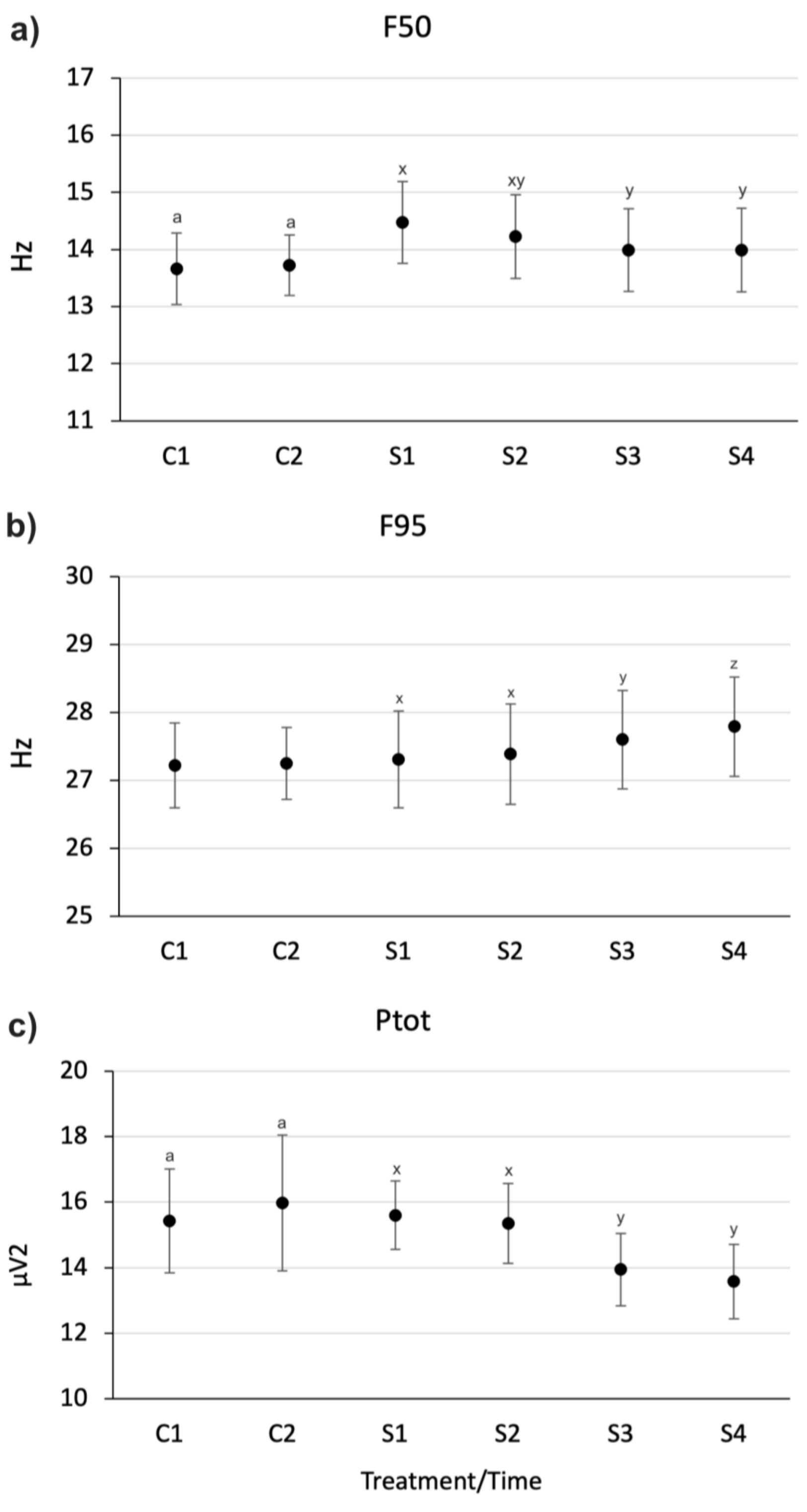

**Figure 18.** Changes in the mean (+/-SEM), obtained from the mixed-effects modelling, for a) median frequency (F50), b) spectral edge frequency (F95) and c) total power (Ptot) of the EEG in lightly anaesthetised piglets subjected to a noxious stimulus (toe clamp) with or without a thoracic squeeze. Squeezed piglets (S; n=10) had a thoracic squeeze applied using an inflation cuff, whereas control piglets (C; n=10) did not. C1 = control mean 1-minute prior to toe clamp; C2 = control mean 1-minute after toe clamp; S1 = squeeze mean 1-minute prior to cuff inflation, S2 = squeeze mean 1-minute after cuff inflation; S3 = squeeze mean 1-minute prior to toe clamp (4 minutes after cuff inflation); S4 = squeeze mean 1-minute after toe clamp. Superscripts denote significant differences between sampling points within treatment group (P<0.06).

## 5.5 Discussion

This study aimed to evaluate the effects of a thoracic squeeze, and the effects of a noxious stimulus applied during the squeeze, on the EEG of healthy neonatal piglets.

### 5.5.1 EEG responses to toe clamping

Noxious stimuli typically elicit an increase in F50 and a decrease in Ptot of the mammalian EEG, often accompanied by an increase in F95 (Murrell and Johnson 2006). In control piglets (not subjected to a thoracic squeeze), no changes in the EEG frequency were observed between the pre-clamp baseline and the 60-seconds after clamp application.

There are two potential explanations for this unexpected finding. Either the toe clamp was not sufficiently noxious to elicit a measurable response in the EEG, or the very young age of the piglets influenced their EEG responses (or a combination of both).

A previous study that characterised EEG responses to tail docking in lightly anaesthetised pigs aged between 1 and 20 days demonstrated that the magnitude of responses to the stimulus increased with increasing postnatal age, with adult-like responses evident from 10 days of age (Kells *et al.* 2017b; Kells *et al.* 2019). A similar phenomenon was observed in lambs subjected to castration (McCracken *et al.* 2010). The authors concluded that the smaller responses observed in younger animals likely indicated qualitative differences in pain perception compared to older animals. It has been postulated that this may be due to the lingering effects of in-utero chemical mechanisms responsible for maintaining behavioural quiescence during birth, which also exert anti-nociceptive effects (Diesch *et al.* 2010; Kells *et al.* 2019). Together, these results suggest that age played a significant role in the EEG responses of piglets to noxious stimuli and thus may explain why EEG responses to a toe clamp in both the control and thoracic squeeze piglets were absent in the current study. Given that responses to a thoracic squeeze have not previously been evaluated in older pigs, one-day-old piglets were selected for the present study based on the demonstrated efficacy of the squeeze at this age in previous research.

Alternatively, it is possible that the noxious stimulus applied was not sufficient to elicit a change in the EEG. Different noxious stimuli are reported to elicit different EEG responses (Reiser *et al.* 2022). Studies that have combined somatic and visceral noxious stimuli (such as tail docking) have demonstrated measurable changes in the EEG, even in young mammals (Kells *et al.* 2017b). A study evaluating EEG responses of anaesthetised lambs at 2 weeks and 4 weeks old to castration (a combined somatic and visceral noxious stimulus) found that younger lambs demonstrated a greater cortical response to the visceral components of castration. In contrast, older lambs exhibited EEG changes consistent with castration being perceived as a somatic noxious stimulus (Johnson *et al.* 2005). Thus, it is possible that the toe clamp used in the piglets may not have been perceived as a somatic noxious stimulus and may explain why changes in the EEG were not observed. As such, a combined visceral and somatic noxious stimulus such as tail docking, or other surgical stimulus, may be better suited for evaluating changes in EEG in response to nociception in young piglets. Tail docking was excluded as a possible noxious stimulus due to the fact that it combines somatic and visceral noxious stimulation, and thus may have overwhelmed any possible antinociceptive effects of the thoracic squeeze. In contrast, toe clamping was not considered as supramaximal as tail docking, and was considered less likely to override any potential antinociceptive effects of the thoracic squeeze. The effects of toe clamping on EEG responses have been previously explored in tammar wallabies (Diesch *et al.* 2010) that showed statistically significant changes in the EEG indicative of noxious stimulation. Additionally, previous research evaulating minimum alveolar concentration using isoflurane in newborn, characterised toe clamping as noxious and therefore stimulating (Eger *et al.* 1988, Satar *et al.* 1996).

No inferences can be made about possible antinociceptive or analgesic effects of the thoracic squeeze, given that there were no responses in the control group to compare to. One other study previously explored behavioural pain responses of conscious neonatal foals after application of a thoracic squeeze (Toth *et al.* 2012). Following application of a graded noxious electrical stimulus to the tail, behavioural responses including withdrawal reflexes, tail, limb, head or neck movements, kicking and/or coordinated head movements towards the electrodes in the tail were evaluated (Toth *et al.* 2012). It was reported that the 8 foals tested showed increased pain thresholds, from 3.11 V pre-squeeze, to 3.85 V during the squeeze (Toth *et al.* 2012). However, this study did not include a control group, therefore it cannot be determined whether the change in threshold was due to the squeeze or due to other factors, such as habituation to the stimulus.

### 5.5.2 EEG responses to application of a thoracic squeeze

There were no initial changes observed in the EEG frequency spectrum immediately after application of the inflation cuff. Five minutes after squeeze application, decreases in total power and median frequency were observed, along with an increase in spectral edge frequency. This pattern of responses is not consistent with changes reported during deepening anaesthesia or sleep-like states. For example, in EEG recordings from young pigs during non-REM sleep, brain activity was characterised as high amplitude, low frequency (consistent with decreased median and spectral edge frequency, and increased total power) with occurrences of k-complexes and sleep spindles (Saito *et al.* 2005). Similarly, EEG during induction of general anaesthesia is characterised by a steady decrease in median frequency and increase in total power with increasing anaesthetic depth (Tonner and Bein 2006). The lack of changes in the EEG characteristic of sleep or an anaesthetised state suggests that either the squeeze has no effect on the state of conscious awareness, or that the model used was not suitable for detecting changes in brain activity associated with a reduced state of awareness.

Previous research has evidenced a reduced state of awareness in non-anaesthetised neonates after application of a thoracic squeeze (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Specifically, neural reflexes were documented to be reduced or completely abolished in piglets, and in some cases, in calves and lambs (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Piglets in particular demonstrated consistently reduced or absent palpebral reflexes (Holdsworth *et al.* 2021). Palpebral reflexes are commonly used as an indicator of anaesthetic depth, as a proxy for changes in states of awareness (Guntheroth and Kawabori 1975; Spyer 2009). The complete absence of a palpebral reflex is considered to reflect a state of unconsciousness (Guntheroth and Kawabori 1975; Spyer 2009).

Only one other study has attempted to characterise the changes in brain activity of mammals after application of a thoracic squeeze (Toth *et al.* 2012). The study used subcutaneous needle electrodes to record EEG from three healthy foals during the thoracic squeeze. Statistical analyses were not carried out, but EEG activity reportedly changed after squeeze application (Toth *et al.* 2012). Visual inspection of the raw EEG reportedly showed characteristics of wakefulness punctuated by short periods characteristic of sleep, such as k-complexes and sleep spindles. However, the lack of a non-squeezed control group meant that no causal inferences could be made (Toth *et al.* 2012). Furthermore, freely behaving, conscious neonates are documented to display sleep characteristics in awake EEG (Mysinger *et al.* 1985), potentially confounding data interpretation. The current study did not attempt to evaluate sleep-like characteristics in the raw EEG, due to the confounding effects of general anaesthesia, which can inhibit sleep patterns in EEG recordings (Akeju and Brown 2017).

A further possible explanation for the lack of EEG responses to a thoracic squeeze under anaesthesia relates to the tonic immobility theory. It may be that induction into a state of TI requires conscious perception and integration of sensory stimuli. The orienting response, as the initial stage of the defence cascade, is understood to require conscious sensory processing in order to trigger emotional responses from the amygdala (Marx *et al.* 2008; Terpou *et al.* 2019). General anaesthesia abolishes conscious perception (Otto 2008), potentially inhibiting the orienting response and consequently preventing induction into TI. As such, it is possible that induction into a state of reduced responsiveness after application of a thoracic squeeze also requires conscious perception. If the thoracic squeeze is in fact a stimulus of TI, then it seems likely that the requirement for conscious perception is equivalent.

Future research exploring brain responses of mammals to a thoracic squeeze, and to noxious stimuli during the squeeze, should consider adopting a modified approach to that used in the present study. For example, the use of pigs aged 10-days or greater would ensure that EEG responses to noxious stimulation are fully mature. This would also enable the use of surgically implanted electrodes, enabling EEG monitoring in non-anaesthetised animals, meaning that characteristics such as sleep spindles and k-complexes could be evaluated alongside changes in the frequency spectrum. Because the aim of the current study was to assess EEG responses in neonatal piglets, surgically implanted electrodes were not an option, as piglets need to be robust enough to tolerate removal from the sow and general anaesthesia for electrode placement. Additionally, a period of recovery after surgery is required prior to testing, making it impossible to evaluate 12-24 hour old piglets. Future studies should also consider using a combined visceral/somatic noxious stimulus.

## 5.6 Conclusion

The current study did not observe significant changes in the piglet EEG in response to a toe clamp in any piglets. Therefore, no definitive conclusions can be drawn regarding the potential antinociceptive or analgesic effects of the thoracic squeeze. The lack of response in control piglets may be attributable to the type of noxious stimulus, or the very young age of the piglets. Future research should consider using a more potent noxious stimulus, such as tail docking using clippers, which has been demonstrated to elicit EEG changes in young piglets, and/or using piglets greater than 10 days of age.

This study did not identify changes in the EEG consistent with reduced awareness in piglets undergoing a thoracic squeeze. However, based on previous evidence of attenuation of neural reflexes during the squeeze, along with the potential confounding effects of general anaesthesia, this should be interpreted with caution. Additionally, these limitations make it challenging to compare EEG responses to a thoracic squeeze with those reported during TI. Further research is necessary to refine the methodology to explore whether piglets exhibit frequency changes in EEG activity consistent with changes in awareness and nociception during application of a thoracic squeeze.

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# CHAPTER 6

# 6 GENERAL DISCUSSION

Despite recommendations in the veterinary industry to use the thoracic squeeze as a therapy for abnormally behaving neonatal farm mammals and possibly as a ‘low stress’ method of restraint, little has been done to understand the mechanisms underlying the technique and the animal’s experience of being squeezed.

The research presented in this thesis explores whether the responses of neonatal farmed mammals to the thoracic squeeze, as applied in previous studies, are consistent with features of the generalized phenomenon known as Tonic Immobility. It provides the first structured exploration into potential mechanisms underlying responses to the thoracic squeeze and provides novel information about its relation to TI. The major findings of each experiment are summarised below, followed by consideration of their overall consistency with TI. Generally, these studies show that responses to the thoracic squeeze are consistent with features of TI, and so the squeeze may tentatively be classified as a stimulus for eliciting TI. I then discuss the limitations of this body of work for exploring the squeeze as a stimulus to TI in neonatal mammals, followed by suggestions for future work which would extend this investigation of the squeeze as a manifestation of TI and begin to delve into the affective experiences of animals subjected to a thoracic squeeze.

## 6.1 Tonic Immobility

Tonic Immobility is a well-documented phenomenon, whereby sustained physical contact on the body of an animal causes them to enter a state of temporary and reversible immobility (Gilman *et al.* 1950). TI can be characterised by features of the stimulus required to induce the state and by features of the responses that occur while in the state. Following application of the stimulus, a suite of general responses occur, which are characterized into three phases: induction, maintenance, and recovery (Ong 1993) (Figure 19). These general responses have been reported across a range of vertebrate and invertebrate species and can be elicited in young and adult animals.

## 6.2 Major Findings

### 6.2.1 Stimulus

Key features of stimuli that cause animals to enter a state of TI are a brief period of handling, followed by sustained physical contact with the animal’s body (restraint). Inversion of the animal before or following restraint is added to facilitate faster induction into TI (Lalonde and Strazielle 2022). Sustained physical contact is applied either by a hand or using a facilitatory stimulus (objects such as rope, cloth, troughs, sandbags). The restraint is often removed following successful induction into reduced responsiveness. Pressure additional to that caused by restraint is known to facilitate a faster induction into TI, and is often included in the induction process (Lalonde and Strazielle 2022).

The thoracic squeeze, as applied to neonatal mammals in these and previous studies, has features consistent with other stimuli used to induce TI. A thoracic squeeze is applied by handling an animal to limit its movement, followed by application of restraint, i.e. sustained physical contact with pressure. Throughout my three studies, the thoracic squeeze stimulus applied was either a rope or inflation cuff, with an initial period of handling and restraint to apply the stimulus. This period of handling for squeeze application was a similar duration to what has been done in TI studies (Lalonde and Strazielle 2022).

However, the restraint and additional pressure caused by the thoracic squeeze stimulus was significantly longer than that typically required to induce TI (Lalonde and Strazielle 2022). TI can be induced from mere seconds of stimulus application, whereas the thoracic squeeze was maintained for up to 10 minutes in my studies (10 minutes – Chapter 3 & 5; 4 minutes – Chapter 4). Despite the wide range in the duration of stimulus application, nearly all animals in my studies induced into reduced responsiveness, similar to what has been observed in TI. Thus, it suggests that the initial handling prior to stimulus application has more of an effect on induction into reduced responsiveness (or TI) than restraint (sustained physical contact). This is supported by the results from the cortisol study (Chapter 4), where the thoracic squeeze piglets and sham squeeze piglets demonstrated a similar increase in cortisol concentrations at 15 minutes compared to control piglets, perhaps due to the initial human handling prior to application of the treatment.

Unlike other stimuli used to induce TI, the thoracic squeeze does not include forced inversion of the animal. Induction of TI using other methods involves forced inversion of the animal onto its side or back. In contrast, application of a thoracic squeeze in both previous research (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022) and in my studies caused spontaneous inversion in most animals of multiple species, whereby the animal collapsed on to its side when it lost posture. Thus, inversion still occurs as part of the induction process, but arises as part of a response rather than as a feature of the stimulus.

The finding in my studies that most neonatal lambs and piglets induced into reduced responsiveness without being inverted supports the possibility, suggested by Lalonde and Strazielle (2022), that inversion is not required for induction into TI. The literature supports this possibility, with a study in zebra sharks utilising only handling and restraint of the tail with additional pressure for inducing TI (Williamson *et al.* 2018). The sharks became immediately immobile without forced inversion of the body and with no spontaneous inversion happening following restraint (Williamson *et al.* 2018). Likewise, wild scorpions picked up by experimenters rapidly induced into TI, with most scorpions assuming a ventral immobility posture (Triana *et al.* 2022). In addition, TI was induced by restraint in the ventral position in young domestic chicks, and inversion was neither forced nor spontaneously occurring after induction into TI in this instance (Rovee *et al.* 1973).

This proposal is further supported by reports of TI induction in natural settings involving capture and restraint of a prey animal by a predator (Klemm 1966b; Gallup *et al.* 1971a; Gallup 1977; Jones 1986b; Monassi *et al.* 1999; Humphreys and Ruxton 2018). In such cases, induction does not apparently require inversion of the prey animal, and restraint from predators often prevents spontaneous inversion. In a bid to recreate a 'natural' TI scenario, wild red foxes were penned with wild-caught and pen-reared ducks and the reactions of captured ducks observed (Sargeant and Eberhardt 1975). Nearly all captured ducks were induced into TI when restrained around the thorax by the foxes' mouths and assumed ventral immobility without inversion.

It can therefore be concluded that the thoracic squeeze, as applied in my three studies, meets the relevant criteria for a stimulus to induction into tonic immobility.

### 6.2.2 Responses

#### 6.2.2.1 Age-related effects on responses to a thoracic squeeze

My first study (Chapter 3) aimed to evaluate whether responses to the thoracic squeeze could be elicited in older animals, i.e. those outside the early postnatal period. This aim was developed to support differentiation between two main theories about the mechanisms underlying responses to the thoracic squeeze. It was originally posited that responses of neonatal mammals to a squeeze relate to the natural compression of the head and chest that occurs during the fetus's passage through the birth canal (Aleman *et al.* 2017). During the birth process, this compression is thought to facilitate a neurohormonally-driven transition into a deeper state of unconsciousness and motor quiescence to facilitate safe labour. Once the animal is born and compression is relieved, a different set of hormones and environmental factors support the newborn becoming conscious and behaviourally active in order to express important survival behaviours (Aleman *et al.* 2017). Mimicking this compression on neonatal animals was proposed to act as a therapy for abnormally behaving newborns, by temporarily re-invoking a state of reduced or absent consciousness. This was thought to 'reset' their brains to facilitate normal consciousness and expression of healthy newborn behaviours following removal of the squeeze. As this theory centres around the birth process and associated peri-natal neurohormonal environment, it predicts that observed responses to a thoracic squeeze would become extinct with increasing postnatal age.

I found that lambs of 3 weeks and 3 months of age showed induction behaviours consistent with those observed in neonatal lambs and with what has been reported in healthy neonatal mammals of other species (Holdsworth *et al.* ; Toth *et al.* 2012; Stilwell *et al.* 2019; Holdsworth *et al.* 2021). Therefore, responses to a thoracic squeeze appear to persist past the neonatal period, at least in this species, and a specific birth-related mechanism is less likely to explain the effects of a thoracic squeeze than a more generalized mechanism. A larger study and reliable method of applying squeeze to older lambs (see section 6.5.1 below) is needed to confirm these findings.

TI is a phenomenon that is documented to occur in young and adult animals (Gilman *et al.* 1950; Rovee and Luciano 1973; Prestrude 1977). In particular, induction behaviours, as well as the associated neural and physiological responses characteristic to each phase of TI, are reported to remain qualitatively consistent across different ages (Gilman *et al.* 1950; Rovee and Luciano 1973; Prestrude 1977). Thus, the findings in lambs are consistent with the idea that responses of neonatal mammals to a thoracic squeeze are a manifestation of TI, which remains consistent in older animals.

#### 6.2.2.2 Further evidence of generalized mammalian response to thoracic squeeze

A prominent feature of TI is that is has been observed to occur in most species studied to date - it is a generalised phenomenon that spans multiple taxa (Ong 1993). Prior to this research, the thoracic squeeze had only been applied to healthy neonatal foals, piglets and calves, as well as unhealthy foals, calves and lambs (Toth *et al.* 2012; Aleman *et al.* 2017; Stilwell *et al.* 2019; Flora *et al.* 2021; Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Previously, there was no information about the responses of healthy neonatal lambs to a thoracic squeeze. In my first study (Chapter 3), I found that neonatal lambs responded to a thoracic squeeze with the same induction behaviours as observed in healthy piglets and calves previously. These behaviours were generally consistent with what has been reported for mammals induced into TI. This provides further support for the generalizable nature of responses to a thoracic squeeze and their consistency with what has been reported during TI.

The lambs' reactions to a thoracic squeeze differed somewhat from those seen in other squeezed mammals, but this does not discount the possibility that their response was indicative of TI. Ninety five percent of the neonatal lambs squeezed with a rope or inflatable cuff were induced into a state of reduced responsiveness, characterised by loss of posture and cessation of movement that lasted for up to 10 minutes. Animals induced into TI express similar behaviours to those observed in the lambs of this study. Generally, the application of handling and restraint with or without inversion causes animals to cease moving along with loss of righting reflexes that is maintained beyond the removal of restraint (Lalonde and Strazielle 2022).

None of the lambs in my study closed their eyes, suggesting that the reduced responsiveness was qualitatively different from what has been previously reported for piglets of the same age that consistently closed their eyes during the squeeze (Holdsworth *et al.* 2021), though was consistent with calves where eye closure reportedly did not occur (Holdsworth *et al.* 2022). While eye closure is sometimes reported to occur during TI, this is not consistent among individuals or species (Hoagland 1928b; Rovee and Kleinman 1974; Webster *et al.* 1981; Gentle *et al.* 1989). This suggests that eye closure is not a requirement for the classification of the thoracic squeeze as a stimulus for eliciting TI.

Additionally, most lambs subjected to the thoracic squeeze in my study retained their palpebral reflexes following induction, a finding that is also consistent with observed responses of neonatal calves to a thoracic squeeze (Holdsworth *et al.* 2022) but contrasts with piglets (Holdsworth *et al.* 2021). These results align with what has been reported for neural reflexes during the maintenance phase of TI. Pedal reflexes usually become reduced or absent, though palpebral reflexes often remain present for the duration of TI (Crawford 1977; Carli *et al.* 1984; Porro and Carli 1988; Gentle *et al.* 1989; Overeem *et al.* 2002). In particular, palpebral reflexes have been reported as present during TI for animals that do not display eye closure, suggesting that the quality of reduced responsiveness may vary at individual and species levels (Ong 1993). Muscle tone has also been reported to reduce during the maintenance phase of TI in vertebrate species, and is associated with reduced or absent spinal reflexes (Crawford 1977; Carli *et al.* 1984; Porro and Carli 1988; Gentle *et al.* 1989; Overeem *et al.* 2002). The documented variability in responses during TI further support the notion that the thoracic squeeze may be characterised as a stimulus for inducing TI.

#### 6.2.2.3 Hypothalamic-Pituitary-Adrenal (HPA) responses to a thoracic squeeze in piglets

A number of studies have demonstrated characteristic changes in HPA activity in response to application of TI induction stimuli (handling, restraint and inversion) (Braud and Ginsburg 1973; Campo and Davila 2002; Jones *et al.* 2005; Brooks *et al.* 2011; Baker *et al.* 2019). TI is considered a fear-induced state, and the increase in cortisol concentration has been associated with TI being perceived as stressful by animals (Marx *et al.* 2008). The aim of the study presented in Chapter 4 was to evaluate the HPA responses of neonatal piglets to a thoracic squeeze. If the thoracic squeeze can be classified as a stimulus for inducing TI, then HPA responses to a thoracic squeeze would reflect patterns observed during TI.

All 16 squeezed piglets lost posture and ceased movement within 2 minutes of application of the squeeze (using an inflatable cuff) and were maintained in this state for the full 4 minutes of squeeze application. Squeezed piglets had significantly higher plasma cortisol 15 minutes after removal of the squeeze when compared to control piglets. At 30 minutes after the end of the squeeze, plasma cortisol concentration of the squeezed piglets was not different from controls. These results suggest that a 4-minute squeeze caused a marked but relatively brief HPA response in neonatal piglets. Likewise, animals of other species induced into TI using methods such as restraint (sustained physical contact with or without additional pressure) and inversion have been reported to mount significant cortisol responses (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011).

In support of this, sham squeezed piglets also had higher plasma cortisol at 15 minutes after capture and handling compared to controls, a response which had apparently resolved by 30 minutes; no significant differences were observed between the squeezed and sham squeezed piglets. This response was unexpected, given that sham squeezed piglets received only a handling stimulus, whereas both the thoracic squeeze and stimuli inducing TI involve handling and sustained physical contact (restraint) as part of the induction process.

Carli and Farabollini (2022a) note that HPA activity plays a significant role in the induction phase of TI responses and is specifically related to the handling and restraint (sustained physical contact) that precedes immobility and behavioural quiescence. A study evaluating corticosterone responses in quail subjected to TI induction or routine weighing found that both treatments elicited increased adrenocortical responses detectable around 9 minutes later, when compared to control animals (Jones *et al.* 2005). Quail were handled for approximately 1 minute before either being induced into TI (15s restraint and inversion followed by 1 minute of TI), or restrained in a cylinder weigh tube for 30s. Despite the differences in handling and restraint between the two treatments, there was no significant difference in corticosterone concentration (Jones *et al.* 2005). These results provide further support that the handling preceding TI induction is responsible for HPA activation, and suggests that while HPA activation is a feature of TI, further research is required to determine whether it is responsible for induction as has been previously claimed (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011).

Also unexpectedly, nearly all the sham squeeze piglets demonstrated some characteristic induction behaviours. Typically, neonatal piglets that are handled briefly and then returned to the pen do not collapse into lateral recumbency and cease movements (Hemsworth *et al.* 1981; Morrison and Hemsworth 2020a, 2020b). However, roughly 62% of sham squeezed piglets showed behaviours characteristic of induction (loss of posture and cessation of movement) following a brief 30 second handling period to secure the (uninflated) cuff before being returned to the pen for the 4 minute treatment. This unexpected response could be taken to mean that the additional pressure applied to the thorax when the cuff was inflated during restraint (sustained physical contact, as described by Lalonde and Strazielle (2022)) is not necessary to induce an animal into TI. However, the behaviours of the sham squeezed piglets suggests they entered into a type of reduced responsiveness that was qualitatively different to that of the thoracic squeezed piglets. Specifically, none of the sham squeezed piglets displayed eye closure during the 4 minute treatment, which is expected for induced piglets (Holdsworth *et al.* 2021). However, the reason for the unusual behavioural response of the piglets is unknown.

Further research is needed to assess the degree of restraint (sustained physical contact with additional pressure) required for TI induction and whether additional pressure is necessary to induce animals into a state of reduced responsiveness. Pressure additional to that caused by restraint has been demonstrated to elicit a faster induction into TI, as well as a longer maintenance phase (Klemm 1966b; Ong 1993). As such, it may be that the pressure caused by the thoracic squeeze acts as a facilitatory stimulus for inducing TI that produces a deeper state of reduced responsiveness. To assess this, research could focus on evaluating the behavioural and physiological responses of animals to varying degrees of cuff inflation, from no inflation to full cuff inflation. This would enable measurement of the effects of varying intensities of restraint on induction.

The time course of cortisol responses across the different phases of TI has not been explicitly studied, though the initial increase in cortisol/corticosterone concentration reported following induction suggests this is related to the restraint and handling of animals prior to TI induction (Carli 1977; Gallup 1977; Porro and Carli 1988; Hazard *et al.* 2008; Zulkifli *et al.* 2009; Brooks *et al.* 2011). Additionally, studies of TI indicate that plasma cortisol concentration plateaus before returning towards baseline in animals that enter the maintenance phase of TI (Braud and Ginsburg 1973; Campo and Davila 2002; Jones *et al.* 2005; Brooks *et al.* 2011; Baker *et al.* 2019). Rabbits that were induced into TI (using restraint for 30s coupled with inversion) and maintained (TI lasting longer than 30s) had a greater corticosterone concentration than rabbits that were induced but not maintained (TI lasting less than 30s) (Carli *et al.* 1979). Thus, further research is needed to tease apart the time course of adrenocortical responses to both TI-inducing stimuli and the thoracic squeeze. It is thought that the maintenance phase physiologically prepares animals for recovery out of the state, as demonstrated by decreases in HPA activity and increased parasympathetic activity during maintenance of TI (Carli and Farabollini 2022a).

In my study, the effects of induction and maintenance on plasma cortisol concentration in the piglets could not be separated, due to the short 4 minute duration of the thoracic squeeze relative to the time of blood sampling and the fact that the induction stimulus was applied for the full 'maintenance' period. However, the cortisol responses observed in piglets in the thoracic squeeze group demonstrated a similar pattern to HPA responses of animals induced into TI, and together with the behavioural responses observed, provides further support that the thoracic squeeze may be a stimulus for inducing TI. Furthermore, consistent with the notion that induction into TI is reportedly perceived as stressful, the acute rise in cortisol concentration of squeezed piglets suggests that induction into reduced responsiveness may be perceived as stressful to the animals.

In the second study, it is possible that the thoracic squeeze elicited a peak in cortisol concentration that was not reflected at the 15 minute sampling time point and that squeezed piglets were sampled when that peak was already resolving as has been proposed to occur in TI studies (Carli and Farabollini 2022a). Previous studies on TI in other species such as quail have demonstrated a rapid increase in corticosterone concentration following induction, with a detectable increase occurring as early as 2 minutes after treatment and as late as 15 minutes, though this could be due to the timing and frequency of sampling (Jones *et al.* 2005; Hazard *et al.* 2008). It is thus possible that squeezed piglets mounted a peak cortisol concentration higher than that of the sham squeezed piglets but that this was no longer detectable at 15 minutes when both groups were sampled. This provides an alternative reason for the finding that there was no significant difference in cortisol concentration between squeezed and sham squeezed piglets. However, this needs further exploration.

#### 6.2.2.4 Effects of a thoracic squeeze on nociception

Previous studies of TI have demonstrated antinociceptive and analgesic effects during the maintenance phase in some species. For example, behavioural and electrical brain activity (EEG) responses to noxious stimuli have been reported to attenuate during the maintenance phase relative to before induction into TI (Wallnau and Gallup Jr 1977; Porro and Carli 1988; de Oliveira *et al.* 1997; da Silva and Menescal-de-Oliveira 2006; Baker *et al.* 2019). To illustrate, rabbits induced into TI failed to show expected pain behaviours following repeated nociceptive stimulation, i.e. formalin injection (Carli *et al.* 1976). Furthermore, rabbits' EEG activity during TI changed from low amplitude, fast activity to high voltage slow wave patterns (consistent with a quantitative increase in total power and a decrease in median frequency and spectral edge frequency) despite repeated formalin injections (Carli *et al.* 1976).

Likewise, successive removal of feathers in adult hens elicited TI behaviours and spontaneous EEG activity showed high amplitude, low frequency patterns (increased total power, and a decrease in median frequency and spectral edge frequency), a pattern consistently seen in animals during the maintenance phase of TI (Gentle and Hunter 1991). In contrast, EEG recordings taken before hens were subjected to successive feather removal demonstrated low amplitude, high frequency patterns consistent with states of alert awareness (Gentle and Hunter 1991). Together, these results provide evidence that induction into a state of TI inhibits both behavioural and EEG responses to nociceptive stimulation, and that EEG activity during TI differs to that prior to induction.

It is possible that the thoracic squeeze has the capacity to induce analgesia and antinociception in a similar way to TI. However, the methods used in the study in Chapter 5 hindered acquiring evidence to assess this. In order to further evaluate whether the thoracic squeeze is a stimulus for inducing TI, I aimed to evaluate EEG responses of lightly anaesthetised piglets to noxious stimuli after application of a thoracic squeeze. Given that the EEG recordings collecting during my lamb study could not be analysed due to movement artefact, I decided to use the 'minimal anaesthesia model' in order to reduce data contamination. The model has been successfully used to detect electrical brain responses to noxious stimuli in a range of mammalian species including piglets (Murrell and Johnson 2006; Kells *et al.* 2017b) and was thus considered a suitable method to achieve this aim. However, I found no changes in any of the summary variables reflecting electrical activity of the cortex (median frequency, spectral edge frequency or total power) following a toe clamp in either the control piglets or thoracic squeeze piglets. The lack of response in the non-squeezed control group was somewhat unexpected and makes it challenging to make inferences about the potential antinociceptive or analgesic effects of the thoracic squeeze.

The only previous study exploring pain threshold responses of neonatal mammals after a thoracic squeeze found an increase in pain thresholds after application of a graded noxious electrical stimulus applied to the base of the tail, from before the squeeze to during the 20 minute squeeze. However, the study did not have a control group, making it difficult to determine if the observed changes in pain thresholds could be attributed to the actions of the squeeze or were confounded by other factors such as habituation to the repeated stimulus (Toth *et al.* 2012).

In the third study, it is likely that the age of the piglets used in the study influenced the EEG responses to the toe clamp and impaired our ability to detect an antinociceptive effect of the squeeze. Previous research has demonstrated that electrocortical responses of piglets to a noxious stimulus such as a toe clamp become more marked and begin to follow a consistent adult pattern from about 7 days of age (Kells *et al.* 2019). Therefore, the lack of responses in the control piglets suggests they were too young for meaningful nociceptive responses to be seen in the EEG. At the time of this study, the results from the first experiment in older lambs (Chapter 2) had not yet been analysed, and previous research had already demonstrated susceptibility of newborn piglets to a thoracic squeeze. Thus, I decided to use newborn piglets in order to be confident that the thoracic squeeze would be effective. Now that we have evidence that the response to a thoracic squeeze persists in older animals, at least in lambs (Chapter 2), future research could use older piglets to evaluate EEG responses to noxious stimuli after a thoracic squeeze.

Another possible explanation for the lack of EEG responses to the thoracic squeeze under anaesthesia is that induction into a state of TI may requires conscious perception and integration of sensory stimuli. Anaesthesia abolishes conscious perception of stressors (Otto 2008), inhibiting sensory processing that is likely necessary for induction into TI. If the thoracic squeeze is indeed a stimulus for TI, it is likely that conscious perception of the sensory event is required for induction into reduced responsiveness. As such, it is probable that the minimal anaesthesia model used to collect EEG recordings after application of a thoracic squeeze was inappropriate for exploring similarities in brain responses between TI and the thoracic squeeze.

This idea provides further support for the conclusion that responses to a thoracic squeeze are *not* caused by a birth-related mechanism specific to neonatal mammals. According to that theory, fetuses are still unconsciousness during the compression that occurs during birth, and thus would be unable to consciously perceive or process the sensory information associated with this squeezing (Aleman *et al.* 2017). Following that line of thinking, conscious perception would not be required for the thoracic squeeze to induce postnatal animals into a state of reduced responsiveness. In the current study, the absence of a detectable EEG response to application of the squeeze itself in anaesthetised neonates suggests that conscious perception is required for induction, favouring TI as the more plausible of the two theories.

#### 6.2.2.5 Effects of a thoracic squeeze on indicators of state of awareness or arousal

Studies on conscious birds and mammals have shown qualitatively assessed changes in EEG patterns consistent with changes from states of behavioural arousal and awareness during induction of TI to states of relaxation and reduced responsiveness during the maintenance phase (Schwarz and Bickford 1956; Kumazawa 1963; Klemm 1966a; Klemm 1971). Quantitatively, these changes would be reflected in an increase in high frequency activity (e.g. increased median frequency and spectral edge frequency) in the EEG trace, with a decrease in total power during induction. Such changes are reflective of a desynchronisation in brain activity on the EEG, which occurs in situations of behavioural arousal and alertness (Schwarz and Bickford 1956; Kumazawa 1963; Klemm 1966a; Klemm 1971). Following such changes during induction, the maintenance phase is considered to cause a shift in neural activity towards synchronised EEG patterns related to states of reduced awareness, characterised by a decrease in high frequency activity (e.g. lower median frequency and spectral edge frequency) and an increase in total power (Schwarz and Bickford 1956; Kumazawa 1963; Lefebvre and Sabourin 1977b).

In my EEG study, I found no evidence of a change in the piglets' state of awareness following application of a thoracic squeeze; changes similar to those previously observed in animals during or after induction into TI were not statistically detected. After 4 minutes of thoracic squeeze application, small changes were observed in EEG summary variables. Specifically, median frequency and total power decreased, and spectral edge frequency increased, relative to pre-squeeze baseline. However, these changes followed no biologically sensible pattern when compared to previous EEG research related to changing depth of anaesthesia (Saito *et al.* 2005; Tonner and Bein 2006) or to patterns observed during induction and maintenance of TI.

In contrast, a previous study in non-anaesthetised neonatal foals reported changes in EEG activity after a thoracic squeeze applied using a rope. The raw EEG recordings from three foals were not statistically analysed, but visual inspection reportedly showed characteristics of wakefulness interspersed with sleep patterns (Toth *et al.* 2012). However, the lack of a non-squeezed control group in that study made it difficult to draw conclusions about the causal relationship between the squeeze and the state of awareness and whether the actions of the thoracic squeeze were responsible for the sleep-like characteristics on the EEG (Toth *et al.* 2012). Conscious, freely-behaving neonatal foals are also documented to exhibit sleep-like characteristics on awake EEG recordings (Mysinger *et al.* 1985), thus confounding interpretation of the thoracic squeeze results. The use of anaesthesia in my study also complicates interpretation of the EEG results. In particular, EEG patterns characteristic of sleep (e.g., K-complexes and spindles) do not occur under anaesthesia (Tonner and Bein 2006; Otto 2008; Akeju and Brown 2017) and therefore would not have appeared in the EEG from piglets in this study. As a result, few comparisons can be made between the results of my study and those of the previous study in foals.

While the results of the EEG study do not provide support for the overall thesis hypothesis that the thoracic squeeze is a stimulus for inducing TI, previous research has evidenced a state of reduced responsiveness in neonates after application of a thoracic squeeze (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Specifically, neural reflexes have been documented to become reduced or completely absent in piglets, and in some cases, in calves and lambs following induction using the squeeze (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Piglets in particular have demonstrated consistently reduced or absent pedal and palpebral reflexes (Holdsworth *et al.* 2021). While pedal reflexes are indicative of responsiveness at the spinal cord level, palpebral reflexes are commonly used as an indicator of anaesthetic depth, as a proxy for changes in states of awareness (Guntheroth and Kawabori 1975; Spyer 2009) and absence of a palpebral reflex is thus considered to reflect an animal that is in a state of unconsciousness.

Similarly, studies measuring the neural reflexes of a range of species (e.g. chickens and rabbits) during TI found reflex responses became reduced or absent during the maintenance phase, consistent with EEG changes reflecting reduced states of awareness (for a list of species studied, see the following reviews: (Crawford 1977; Carli *et al.* 1984; Porro and Carli 1988; Gentle *et al.* 1989; Overeem *et al.* 2002). Given that neural reflexes can provide some information about level of awareness in animals, the reduced or absent reflexes observed previously in neonatal piglets following application of a thoracic squeeze suggest a change in the state of awareness that is consistent with that observed after induction into TI.

## 6.3 Other Findings

Chapter 3 presents the first study to systematically characterise the behavioural responses of healthy neonatal lambs to a standardised application of a thoracic squeeze. Before this, only one study had looked at the efficacy of a rope squeeze in unhealthy newborn lambs (Flora *et al.* 2021). In that study, the squeeze was applied to lambs showing pathophysiology, such as hypoxaemia, chronically low heart rate and respiratory rate, or no responsiveness to external stimulation, features that are not consistent with reports of neonatal maladjustment syndrome in foals and calves (Aleman *et al.* 2017; Stilwell *et al.* 2019). In that study, the squeeze reportedly induced all lambs into a “sleep-like” state (Flora *et al.* 2021). However, there was no characterisation of behavioural or physiological responses during the squeeze.

My study provided detailed characteristics of induction behaviours, including the success rate for induction in neonatal lambs and older lambs, latency to induction (i.e. latency to loss of posture and cessation of movement), detailed behavioural responses, duration of the maintenance phase following induction (i.e latency to discontinuation of the squeeze) and information about the physiological stability of lambs during the squeeze. I also evaluated the effect of different methods of application on the success and safety of the squeeze on lambs. These results provide detailed information about the behavioural and physiological effects of a thoracic squeeze on both neonatal lambs, and lambs at 3 weeks and 3 months old. This information therefore provides a foundation to explore the application of the thoracic squeeze technique in industry settings. For example, there have been suggestions that the squeeze may be useful as a low stress form of handling and restraint, for the purpose of applying routine or painful husbandry procedures to animals that prioritises their safety through immobilisation and reduced responsiveness. However, given the cortisol findings in Chapter 4, and the similarities between TI and responses to a thoracic squeeze, the thoracic squeeze is unlikely to be a low stress technique.

## 6.4 Methodological considerations

There were a number of alternative methods that required consideration for the three experiments outlined in Chapters 3, 4 and 5. There were also methods used that created limitations for interpretation of data. Here, I discuss the considerations for particular methods used in each study.

### 6.4.1 Application of the thoracic squeeze to lambs using the inflatable cuff

As in my previous studies on piglets and calves (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022), the thoracic squeeze was applied to lambs using a rope or an inflatable cuff. The inflation cuff did not fit the thorax of each lamb correctly, and thus caused problems that were more likely related to the squeeze method than due to species susceptibility. Maintenance of reduced responsiveness was lower in lambs than in any other species studied to date, with 68% of induced lambs having the squeeze discontinued before the end of the 10 minute squeeze due to spontaneous arousal or physiological instability, compared to 5% in piglets and 11% in calves (Holdsworth *et al.* 2021; Holdsworth *et al.* 2022). Interestingly, physiological instability occurred only in lambs squeezed with the cuff, a finding that is consistent across species. In piglets, only one animal out of 17 squeezed was discontinued due to physiological instability and it was in the cuff group (Holdsworth *et al.* 2021). Similarly in calves, two of 16 in the cuff group were discontinued due to physiological instability (Holdsworth *et al.* 2022). Issues with cuff sizes may therefore have been the cause for a higher proportion of lambs becoming physiologically unstable during the squeeze.

These findings suggest that the cuff design influenced the proportion of lambs maintained in reduced responsiveness for the full 10 minutes. During preparation for this study, the thoracic girth and length of lambs within the experimental age groups could not be directly measured due to a lack of lamb availability. Instead, anatomical dimensions were taken from the literature to develop the different sized cuffs. As a result, there were issues with the cuffs being too short to fit around the thoracic girth, or being too wide so that the cuff impeded abdominal breathing which is often observed when the thorax is compressed (Huff *et al.* 2020; Marini and Gattinoni 2021). Therefore, further research should refine the design of the inflation cuff in order to re-evaluate behavioural and physiological responses of this species.

### 6.4.2 Study design and practicalities of measuring cortisol in neonatal piglets

In Chapter 4, I analysed plasma cortisol concentrations obtained from blood samples collected from one day old piglets. The results of the second study were limited to some degree by the cortisol sampling method used, which required restraining the animals to obtain blood samples. Restraint itself has been demonstrated to elicit increased cortisol secretion (Blackshaw and Blackshaw 1989) but as long as the sample can be taken within 2 minutes of the start of handling, the amount of cortisol in the sampling should not be affected (Stilwell *et al.* 2008). In this case, blood sampling took less than one minute per piglet. However, the effect of sampling itself meant that repeated samples could not be taken from the same piglet over time, limiting direct comparison of timepoints. Based on evaluation of previous literature and the known effects of repeated blood sampling on cortisol responses, each piglet was sampled at a single timepoint. As such, it is not possible to evaluate the time course of cortisol responses after application of a thoracic squeeze directly, and individual differences in baseline cortisol or responses to treatment could have reduced our ability to detect biologically significant treatment effects. However, this seems unlikely given the large differences between the 15-minute values and the controls and the very small differences between sham and squeezed groups at 15 and 30 minutes.

Non-surgical catheterisation of the jugular vein for repeated sampling was considered. However, this method still requires some degree of restraint for sample extraction and is not suitable for very young animals moving freely around a pen. It is also known to affect cortisol concentrations between samples (Flournoy and Mani 2009), and thus was not a viable option for the study.

Nor was it possible to evaluate cortisol levels via saliva and urine sampling techniques in this study. Saliva sampling involves placing a swab into the mouth of the animal for at least 30 seconds and up to 5 minutes (Blackshaw and Blackshaw 1989; Rodarte *et al.* 2004). Older pigs will chew the swab to elicit saliva secretion that coats the swab for later analysis (Němečková *et al.* 2022). This method was considered for the piglets in the study in order to provide a non-invasive method of cortisol sampling that did not require restraint, enabling repeated sampling of cortisol from the same individuals over time. However, at 24 hours old, piglets do not have the instinct to chew when objects are placed into their mouths (Chidgey, K., personal communication, August 2021). As a result, sampling takes longer, and piglets must be restrained to place and hold the swab in the mouth, negating any benefits of the technique.

Cortisol sampling via urine collection is a particularly difficult method. Urination, particularly in 24 hour old piglets, is inconsistent and unpredictable (Cook *et al.* 1997). In some cases, urine sampling requires specialised collection devices (Cook *et al.* 1997). Urine collection is also often constrained by time, and therefore limits the number of animals that can be used in a study. In addition, cortisol concentrations vary depending on the volume of urine, thus requiring collection over a period of time. Due to the time course of the response to a thoracic squeeze, this kind of chronic cortisol collection via urine would have been unsuitable for the study (Cook *et al.* 1997).

### 6.4.3 General Anaesthesia

In the final experiment in Chapter 5, piglets were placed under light anaesthesia using the minimal anaesthesia model. This model involves placing animals in a light plane of anaesthesia that is equivalent to light sedation with accompanied immobility. The resulting effect on brain activity is minimal cortical depression. This model has been used to study responses to noxious stimuli and the efficacy of various analgesic agents in a range of mammals (Murrell *et al.* 2003; Diesch *et al.* 2010; Kells *et al.* 2017b; McIlhone *et al.* 2018). Under minimal anaesthesia, the cerebral cortex is still active and responsive but conscious perception of pain is prevented, making this an ethical model for the study of pain in animals (Murrell and Johnson 2006). Moreover, changes in the EEG under minimal anaesthesia are comparable to those recorded in conscious mammals (Murrell and Johnson 2006). This technique is particularly advantageous for the collection of EEG recordings without data contamination such as movement artefact. However, as discussed in section 6.2.2.4 it may not have been appropriate for evaluating EEG and nociceptive responses to the thoracic squeeze.

### 6.4.4 EEG Analysis

Fast Fourier Transformation (FFT) is one of a number of methods used to quantitatively analyse raw EEG data. An alternative method for analysing EEG is wavelet transformation (WT). WT provides a similar function to FFT, but breaks down signals in the EEG recording to oscillations between time and frequency simultaneously, so that WT data are based on wavelets within a limited and specified timeframe called a ‘window’ (Farge 1992; Sifuzzaman *et al.* 2009). In comparison, FFT works by analysing the frequency components of an EEG wave, but does not have the capacity to analyse time signals (Sifuzzaman *et al.* 2009). As a result, WT was originally introduced for seismic wave analysis, with applications for more complex signals such as those seen with turbulence, speech processing, image processing and medical imaging (Farge 1992; Sifuzzaman *et al.* 2009). Research in animals utilising WT has increased in the last decade, but has predominantly focused on seizure detection, with animals as a model for humans (Sitnikova *et al.* 2012; Tieng *et al.* 2016; Abbasi and Unsworth 2020). To date, no research has used WT to evaluate changes in EEG in animals for purposes such as nociception.

Another method used to analyse human EEG is the Bispectral Index (BIS), which is used in real time to assess the degree of central depression by anaesthesia in humans (Barnett *et al.* 1971; Miller *et al.* 2004; March and Muir 2005). BIS assigns EEG waveforms a number ranging from 0-100, based on mathematical equations applied to EEG recordings (March and Muir 2005). The index was created using specific parameters in EEG data that correlated with features of conscious and unconscious states in humans under anaesthesia. BIS scores inversely correlate with increasing anaesthesia and deeper states of unconsciousness (Barnett *et al.* 1971; Bouafif 2021). When it comes to evaluating EEG responses of animals to a noxious stimulus, a single value such as BIS would provide inadequate information about nociception and potential changes over the time course of the thoracic squeeze. As a result, this method of EEG analysis was considered to be unsuitable for the study. Furthermore, its use predominantly in humans means that comparisons for data interpretation would be limited.

To date, FFT is the only method that has been used to study EEG changes in animals in response to nociception and analgesia. Using the same analysis method as has been used in previous research in piglets (Kells *et al.* 2017a; Kells *et al.* 2017b) would support interpretation of the data. Therefore, FFT was the chosen analysis method for EEG data in the third experiment in this thesis.

## 6.5 Future research

The results of each experimental chapter raise further questions about aspects of the thoracic squeeze that need further exploration. In particular, suggestions for future research were developed to build upon existing preliminary results (Chapter 3 and 4), producing more appropriate methods for addressing research questions/objectives (Chapter 5), or exploring new methods to extend our knowledge about the effects of a thoracic squeeze on animals.

### 6.5.1 Effects of a thoracic squeeze on non-mammalian species

Given that the results from my first study demonstrate that responses to a thoracic squeeze may be generalised across multiple mammalian species, future research should investigate whether these responses are also observed in non-mammalian animals. TI has been studied in numerous vertebrate and invertebrate species, and responses appear to be generalised across taxa. Thus, exploring whether non-mammalian animals respond similarly to a thoracic squeeze as what has been previously observed in mammalian species would provide further support that responses to a thoracic squeeze are equivalent to TI.

### 6.5.2 Time course of cortisol responses to a thoracic squeeze

Future research should investigate the time course of HPA responses to a thoracic squeeze by sampling frequently at much shorter intervals (such as 2 minutes). A single sampling design could still be feasibly used, but consideration should be given to the limitations of single sampling study designs on inferences about individual and group variation in cortisol responses over time. However, single sampling circumvents the potential confound of repeated blood sampling on cortisol concentrations, and would enable more frequent sampling to be done without potentiating stress to the animals. Should a repeated measures design be used, consideration should be given to the effects of repeated blood sampling on cortisol concentration and the perceived stressfulness of blood sampling to animals. A way to accommodate this is to use older animals and utilise catheterisation for blood sampling. Larger volumes of blood can be obtained from older animals over time, and catheterisation is a method commonly employed for repeated sampling studies (Lasbennes *et al.* 1986; Blackshaw and Blackshaw 1989; Moe and Bakken 1996; Buzzard *et al.* 2012) and is known to have a smaller impact on cortisol concentrations. Additionally, the impact could be further lessened by ensuring that physical contact with the animal and obtaining the blood sample takes less than 2 minutes (Stilwell *et al.* 2008). Collectively, these methods could provide detailed information about cortisol concentration changes throughout induction, maintenance and recovery after application of a thoracic squeeze.

### 6.5.3 Exploring the effect of anaesthesia on induction into reduced responsiveness

The results in Chapter 5 highlight the need for further investigation into the potential confound of anaesthesia. In particular, there is a need to determine whether conscious perception is a requirement for induction using a thoracic squeeze. One way to explore this is to evaluate changes in EEG patterns of non-anaesthetised animals after application of a thoracic squeeze using implantable electrodes. Implantable electrodes in older mammals would overcome both the potential confound of age on EEG responses, as well as circumvent the requirement for anaesthesia during EEG recordings in order to minimise movement artifact. The EEG responses during each phase (induction, maintenance and recovery) could then be compared to anaesthetised animals to determine whether there are similarities in EEG responses.

Additional physiological measurements that are documented to change during each phase of TI (e.g. decreases in cardiorespiratory values relative to pre-squeeze baseline, increase in cortisol concentration following induction) would provide supporting information about whether induction and maintenance occurs in conscious animals. If responses are quantitatively different, this would suggest that anaesthesia may be a potential confound for interpretation of results. In particular, if non-anaesthetised animals exhibit EEG changes consistent with what has previously been reported for animals during TI and in contrast to the absence of EEG changes in my anaesthetised animals, then this would suggest that conscious perception is required for induction into a state of reduced responsiveness following application of a thoracic squeeze.

### 6.5.4 Conditioned place aversion to evaluate emotional effects of a thoracic squeeze

There is clear evidence that TI causes a stress response in various animal species. Likewise, my second study and one other study on foals (Toth *et al.* 2012) indicate that the application of a thoracic squeeze causes an HPA response. In addition, it has been demonstrated in TI that the HPA response during induction is associated with 'stress-induced analgesia' during the maintenance phase due to activation of endogenous opioids (Baker *et al.* 2019). Together, these findings suggest that the experience of a thoracic squeeze may be unpleasant. Unfortunately, the results of my final study (Chapter 5) did not provide more direct information on whether the piglets perceived the thoracic squeeze to be stressful or unpleasant. Thus, there is a need to further explore affective states of animals to application of a thoracic squeeze and whether the squeeze is actually aversive or unpleasant. Future studies could use conditioned place aversion test (Kc *et al.* 2016). This kind of study could evaluate the willingness of animals to return to a defined environment where a squeeze previously took place, compared to a defined environment where animals were left unhandled. This would provide more compelling behavioural information about whether the squeeze is aversive to animals and inform the application of the squeeze as a 'low stress' restraint technique.

## 6.6 Overall conclusions

On the balance of evidence developed in the three studies presented in this thesis, the thoracic squeeze meets many of the criteria to be classified as a stimulus for inducing TI (Figure 20). The features of responses to the thoracic squeeze that meet the criteria for TI are as follows:

- Healthy lambs responded similarly to a thoracic squeeze as do piglets, calves and foals, suggesting behavioural responses to thesqueeze may be a generalised phenomenon across multiple mammalian species.
- Older lambs up to 3 months of age expressed induction behaviours consistent with what was/has been observed in neonates. This findings refutes the special birth-related mechanism theory and provides support for TI behaviours that are known to persist beyond the neonatal period.
- Piglets demonstrated HPA responses to a thoracic squeeze in a similar way to animals that are induced into TI.
- No inferences can be made about the effect of a thoracic squeeze on nociception as the control piglets did not show EEG responses to application of a noxious stimulus. Further research on older piglets is required to explore possible similarities in the effects of the thoracic squeeze and TI on nociceptive responses.
- No inferences can be made about the effect of a thoracic squeeze on state of awareness in piglets, as indicated by EEG. The methodology may have been inappropriate due to the inability of animals to consciously perceive the squeeze when anaesthetised . However, reduced neural reflexes have been observed in neonates after application of a thoracic squeeze and suggest that the squeeze does induce changes in states of awareness that are consistent with TI. Further research is required to determine the effects of a thoracic squeeze on the state of awarness in animals relative to what is reported for TI.

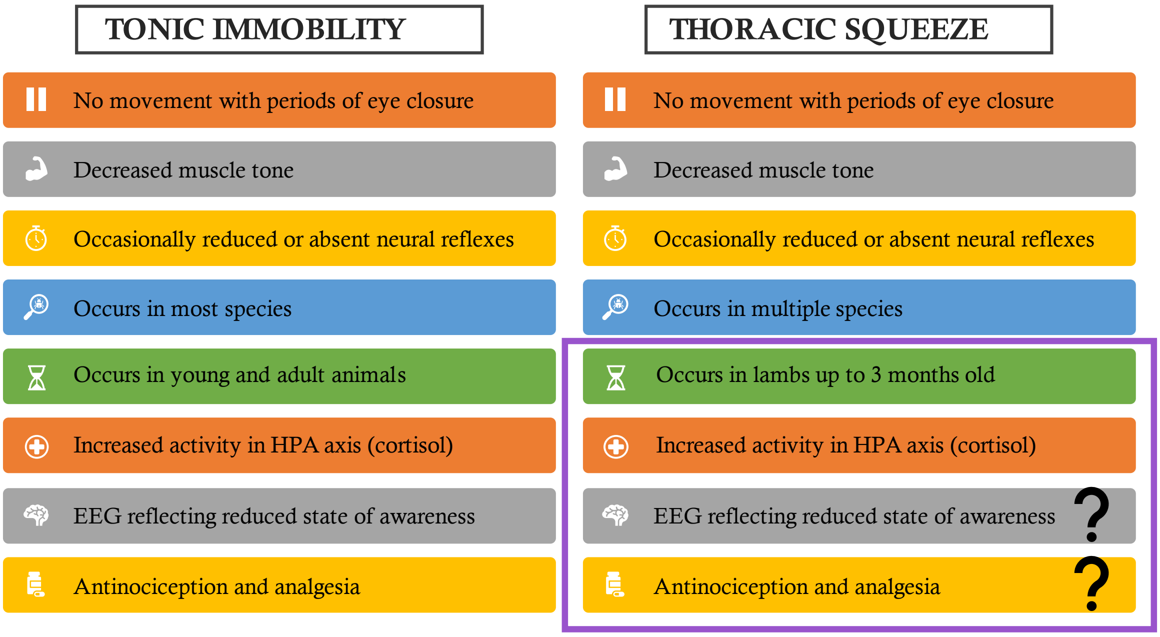

**Figure 19.** Summary of responses observed during TI and how they compare to current observations during the thoracic squeeze. Question marks refer to areas where evidence is still required.

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