A study of brain injury

in

New Zealand sea lion pups

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Abstract

The New Zealand sea lion (*Phocarctos hookeri*) is a threatened species endemic to New Zealand. The majority of breeding in this species occurs on the Auckland Islands in the sub-Antarctic, and recent population estimates indicate that pup production is declining. Trauma is a significant cause of mortality in New Zealand sea lion pups, and much of this is believed to be caused by adult and subadult males, that bite, crush, shake and throw young pups.

In this thesis, a number of techniques are used to determine the role played by traumatic brain injury in the mortality of NZ sea lion pups. The findings of gross necropsy examinations show that pups have numerous lesions indicative of traumatic brain injury, including skull fractures and subdural haemorrhages, and that pups die due to crushing and impact injuries. Although some pups have gross lesions considered in human paediatric medicine to be indicative of shaking injury, detailed histological and microbiological studies of sea lion pups show that most of these are associated with meningitis due to *Klebsiella pneumoniae*. This bacterium is a common cause of pup mortality.

Immunohistochemical techniques are used to demonstrate that axonal injury is common in sea lion pups, but show that shaking is not a common mechanism of this pathological process. Instead, most axonal injury is found to be due to hypoxia-ischaemia, and evidence that raised intracranial pressure has occurred is comparatively common in dead pups. The combined findings of histological and immunohistochemical studies suggest that lesions such as optic sheath haemorrhage, intracranial subdural haemorrhage, spinal sub-meningeal haemorrhage, and optic nerve axonal injury could be caused by pertubations to vascular, intra-ocular, intracranial and subarachnoid pressure rather than being a direct result of trauma as is proposed in shaken baby syndrome.

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Glossary

acceleration	the change in velocity over time. Can be positive or negative (deceleration)
AMPA/KA receptors:	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid/ kainic acid receptor. A membrane-bound glutamate receptor
ATP:	adenosine triphosphate. An energy-containing nucleotide produced by ATP-synthase
axotomy:	physical separation/tearing of an axon
biomechanics:	the study of mechanical forces as they apply to biological systems
blood-brain barrier:	the anatomical structures responsible for prevention of uncontrolled movement of fluid and solute between the cerebral blood vessels and the brain parenchyma
brain herniation:	movement of brain tissue from one anatomical compartment into another
brain swelling:	enlargement of the brain that occurs due to increased intravascular or extravascular fluid (e.g. cerebral oedema), resulting in flattening of gyri, shallow sulci, and herniation of brain tissue

bridging veins:	the veins that pass from the leptomeningeal surface through the subarachnoid space and dura mater, to empty into the intradural venous sinuses
calvarium:	the domed upper part of the skull comprising the frontal, parietal and occipital bones
cerebral autoregulation:	the maintenance of cerebral blood flow within homeostatic limits in response to changes in central venous pressure, intracranial pressure and metabolic demands
cerebral oedema:	accumulation of excessive amounts of extravascular fluid within brain tissue
compressive strain:	a force that results in tissues or structures being moved closer together
concussion:	transient loss of consciousness following a head injury
contre-coup contusion:	a contusion occurring remote to the site of impact. Historically these were said to occur opposite to the site of impact, but in human beings most occur on the frontal and temporal lobes, irrespective of impact site. The pathogenesis may involve tearing of parenchyma as it moves against bony protuberances or meningeal structures such as the falx cerebri
contusion:	disruption of the brain parenchyma following trauma, leading to haemorrhage and necrosis
coup contusion:	a contusion that occurs at the site of impact

cytoskeleton:	the microtubules, neurofilaments and microfilaments that make up the structural elements of a cell
cytotoxic oedema:	swelling of glial cells that results from damage to the energy-requiring membrane ion pumps, with subsequent influx of ions and fluid
depressed fracture:	the result of a focal impact where the outer table of the skull is depressed below the surface of the unaffected bone, and the inner table of the skull is pushed into the parenchyma
diffuse axonal injury:	both a neuropathological and clinical entity. The clinical syndrome injury involves immediate loss of consciousness followed by prolonged coma in the absence of a mass lesion. The neuropathological entity comprises widespread damage to axons throughout the parasagittal cortex, corpus callosum, internal capsule and long tracts of the brainstem
dynamic loading:	rapid application of a force, usually over a period of between 2 and 25 milliseconds
excitotoxicity:	excessive activation of excitatory glutamate receptors in the brain, resulting ultimately in cell death
fast axonal transport:	rapid (up to 400mm per day) transport of intracellular substances along microtubules
global ischaemia:	inadequate blood supply to the entire brain
hypoxia:	decrease in availability of oxygen to a tissue

impact loading:	the force resulting from direct contact between a tissue and a rigid object
impulse loading:	the force resulting from sudden acceleration or deceleration of tissue
inertia:	acceleration or deceleration forces without impact
intracranial pressure:	pressure within the cranial cavity, dependent upon central venous pressure, cerebral perfusion pressure and soft tissue/fluid volume of the brain and vasculature
intradural haemorrhage:	haemorrhage that originates within the dense fibrous layer of the dura mater
ischaemia:	lack of blood supply to a tissue relative to its needs
lamina cribrosa:	part of the sclera that is penetrated by axons of the optic nerve
mechanical loading:	the application of force(s) to a tissue
mechanoporation:	rapid movement of calcium ions into a neuron following transient membrane depolarisation which occurs as a result of mechanical deformation of the neuronal soma or axon
microtubule:	polymers of tubulin that make up part of the neuronal cytoskeleton and play a role in intra-cellular transport

mitochondrial membrane permeability transition pore:

a protein-lined 'hole' (pore) in the inner membrane of the mitochondria, which can open following traumatic brain injury and allow flux of ions across the membrane

neurocranium: the portion of the skull that encloses the brain, including the calvarium as well as the temporal and basisphenoid bones.

neurofilament: the class of intermediate filament found in neurons

NMDA receptors: *N*-methyl *D*-aspartate receptor. A voltage-dependent membrane-bound glutamate receptor

node of Ranvier: unmyelinated portions of axons found between myelinated internodes

otariids: eared seals (fur seals and sea lions)

pan-necrosis: Death of neurons as well as other cellular elements in an area of brain affected by focal ischaemia

phocids: 'true' seals. These species do not have pinnae

pinnipeds: a suborder of carnivorous marine mammals with limbs specialised as flippers

polygynous: an hierarchical social system whereby mature males defend a territory within which a group of females is sequestered. Females are comparatively free to enter and leave the territory

pond fracture: similar to a depressed fracture, but with no displacement of the inner table of bone

rotational acceleration: acceleration about an axis

selective neuronal necrosis: necrosis of specific populations of neurons that are particularly susceptible to ischaemia. Occurs with global ischaemia

shaken baby syndrome: a syndrome seen in human infants with inflicted brain injury proposed to be due to shaking. The typcial syndrome comprises encephalopathy with subdural and retinal haemorrhages

shear forces: sliding forces involving differential movement between layers of tissue

skull: the bones of the head exclusive of the mandibles but including the neurocranium, zygomatic processes and the facial, maxillary, and palatine bones

spinal sub-meningeal haemorrhage:

haemorrhage between the spinal dura mater and the spinal cord. Can involve haemorrhage between the dura mater and the parietal layer of the arachnoid mater (subdural haemorrhage) or haemorrhage between the parietal layer of the arachnoid mater and the spinal cord parenchyma (subarachnoid haemorrhage)

static loading:	the gradual application of a force over a period of greater than 200 milliseconds
strain:	deformation of tissue resulting from the application of a force
stress:	application of force to a tissue
subdural haemorrhage:	haemorrhage into the subdural compartment, which is made up of loose dural border cells at the inner aspect of the dura mater
tensile strain:	a force that results in tissues or structures being moved further apart
translational acceleration:	acceleration in a linear plane
vasogenic oedema:	loss of intravascular fluid into surrounding parenchyma following damage to cerebral blood vessel walls

Chapter one

Prologue

1.1 Problem statement

The New Zealand (NZ) sea lion is a threatened species endemic to New Zealand. The majority of breeding in this species occurs in a geographically limited area of the sub-Antarctic Auckland Islands, and pup production in this area has declined by over 40% in the last twelve years (Robertson and Chilvers 2011). While there are likely to be multiple reasons for this decline, data from Enderby Island, the most well-studied breeding site, implicate trauma as the main cause of early pup mortality (Castinel *et al*. 2007). From discussions with scientists who regularly visit the island it is apparent that a reasonable proportion of these deaths are due to attacks by subadult male NZ

sea lions that have been observed to bite, shake and throw young pups. Observers also suggest that certain subadult males become habitual attackers, and are responsible for clusters of pup deaths, particularly in the later part of the breeding season when larger dominant males have moved away from the breeding site. Both published (Castinel *et al*. 2007) and unpublished reports from necropsy investigations conducted on Enderby Island describe lesions indicating traumatic injury, including subcutaneous bruising of the head and neck, skull fractures, neck injuries, intra-ocular and peri-orbital haemorrhages, brain contusions and subdural haemorrhages. If a few subadult males were indeed responsible for a significant number of pup deaths, then it is possible that intervention strategies could become worthwhile in this declining population.

Shaking of pups by subadult males is of particular interest. In human paediatric medicine a specific form of inflicted injury known as shaken baby syndrome is believed to result from violent or repeated shaking of young children (Caffey 1974), and is characterised by the presence of subdural and retinal haemorrhages (Caffey 1974; Donohoe 2003). In the sea lion colony, subadult males have been seen shaking young pups, and subdural haemorrhages have been described in dead pups, introducing the intriguing possibility that a similar syndrome exists in NZ sea lions. A central aim of this thesis, therefore, is to test the hypotheses that attacks by subadult males cause brain injury resulting in morbidity and mortality in NZ sea lion pups, and that subdural haemorrhages seen at necropsy are due to shaking.

While a better understanding of the importance of trauma inflicted by subadult males could benefit management decisions in this endangered species, this study also has wider application. The pathogenesis of naturally occurring traumatic brain injury in non-human mammalian species has received scant attention in the veterinary literature. In contrast, extensive research into human head injury over the past few decades has resulted in substantial advances in the understanding of brain injury, and has helped improve preventative and therapeutic strategies. Both experimental animal models and

large scale studies of accidental and inflicted human head injury have played important roles in this process. It is, however, well recognised that there are many limitations to applying data obtained in strictly controlled animal experiments to uncontrolled situations under which head injuries occur in humans, in terms of both the nature of the forces applied, and the morphological differences between species. In turn, much of the current theory of the neuropathology of head injury in veterinary medicine has been derived from the human literature, and these processes may very well be different in animals. In order to effectively and rationally diagnose and treat animals with head injuries, we need to have a better understanding of the nature and consequences of traumatic brain injury occurring in animals in a natural setting.

Traumatic brain injury occurs in response to mechanical forces transmitted to the brain parenchyma. These forces can be separated into contact (or impact) and inertia forces, and most non-experimental head injuries involve a combination of the two. The outcome of a head injury will depend on the location and size of focal brain lesions as well as the severity of diffuse lesions, including secondary brain damage. While focal lesions are comparatively simple to identify and quantitate, diffuse brain damage can be more difficult to evaluate. Over the past few decades it has become increasingly apparent that diffuse axonal injury caused by shear forces is an important component of many types of human head injury. This is important both academically and clinically, as some axonal injury is reversible and potentially treatable. Surprisingly, it is currently not known whether diffuse axonal injury occurs in non-experimental head injury in non-human mammals. Since trauma causes axonal injury primarily as a result of inertia forces, investigation of brain injury in a mammalian species that sustains brain damage due to inertia obviously would be valuable.

1.2 Research aim

This project aims to investigate aspects of the importance and pathogenesis of brain

injury as a cause of death in NZ sea lion pups, with a particular focus on determining the role played by shaking.

1.3 Overview of thesis structure

In order to achieve the aim outlined above, this thesis uses a series of investigative techniques to identify brain injury, and to attempt to determine the pathogenesis and likely effects of the injury. Chapter Two provides background information covering relevant aspects of NZ sea lion reproductive biology. This chapter also summarises current knowledge on con-specific trauma to pinniped pups, concentrating on otariids (eared seals), the taxonomic group to which NZ sea lions belong. Concepts of brain injury, including hypoxic-ischaemic injury and traumatic injury, are reviewed, and biomechanical aspects of traumatic brain injury are discussed. Paediatric brain injury and experimental animal models of injury are reviewed, and the difficulties of extrapolating findings between species are highlighted. The veterinary literature on non-experimental traumatic brain injury is summarised.

In Chapter Three, archived necropsy records from Enderby Island are reviewed in order to identify patterns and types of lesions that are consistent with traumatic brain injury. The limitations of using historic records are highlighted, and in Chapter Four, a prospective necropsy study based on pup mortalities at Enderby Island in 2007/08 is described. In this chapter the cause of death of each pup is established based on gross lesions, histological findings and microbiological culture, and the role and causes of traumatic brain injuries are assessed. In Chapter Five, more detailed histological evaluations of the brains, eyes, cervical spinal cords and meninges of pups are described, and both traumatic and non-traumatic causes of lesions are considered. Chapters Six and Seven use immunohistochemical techniques to identify axonal and dendritic damage. The results of these studies are considered with respect to the pathogenesis of the damage identified. In Chapter Eight, knowledge gained in earlier chapters is brought

together, and the importance of brain injury and the role of subadult male attacks in NZ sea lion pup mortality are clarified. In addition, a theory of the pathogenesis of subdural haemorrhages in these pups is proposed.

1.4 Presentation of thesis

A compact disc (CD) containing an electronic version of this thesis is included inside the back cover. This CD also contains the appendices.

1.5 References

Caffey J. Whiplash shaken infant syndrome - manual shaking by extremities with whiplash-induced intracranial and intraocular bleedings, linked with residual permanent brain damage and mental retardation. *Pediatrics* 54, 396-403, 1974

Castinel A, Duignan PJ, Pomroy WE, Lopez-Villalobos N, Gibbs NJ, Chilvers BL, Wilkinsons IS. Neonatal mortality in New Zealand sea lions (*Phocarctos hookeri*) at Sandy Bay, Enderby Island, Auckland Islands from 1998 to 2005. *Journal of Wildlife Diseases* 43, 461-74, 2007

Donohoe M. Evidence-based medicine and shaken baby syndrome part 1: Literature review, 1966-1998. *American Journal of Forensic Medicine and Pathology* 24, 239-42, 2003

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Chapter two

Background and literature review

2.1 Introduction

This chapter provides the necessary background information relevant to the study of brain injury in NZ sea lion pups. The first part summarises information on the breeding behaviour of NZ sea lions, and reviews the published literature on con-specific trauma in pinniped pups, concentrating on otariids (eared seals), the group to which NZ sea lions belong. In the second part of this chapter, the main types of brain injury are examined, and the biomechanics of blunt traumatic brain injury are discussed. Infant brain injury is compared and contrasted with brain injury in adults, and the difficulties

of extrapolating from experimental models and human data to naturally-occurring animal brain injury are discussed.

2.2 New Zealand sea lions

2.2.1 General information

The NZ sea lion (*Phocarctos hookeri;* previously known as Hooker's sea lion) is endemic to New Zealand, and is classified as 'threatened in decline' by the IUCN red list (IUCN 2010) and 'nationally critical' under the New Zealand Threat Classification System (Baker *et al*. 2010). Most breeding occurs within a restricted range within the sub-Antarctic Auckland Islands (Figure 2.1), although a small number of females have recently begun to breed on the New Zealand mainland (Chilvers *et al*. 2007). The Auckland Islands population accounts for the majority of breeding (71% of annual pup production), but pup production at this site has declined by 40% since 1998 (Robertson and Chilvers 2011). The most recent published studies estimate a population of 10,000 - 14,000 individuals (Gales and Fletcher 1999), but these figures are based on surveys completed in the 1994/95 and 1995/96 breeding seasons, prior to the occurrence of three separate mass mortality events (Baker 1999; Wilkinson *et al*. 2006). Perceived threats to the population include severely restricted breeding range, disease epidemics, and interactions with the Auckland Islands commercial squid fishery (Robertson and Chilvers 2011).

Due to the remote location of the majority of the species and to the widespread dispersal of individuals in the non-breeding season, investigations into mortality are limited to those conducted during annual summer visits to the Auckland Islands. The breeding site at Sandy Bay on Enderby Island has been monitored annually since 1994/95. Currently there is a small science station on site, and a team of scientists visits during each breeding season (from early December until late February/early March). Observational studies, pup counts, satellite tagging, and mortality investigations are conducted during this time.

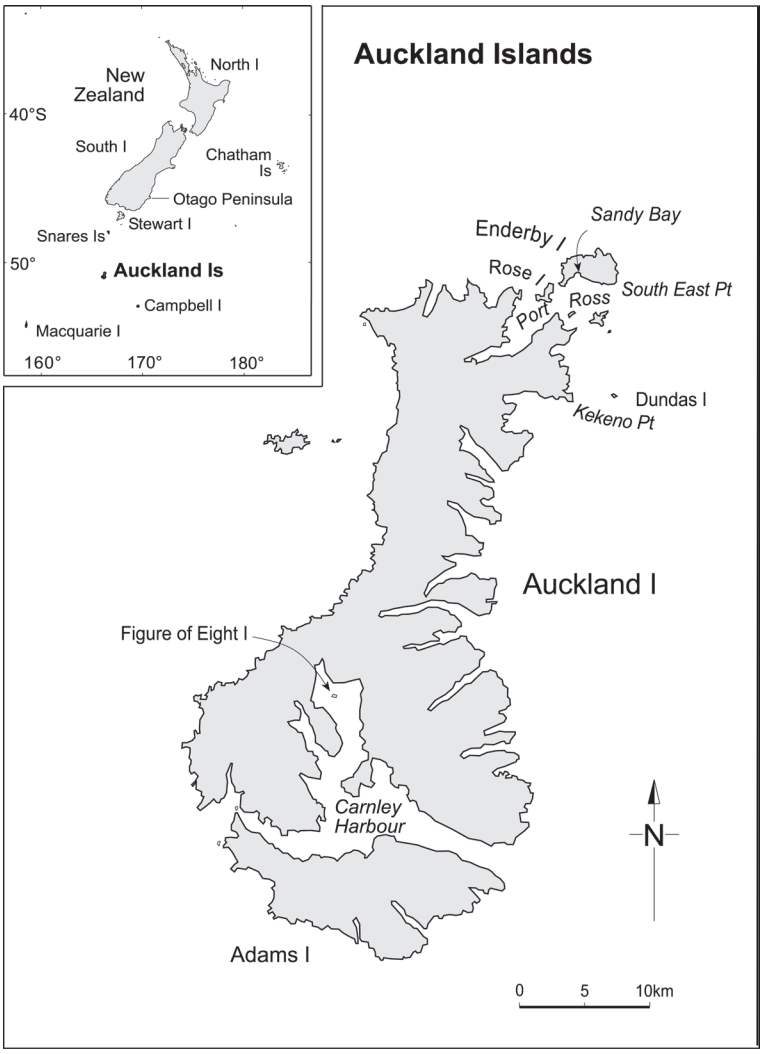

Figure 2.1. Map showing location of Auckland Islands relative to New Zealand. New Zealand sea lions breed mainly at Sandy Bay on Enderby Island, Dundas Island, and Figure of Eight Island in the Auckland Islands. A small breeding group is also located at Otago Peninsula on the New Zealand mainland. Map supplied by Louise Chilvers, Department of Conservation.

Mature male NZ sea lions begin to congregate at Sandy Bay in November each year. Pregnant females arrive in the first few weeks of December, and males compete for and defend breeding groups of females within set territories. The females give birth within a week of landing, and oestrus occurs 7 - 10 days later. The mean pupping date is 25th December, and all pups are born by the end of the third week in January. Mating occurs from mid December until mid January. By the end of January most dominant males have moved away from the breeding site. At this time aggression between competing males has decreased, and subadult males begin to occupy the positions vacated by

mature males, maintaining territories containing groups of females, although not usually managing to mate (Cawthorn 1993).

2.2.2 Evidence of traumatic brain injury in NZ sea lion pups

A study by Castinel *et al.* (2007) is currently the only published information on the causes of mortality in young (newborn to approximately two months old) NZ sea lion pups. In that study the authors found trauma to be the most common cause of mortality in this age group, and ascribed most trauma-related deaths to injuries inflicted by adults, particularly males. Both published and unpublished reports resulting from necropsy investigations conducted on Enderby Island describe subcutaneous bruising of the head and neck, skull fractures, neck injuries, intra-ocular and peri-orbital haemorrhages, brain contusions and subdural haematomas. Similarly, there are both published (Marlow 1975; Wilkinson *et al.* 2000) and unpublished (Cawthorn, Chilvers, Meynier pers. comms, 2009) reports of pups being crushed by fighting adults, as well as being bitten, shaken and thrown during more directed attacks. Some observers believe that individual subadult males may be responsible for repeated pup attacks in any one season.

2.3 Neonatal abuse and infanticide in pinnipeds

Trauma is considered to be a significant cause of neonatal mortality in many pinniped species (Baker and Doidge 1984; Higgins and Tedman 1990; Reid and Forcada 2005; Castinel *et al.* 2007; Maloney *et al.* 2009) . The perpetrators are most commonly adult or subadult con-specifics, and pups can be injured both directly (in targeted attacks) and indirectly (for example being crushed by fighting males). The prevalence of neonatal trauma varies between the major pinniped groups, being more common in phocids ('true' seals) than in otariids (Le Boeuf and Campagna 1994). In general, pup abuse is more common in the more social species, particularly those that form large

congregations during the breeding season (Le Boeuf and Campagna 1994).

2.3.1 The influence of social behaviour and reproductive biology on neonatal trauma

Pinnipeds are seasonal breeders, with varying reproductive strategies. Phocids are relatively variable in their social structure during the breeding season, with some species forming large social groups while others breed in small family groups. Large groups may be either territorial and controlled by a single male, or hierarchical, where several males within the group compete for dominance (Le Boeuf and Campagna 1994). Phocid females remain with and protect their pups throughout the lactation period. While this would seem likely to decrease the incidence of trauma to pups, the females tend to be aggressive towards pups that are not their own, and will attack and injure 'foreign' pups. In addition, mother-pup recognition is poorly developed in these species, so that reunion is difficult when the pair become separated, for example during the frenetic activity that occurs during fights between males. Lost pups are more vulnerable to subsequent attacks by adults of either sex (Le Boeuf and Campagna 1994).

Otariids (fur seals and sea lions) are polygynous, and sexually mature otariid males form and defend groups of females during the breeding season. Males tend to be much larger than females, and in most species there is a defined hierarchy where the largest males (up to 450kg in NZ sea lions) are dominant. These dominant males exclude smaller subordinate animals from access to females.

Female otariids nurse their young for a relatively long period (up to 12 months) in comparison with phocids (4 - 60 days) (Robeck *et al.* 2001). They remain with their pup for the first few days following birth, but then go to sea to forage for several days before returning to feed the pup. While the female is away pups are unprotected, and are particularly vulnerable when very young (Le Boeuf and Campagna 1994).

2.3.2 Nature and severity of neonatal trauma

During the breeding season male otariids frequently engage in fighting behaviour which varies between species from stereotypical aggressive posturing and bluff charges to overt attack. Pups can be inadvertently crushed during bouts of fighting between adult males, particularly in the early neonatal period when they are less mobile (Castinel *et al.* 2007).

In several otariid species targeted 'attacks' on pups have also been observed. This behaviour, sometimes known as abduction, has been recorded in Southern sea lions (Campagna *et al.* 1988), New Zealand sea lions (Marlow 1975; Wilkinson *et al.* 2000; Chilvers *et al.* 2005) and Australian sea lions (Marlow 1975; Higgins and Tedman 1990). Abducted pups were observed to be bitten, shaken, tossed, held down (both on land and in water) and mounted in apparent copulation attempts. In the majority of cases the attackers were adult (Higgins and Tedman 1990) or subadult males (Campagna *et al.* 1988). In general, female otariids were less likely than males to attack pups, and when they did, the attacks rarely ended in injury or death (Le Boeuf and Campagna 1994). An exception to this rule may occur in the Australian sea lion. Marlow (1975) found that 11 of 20 severe attacks in that species were carried out by females. Higgins & Tedman (1990) however, observed the same colony (Kangaroo Island, Australia) for two seasons 15 years later, and found that while females would attack pups that came too close to them, female attacks were less aggressive than those of males, and were not observed to cause injury. Marlow (1975) also carried out observational studies at Sandy Bay on Enderby Island during the summer (1st December to 24th February) of 1972. In 400 hours of watching, he observed only four attacks on NZ sea lion pups, all minor and all conducted by females.

There are few published reports describing the nature of pup attacks in otariid species in any detail. Campagna *et al.* (1988) observed 283 abductions of southern sea lion pups by subadult males over four consecutive breeding seasons. In the majority of

these abductions the male grabbed a pup, carried it out of the breeding area and then dropped it. Most pups would attempt to escape, but were either restrained by the male holding the pup down against the substrate or, less frequently, grabbed and shaken violently from side to side before being thrown into the air. Mounting of pups was also observed. Some pups were carried out to sea and fought over by several males, when they were bitten, submerged, shaken and thrown multiple times. The authors estimated that 5.6% of abducted pups died, although they noted that this was likely to be an underestimate of mortality as some pups were lost to observation. In addition, an unknown number of the pups that were seen alive at the end of the attacks may have died later, as ongoing monitoring was not possible.

Higgins and Tedman (1990) reported eight pup attacks in Australian sea lions. Although six of the attacks were observed, the authors did not provide detailed descriptions, but stated that pups were shaken, bitten, and thrown up to 2-4m by males. They also noted that females were seen to throw strange pups 1-2m, but that these pups did not appear to be injured.

Marlow (1975) described both male and female Australian sea lions as holding and 'violently shaking' as well as throwing pups. Some of these pups were killed or seriously injured. In the same paper Marlow reported attacks in NZ sea lions. He found that attacks by female NZ sea lions were less aggressive, involving only lifting and throwing of pups 1 - 2 m without shaking. No male NZ sea lions were seen to attack pups.

In contrast, during a visit to the NZ sea lion breeding colony on Dundas Island in the summer of 1999, Wilkinson *et al.* (2000) saw one pup being seized by a subadult male. This pup was taken out to sea and was later seen being shaken violently from side to side before being eaten. Nine other episodes of males shaking and eating dead pups were observed. Chilvers *et al.* (2005) stated that male NZ sea lions killed pups via 'biting, shaking, crushing and suffocation' at the Sandy Bay colony on Enderby Island, and were believed to be responsible for 21 – 42 % of pup mortality each season (Castinel

et al. 2007).

Cassini (1998) described a series of attacks by South American sea lions against South American fur seal pups. The author observed 31 attacks over a period of 86 hours. Eight affected pups died, 11 survived and 12 had an undetermined outcome (pups not observed immediately following attack). Although 11 pups were alive immediately after the attack, some appeared to be lethargic and poorly responsive, suggesting they may have sustained severe injuries.

Similar attacks by males have been described in fur seal species, including biting, shaking and mating behaviour in Alaskan fur seals (Bartholomew and Hoel 1953), and grabbing, shaking, throwing and mounting behaviour in Northern fur seals. The authors recorded three mortalities directly due to these attacks. (Kiyota and Okamura 2005).

2.3.3 Role of head trauma in pup morbidity and mortality due to attacks by adult otariids

Using only information from published reports, it is impossible to assess the true importance of head injury in pup morbidity and mortality seen as a result of attacks by adult and subadult otariids. What does seem evident is that some do vigorously shake and throw pups, and that severe injury and death can occur as a result (Bartholomew and Hoel 1953; Wilkinson *et al.* 2000; Kiyota and Okamura 2005). In most studies of pup attacks necropsy examinations are either not conducted or the results are not fully described. In those where necropsies were conducted on a few animals, full details of examination of the central nervous system are not given (Higgins and Tedman 1990; Kiyota and Okamura 2005), and no studies have reported microscopic examination of any tissues.

Traumatic brain injury is likely to be a consequence of some attacks on pups, for

example due to impact against hard substrates such as rocky shelves or large stones. It is also possible that the rotational accelerative forces generated during shaking could be sufficient to cause inertial brain injury and/or spinal cord damage.

Several papers investigating causes of pup mortality in otariid populations have been published. Baker and Doidge (1984) conducted necropsies on 135 Antarctic fur seal pups, and found that head trauma was responsible for 25.2% of all deaths. They described a range of lesions, including subcutaneous bruising, skull fractures, and "*contre-coup* haemorrhages on the ventral surface of the brain". Spraker *et al.* (2007) carried out gross and histological examinations on 186 dead California sea lion pups and found three that had died of trauma presumably inflicted by adult or subadult conspecifics. One was described as having severe blunt trauma, while the other two had bite wounds to the neck and thorax. Specific central nervous system lesions associated with these injuries were not discussed. Spraker and Landers (2010) found lesions of head trauma in 18% of 2,735 Northern fur seal pups, including skull fractures, subdural haemorrhages, epidural haemorrhages and bite wounds. Castinel *et al.* (2007) reported the results of over 400 necropsies on NZ sea lion pups conducted over a period of seven breeding seasons. The authors ascribed trauma as the main cause of death each year, and described cranial and thoracic haemorrhage in pups under two weeks of age as being a common presentation of trauma caused by adult animals.

2.4 The nature of brain injury

Damage to the structures of the brain can occur due to physical disruption of tissues (e.g. trauma) or to failure of oxygen or energy supply (e.g. hypoxia and ischaemia). The following sections examine these mechanisms of injury.

2.4.1 Hypoxic and ischaemic brain injury

The terms hypoxia and ischaemia are not interchangeable, although the concepts are

often combined as 'hypoxic-ischaemic injury' when considering effects on the central nervous system. Hypoxia implies a decrease in oxygen delivery to the brain, and can be due to anaemia, hypoxaemia, or impaired ability of tissues to utilise oxygen. In general, hypoxia tends to result in a compensatory increase in cerebral blood flow (Auer *et al.* 2008). Ischaemia, on the other hand, occurs when there is decreased blood flow to the brain, and therefore involves a lack of energy supply and impaired removal of metabolic waste products in addition to a decrease in oxygen delivery (Auer *et al.* 2008).

Focal ischaemia in the brain usually involves blockage of a single vessel, whereas global ischaemia results from decreased overall cerebral blood flow. In the normal brain, cerebral blood flow is coupled to metabolic demand and to systemic blood pressure, and is maintained within certain limits in a process known as cerebral autoregulation (Pryds 1991; Mayer and Chong 2002; Auer *et al.* 2008; Udomphorn *et al.* 2008). Decreased cerebral blood flow can occur when cerebral autoregulation is disrupted, when systemic blood pressure falls outside the normal limits of pressure autoregulation, or when vascular supply is obstructed. The latter can occur due to increased intracranial pressure or to physical compression or obstruction of the arterial supply to the brain (Graham *et al.* 1987; Mayer and Chong 2002; Auer *et al.* 2008).

The mechanisms of injury in cerebral ischaemia involve lack of oxygen supply, lack of energy supply, and accumulation of metabolic toxins such as lactate. In focal ischaemia, a combination of local lactic acidosis and oxygen and energy substrate deprivation lead to pan-necrosis (Auer *et al.* 2008), resulting in the death of neurons as well as of other cellular elements in the affected area. In global ischaemia specific neuronal populations undergo selective neuronal necrosis, due to differing sensitivities of various neural populations to oxygen deprivation. Vulnerable neuronal populations are well studied in humans, and include the hippocampus, cortical pyramidal neurons, cerebellar Purkinje cells, and some thalamic and brainstem nuclei. The molecular

basis of selective vulnerability probably involves regional differences in distribution of AMPA/KA and NMDA receptors, and may be modulated by regional variations in zinc concentrations (Koh *et al.* 1996; Dugan and Kim-Han 2006).

Excitotoxicity

Excitotoxicity is an important mechanism of neuronal loss in ischaemia, and results from overactivation of excitatory glutamate receptors. Oxygen deprivation in the brain results in decreased generation of ATP, leading to failure of cytoplasmic membrane ion pumps, particularly the Na-K ATPase pump. This in turn causes loss of normal transmembrane ion gradients with consequent prolonged membrane depolarisation and opening of voltage-sensitive ion channels. The overall response to this chain of events is influx of calcium into the cell. Increased levels of intracellular calcium cause a range of damaging events, including release of glutamate from synaptic vesicles, generation of oxygen-derived free radicals, and activation of phospholipases, endonuclease, and proteases. Simultaneously, increased dependence on glycolysis results in accumulation of lactic acid, which can ultimately lead to pan-necrosis (Dugan and Kim-Han 2006; Auer *et al.* 2008).

Cerebral oedema and brain swelling

Hypoxic-ischaemic brain injury can lead to cerebral oedema by a variety of mechanisms. Damage to the blood-brain barrier and to endothelial cells results in vasogenic oedema, while decreased membrane pump function results in accumulation of intracellular fluid (cytotoxic oedema) (Dugan and Kim-Han 2006). Increased fluid volume within the rigid cranial cavity leads to increased intracranial pressure with restriction of cerebral blood flow due to compression of vessels and herniation of brain parenchyma (Dugan and Kim-Han 2006; Auer *et al.* 2008).

2.4.2 Traumatic brain injury

Biomechanics of traumatic brain injury

Biomechanics as a discipline involves the application of mechanical principles to biological systems (Goldsmith and Plunkett 2004). Holbourn was one of the earliest researchers to begin to explain head injury in biomechanical terms (Holbourn 1943). Since then a number of research groups have further expanded our understanding of the mechanical forces that underlie brain injury. The following discussion is summarised from research and reviews by Ommaya, Gurdjian, Thibault, Gennarelli, Smith and colleagues (Gurdjian *et al.* 1955; Ommaya 1995; Graham and Gennarelli 1997; Ommaya *et al.* 2002; Smith 2011) and from Halliday (1999) and Saukko and Knight (2004).

Mechanical loading in closed head injury

Head injury results from application of force to the head, either directly or transmitted via the body. The clinical and pathological consequences of this loading depend on the magnitude and nature of the applied force, the tissue involved, and the duration of load application.

Based on the rate of load application, the forces causing head injury can be divided into static and dynamic loads. Static loading occurs when force is applied gradually over a period of time greater than about 200msec (Graham and Gennarelli 1997). This occurs for example when a head is trapped against a rigid surface and an increasing load is slowly applied, crushing the skull and ultimately compressing the brain. This mechanism is rarely encountered in humans, but is occasionally seen in people who have been trapped under debris during earthquakes or landslides.

More commonly head injury is a result of dynamic loading, where forces are applied more rapidly (generally over a period of 2 – 25 msec) (Graham and Gennarelli 1997). Dynamic loading can be further subdivided into impulse or impact types. Impact loading occurs when a head collides with a rigid surface. Both contact and inertial

damage can occur in this type of injury (see later). In contrast, pure impulse loading occurs when the head suddenly accelerates or decelerates without impact, and the damage that results is due solely to inertia.

Development of brain injuries

Using Holbourn's criteria (Holbourn 1943, 1945), brain injuries can be divided into contact and inertia phenomena. Contact phenomena occur as a result of impact, and include localised deformation of bone, skull fractures, and propagation of shock waves through the brain parenchyma, causing parenchymal haemorrhage. This latter mechanism is believed by many researchers to play a role in the creation of *contre-coup* contusions (Adams *et al.* 1980a; Ommaya *et al.* 2002). Inertial injuries result from acceleration or its negative equivalent, deceleration (both considered here under the term acceleration). The movement of the head and brain during acceleration can occur in either a linear fashion through the centre of gravity of the head (known as translational acceleration), or about an axis (rotational or angular acceleration). Purely translational acceleration is rare, and most cases of inertial injury involve components of both translational and rotational movement. Pure translational movement results in compressive and tensile strains within the brain; rotational movement results in shear effects. Brain and vascular tissue are particularly susceptible to shear forces, and it is these forces that are believed to give rise to subdural, subarachnoid and deep intra-parenchymal haemorrhages as well as diffuse axonal injury (Ommaya *et al.* 2002).

Application of force to the head (stress) results in brain injury via tissue deformation, known biomechanically as strain. Strain can be expressed as a percentage, based on the amount of deformation occurring within the tissue. There are three types of tissue deformation, namely compression, tension and shear. The magnitude of strain occurring in a particular tissue is partially dependent on the physical characteristics of that tissue. The adult human skull, for example, is a rigid tissue, which undergoes minimal deformation during load application but will fracture at a strain of 1 - 2 %

(Graham and Gennarelli 1997; Ommaya *et al.* 2002). The force required to generate a 1-2% strain in bone however, is much greater than that required to generate an equivalent strain in brain tissue. The brain is highly resistant to compression, but is susceptible to tensile and shear strain. In addition to these factors, the response of a tissue to stress will also depend on the rate of load application. In very general terms, biological tissues are more easily damaged by sudden deformation than by slow deformation (Graham 2001).

Damage to the structures of the brain is related to the force loading that occurs during an injury. Pure impulse loading occurs when the head is accelerated or decelerated without impact. In human medicine this is epitomised by 'whiplash' type motor vehicle accidents, where damage to the brain is predominantly inertial. In impact loading such as a fall there is both contact and inertial damage. When a falling head strikes the ground, the direct contact between the skull and the ground results in contact type damage such as fractures or contusions. The extent of damage will depend on the tolerance of the skull (which varies with maturity), the nature of the contacted surface (e.g. rock versus sand) and the impact velocity (which is dependent on the height of the fall). The skull will cease to move at the moment of impact but the brain will continue to move within the skull for a brief period, due to inertia. In most cases this inertial movement will have a rotational component. Pure contact injury is rare under natural conditions, although it forms the basis for some experimental models of brain injury.

Pathogenesis of traumatic brain injury

The transmission of mechanical forces to brain tissue results in damage that can be both vascular and parenchymal. Lesions resulting from trauma may be focal (e.g. contusions, lacerations, intracerebral haematomas) or diffuse (e.g. diffuse axonal injury, diffuse brain swelling). Brain damage as a result of trauma has traditionally been divided into primary damage (immediate) and secondary damage. Secondary changes were reviewed by Blumbergs *et al.* (2008), and include cerebral oedema, brain swelling,

raised intracranial pressure, and hypoxia-ischaemia.

In the past, most brain damage was thought to be immediate and irreversible (Strich 1956, 1961; Nevin 1967; Peerless and Rewcastle 1967). Recently however, many studies have emphasised that brain injury is in fact a process rather than a discrete event (Povlishock and Christman 1995; Povlishock and Jenkins 1995; Gennarelli 1997; Reilly 2001), thus blurring the distinction between primary and secondary events. Understanding this process has vital therapeutic implications, introducing the possibility of interfering with the progress of 'primary' injury, in addition to the more traditional approach of treating secondary effects such as cerebral oedema and ischaemia (Reilly 2001; Sahuquillo *et al.* 2001).

Diffuse axonal injury as a result of trauma

The first person to describe diffuse traumatic damage to cerebral white matter was Rosenblath (1899) (cited in Strich (1956)), who described tears in the corpus callosum and cerebellar peduncle of a 17-year-old tightrope walker who survived in a vegetative state for 8 months after a fall. Subsequently, Strich (1956) described a series of 20 cases of closed head injury where victims were unconscious from the moment of impact and survived for a variable period of time without fully regaining consciousness. In each of these cases there was extensive degeneration of white matter, with no mass lesions or evidence of raised intracranial pressure that could otherwise have explained the clinical signs. Strich surmised that this damage was due to shearing of axons that occurred at the time of injury, and predicted that such lesions could potentially be reversible (Strich 1956, 1961).

Subsequent authors also described diffuse damage to white matter in fatal head injury (Nevin 1967; Peerless and Rewcastle 1967; Oppenheimer 1968; Clark 1974). Various terms were used to describe this type of injury, including 'shearing injury' (Strich 1961) and 'diffuse brain damage of immediate impact type' (Adams *et al.* 1977). Not all authors

accepted Strich's proposition that diffuse white matter injury reflected primary damage to neural processes, with several groups, including that of Jellinger and Seitelberger (1970), suggesting that the observed damage was a consequence of secondary factors such as brain herniation or cerebral oedema.

In 1977 Adams and colleagues looked at a series of 19 fatal head injury cases where the patient had been unconscious from the moment of impact. All had focal lesions in the dorsolateral quadrant of the brainstem and corpus callosum in conjunction with histological evidence of diffuse axonal damage in white matter. Using either clinical data or histological demonstration of raised intracranial pressure (based on criteria developed by Adams and Graham (1976)), they were then able to define a subset of eight of these patients with diffuse white matter damage in the absence of raised intracranial pressure. They concluded that diffuse white matter damage could occur in fatal head injury in the absence of high intracranial pressure, ischaemia or hypoxia, and was therefore a primary event that occurred at the moment of impact. They further surmised that similar, but less severe diffuse axonal damage could potentially play a role in minor head injury, including transient loss of consciousness (concussion).

The term diffuse axonal injury first appears in the literature in the early 1980s (Adams 1982; Adams *et al.* 1982). During the next decade, diffuse axonal injury was more closely characterised in terms of both clinical presentation and neuropathology (Adams *et al.* 1985a; Blumbergs *et al.* 1989). It became obvious that while the general distribution of axonal injury as a result of trauma was fairly consistent (parasagittal cortex, corpus callosum, internal capsule and long tracts of the brainstem), there was marked variation in the severity of the lesions. In 1989 Adams *et al.* proposed three grades of diffuse axonal injury:

Grade I – diffuse damage to axons in the cerebral hemispheres, corpus callosum, brainstem and sometimes cerebellum;

Grade II – as above, with additional focal lesion(s) in the corpus callosum; and

Grade III – as for grade I, with additional focal lesion(s) in the corpus callosum and brainstem (usually the dorsolateral pons). (Adams *et al.* 1989)

The term diffuse axonal injury is now firmly entrenched in the literature as both a neuropathological and clinical entity. The classical clinical syndrome of diffuse axonal injury involves immediate unconsciousness upon injury, followed by prolonged coma in the absence of a mass lesion. This correlates with the pathological syndrome of grade III diffuse axonal injury.

2.4.3 Clinical correlates of diffuse axonal injury

Diffuse axonal injury is believed to be the most common cause of prolonged unconsciousness in severe head injury patients (Gentleman *et al.* 1995), and has been detected in mild head injury as well as in fatal injuries (Blumbergs *et al.* 1994; Blumbergs *et al.* 1995; Gentleman *et al.* 1995; Gennarelli 1996; Oehmichen *et al.* 1998). It seems likely that diffuse axonal injury also underlies most forms of concussive head injury in humans (Povlishock *et al.* 1979; Gennarelli *et al.* 1982; Pilz 1983; Blumbergs *et al.* 1994), and is an almost universal consequence of fatal head injury (Gentleman *et al.* 1995). The clinical spectrum of changes is likely to result from differences in location, quantity and severity of axonal damage (Gennarelli 1996).

2.4.4 Pathogenesis of traumatic axonal injury

Development and severity of axonal injury

Povlishock's group conducted a number of studies that enabled them to describe the morphological appearance of axonal injury and to determine that this injury is progressive (Povlishock and Christman 1995; Povlishock and Jenkins 1995). Using a fluid

percussion model of mild head injury they showed that there was an initial phase of disrupted axonal transport, followed by axonal swelling and lobulation at 3-6hours post injury. Axons fragmented between 12-24hours after injury, and the proximal portion of the axon continued to swell for several hours, presumably due to continued anterograde transport of intracellular substances. They did not detect immediate tearing of axons, in contradiction to the previously held belief that most axonal damage was instant and irreversible. Their findings were later supported by studies using other experimental models and by studies of human head injuries of varying severity (Maxwell *et al.* 1991; Sherriff *et al.* 1994; Gentleman *et al.* 1995). Povlishock and Jenkins (1995) proposed two possible initiating events for this process. Firstly, mechanical loading might alter the cell membrane and allow influx of ions such as calcium, which could then secondarily disrupt axonal transport. Alternatively, the mechanical loading could itself directly damage the cytoskeleton and thus impair axonal transport.

In 1996 Thomas Gennarelli described a spectrum of axonal changes, with increasing damage occurring as the strain applied to an axon increased (Gennarelli 1996). He proposed that primary axotomy represents the extreme of this spectrum, and occurs at over 20% strain. This axonal tearing occurs immediately on impact and is irreversible. With decreasing strain, less severe degrees of axonal injury occur. At less than 5% strain, axons undergo transient membrane injury with short term ionic disturbances. These changes are reversed within minutes of injury. At 5-10% strain there is reversible cytoskeletal damage and more severe ionic disturbances which result in disturbances of axonal transport and axonal swelling. The majority of axons would be likely to recover from this damage. Axons that undergo 15-20% strain suffer more severe structural and ionic disturbances that may develop into secondary axotomy 1-3 days after injury. It is at this level of injury that therapeutic intervention could potentially be targeted (Gennarelli *et al.* 1998).

LaPlaca and Thibault (1998) demonstrated that rapid mechanical loading of neurons

directly results in transient membrane depolarisation followed by ion fluxes, with a net movement of calcium into the cell. The term 'mechanoporation' is now used to describe this phenomenon (LaPlaca and Thibault 1998; Farkas *et al.* 2006).

Numerous authors have contributed to an understanding of the pathogenesis of the events described above, and there have been several comprehensive reviews in the past decade (McIntosh *et al.* 1996; Gennarelli 1997; Gennarelli *et al.* 1998; Reilly 2001; Sahuquillo *et al.* 2001; Marciano *et al.* 2002) which have helped to clarify and consolidate knowledge of the events underlying axonal injury. It now seems apparent that the majority of axonal damage does not occur immediately, but that mechanical loading initiates a sequence of changes that can lead to secondary axotomy in more severely damaged axons. The severity of diffuse axonal injury depends on the number of axons damaged, the proportion of axons that have been exposed to high strain and are therefore more likely to progress to irreversible damage, and the extent of secondary events such as brain swelling and hypoxia-ischaemia.

Biochemical and cellular events associated with axonal injury

The pathogenesis of axonal injury serves as a useful model of brain injury in general, as the majority of the processes that occur in the axon as a result of traumatic injury are similar to those occurring in other cells of the brain.

Mechanoporation results from mechanical injury to both axons and cell bodies (Gennarelli 1997; LaPlaca and Thibault 1998). In axons, this occurs at the node of Ranvier (Maxwell *et al.* 1991). Calcium influx into the neuron has been shown to be a key event in pathogenesis of damage to both the neuronal soma and the axon (LaPlaca and Thibault 1998; McIntosh *et al*. 1998; Buki *et al*. 2000). Mechanical deformation of the neurolemma or axolemma leads to transient depolarisation, which both alters ionic flux and stimulates release of excitatory neurotransmitters including glutamate (McIntosh *et al.* 1998; Sahuquillo *et al*. 2001). As a result of the increased membrane permeability,

calcium, sodium and chloride move into the cell, and potassium moves out into the interstitium (Gennarelli 1997). Calcium influx may also occur indirectly, via NMDA and non-NMDA receptor activation following release of glutamate, which is in turn triggered by mechanoporation-induced depolarisation (McIntosh *et al.*1998).

These changes occur even at the mildest end of Gennarelli's spectrum of axonal injury, the magnitude of the change depending on the magnitude of mechanical loading to the axon. At very mild strains, the cell recovers quickly, while at higher levels of strain, cytosolic calcium levels may rise to a point where intracellular proteases and phospholipases are activated (McIntosh *et al.* 1996; Gennarelli 1997; Buki *et al*. 2000; Sahuquillo *et al.* 2001; Buki and Povlishock 2006). Povlishock's group studied the pathogenesis of the cytoskeletal changes that develop in the more severe levels of axonal injury (Povlishock and Christman 1995; Maxwell *et al.* 1997). There are two possible causes of the neurofilament and microtubular damage that underlies disrupted axonal transport and secondary axotomy: proteolysis of cytoskeletal elements by calcium-activated calpains, a group of intracellular proteases; or mechanically induced misalignment of neurofilaments (Sahuquillo *et al.* 2001). The relative roles played by these two possible factors are not yet fully understood.

Povlishock's group also investigated the role of mitochondrial dysfunction in secondary axotomy (Buki *et al.* 2000). They found that increased cytosolic calcium results in opening of the mitochondrial membrane permeability transition pore, leading to influx of calcium into the mitochondria. This in turn causes mitochondrial swelling and decreased ATP generation, as well as release of cytochrome-c. Decreased mitochondrial energy production further disrupts ionic homeostasis, potentiating calcium influx and contributing to a cascade of worsening axonal damage. In combination, these events can lead to secondary axotomy.

2.4.5 Assessing severity of central nervous system injury using post mortem tissues

The severity of any brain injury depends on the effects of a combination of processes, including contusions, lacerations, meningeal and parenchymal haemorrhage, axonal injury, brain swelling, cerebral oedema and hypoxic-ischaemic injury. Several scoring systems have been developed for focal lesions such as contusions (Adams *et al*. 1980b; Adams *et al*. 1985b; Ryan *et al*. 1994; Finnie *et al*. 2000) as well as for diffuse injury (Blumbergs *et al*. 1995; Lewis *et al*. 1996; AbouHamden *et al*. 1997; Gentleman *et al*. 1999; Gorrie *et al*. 1999; Van den Heuvel *et al*. 1999; Finnie *et al*. 2000). In some studies combined systems have been used to estimate the overall severity of injury (Gorrie *et al*. 1999; Finnie *et al*. 2000; Finnie *et al*. 2001; Graham *et al*. 2004). Despite this, determining the clinical consequences of injuries identified at post mortem is fraught with difficulty (Geddes *et al*. 2000; Graham *et al*. 2004; Reichard *et al*. 2005). Survival time can affect both the presence and magnitude of some lesions. Neuronal necrosis, for example, is not detectable with survival of less than a few hours (Geddes *et al*. 2000; Graham *et al*. 2004), while axonal injury progresses over time, peaking at about 24 hours post-injury, before declining (Graham *et al*. 2004). These factors combine to mean that the clinical significance of brain injury detected at post mortem can be difficult to determine in animals where survival times are short or unknown.

2.5 Paediatric traumatic brain injury

Traumatic brain injury is the most common cause of debility and death in infants and children in many countries (Jennett 1996; Adelson *et al*. 2001; Bruns and Hauser 2003). Differences in mechanisms of injury, variations in case definitions and lack of consensus on definitions of terms (e.g. infant versus child versus youth) make it difficult to determine specific numbers, but published reviews indicate that there are around 80-350 cases of paediatric head injury per 100,000 population with a significant

percentage of these being severe or fatal cases (Berney *et al*. 1993; Finnie *et al*. 1999; Thurman *et al*. 1999; Adelson *et al*. 2001; Bruns and Hauser 2003). The mechanisms of injury vary between countries and with age, but in general, falls are the major cause of injury in children under 4 years of age, while inflicted head injury is the most common cause of fatal traumatic brain injury in children under 1 year old (Duhaime *et al*. 1998; Adelson *et al*. 2001; Bruns and Hauser 2003). The mechanism of injury is not the only variable responsible for different clinical presentations and outcomes in paediatric cases of head injury; there are also differences in morphometric parameters and in physiological responses of the immature brain compared with that of an adult.

2.5.1 Comparative anatomy and pathology: adult versus infant humans

There are several distinct physiological and anatomical features of the paediatric brain that distinguish it from an adult brain. The anatomical features have been summarised by several authors (Duhaime *et al*. 1987; Graham and Gennarelli 1997; Case *et al*. 2001) as follows:

- bones of the calvarium are thinner and more elastic than the thicker bones in adults, but also more prone to fracture on impact. The nature of the calvarium also means that forces are easily transmitted to underlying brain parenchyma;
- the neck muscles are weak and the head comparatively large and heavy;
- incomplete myelination makes the infant brain soft and more susceptible to shear forces;
- the subarachnoid space is large in volume but narrow in depth; and
- the internal surface of the neurocranium is relatively smooth.

In addition to these features, there are also several unique physiological aspects of the developing brain:

- the immature brain has a degree of plasticity in association with an excess of synapses (Huttenlocher 1979);
- cerebral autoregulation is impaired following trauma to young brains (Freeman *et al*. 2008; Udomphorn *et al*. 2008);
- there are age-related changes in the number and function of excitatory amino acid receptors (Kochanek *et al*. 1999); and
- immature brains are particularly prone to development of severe brain swelling (Graham and Gennarelli 1997; Kochanek *et al*. 1999; Blumbergs *et al*. 2008).

Morphological (e.g. suture closure, skull rigidity, myelination) and physiological (e.g. cell receptor numbers, response to ischaemia) features of the paediatric brain mature at varying rates. An important consequence of this is that there are quite marked differences in the response of the brain to injury at various ages within the category of 'paediatric' traumatic brain injury. It follows that studies should closely define what is meant by terms such as 'infant' or 'child' in order to make meaningful comparisons between studies and between age groups; unfortunately this is not always done (Duhaime *et al*. 1992; Berney *et al*. 1994; Geddes *et al*. 2001a; Geddes *et al*. 2001b).

2.5.2 Inflicted head injury and shaken baby syndrome

Inflicted head injury is a major cause of traumatic brain injury in children less than 2 years old, and is the most common cause of mortality in head trauma cases in this age group (Duhaime *et al*. 1992). Early reports of head injury in infants and children as a result of child abuse used the phrase 'battered-child' or 'battered baby' syndrome (Kempe *et al*. 1962), and recognised a common clinical pattern of subdural haematomas in conjunction with long bone fractures (Caffey 1946; Lis and Frauenberger 1950). In 1972, Caffey proposed that the lesions present in many of these children were caused by shaking (Caffey 1972). He hypothesised that the shear forces associated with shaking resulted in injury to the parenchyma of the brain as well as tearing of the bridging

veins, with consequent subdural haemorrhage. He introduced the term 'whiplash shaken infant syndrome' to describe this clinical entity.

Over subsequent years the syndrome has been more closely characterised in terms of clinical and pathological findings, although the underlying mechanisms remain contentious. While the term 'shaken baby syndrome' is now widespread, several other terms are used in the current literature, presumably reflecting the lack of consensus as to cause. These terms include: 'non-accidental head injury/trauma' (Duhaime *et al*. 1998; Kemp *et al*. 2003; Gerber and Coffman 2007); 'inflicted head injury' (Geddes *et al*. 2001a; Geddes and Whitwell 2004; Dolinak and Reichard 2006); and 'shaken-impact syndrome' (Bruce and Zimmerman 1989; David 1999).

Fatal cases of shaken baby syndrome typically have a triad of lesions involving encephalopathy, subdural haemorrhages and retinal haemorrhages. Some also have subarachnoid haemorrhages, epidural haemorrhages at the craniocervical junction, focal damage to the cervical spinal cord, skull fractures, optic nerve sheath haemorrhages, hypoxic brain damage and/or brain swelling (Geddes *et al*. 2001a; Geddes *et al*. 2001b; Leestma 2005; Gerber and Coffman 2007).

Most of the controversy in this form of inflicted head injury is centred on the nature and degree of force required to generate these injuries. Biomechanical analysis shows Caffey's initial contention that severe injuries can be inflicted as a result of repeated mild episodes of shaking to be unlikely (Duhaime *et al*. 1987; Ommaya *et al*. 2002). While some authors conclude that the syndrome can occur as a result of vigorous shaking alone (Alexander *et al*. 1990; Case *et al*. 2001), others believe an impact must occur before the forces generated exceed thresholds for shear injury (Duhaime *et al*. 1987; Bruce and Zimmerman 1989). Furthermore, the roles played by disparate factors including apnoea, hypoxia and spinal cord damage have not been fully elucidated (Hadley *et al*. 1989; Shannon *et al*. 1998; Geddes *et al*. 2001a; Geddes *et al*. 2001b; Geddes and Whitwell 2003). Perhaps the most relevant conclusion is that there are insufficient well designed

studies to enable firm statements to be made about the mechanism of injury in shaken baby syndrome (Donohoe 2003; Leestma 2005).

2.6 Subdural haemorrhage

The intracranial dura mater is composed of variably dense collagenous connective tissue, with an outer endosteal layer that forms the periosteum of the skull, and inner folds (the falx and tentorium) that enclose the dural venous sinsues. The dural connective tissue is well innervated, and also contains an extensive venous plexus. The inner aspect of the dura mater is continuous with the outer layer of the arachnoid mater. As these two layers are derived embryologically from the same structure, the meninx primitiva, there is no true space between the two, although they are easily separated at surgery or at necropsy. This separation is due to the presence of an easily-disrupted layer of loosely-connected fibroblasts along the inner aspect of the dura mater, known as the dural border cell layer (Weller 2005; Mack *et al.* 2009). There is thus no true 'subdural space', and subdural haemorrhage in fact accumulates *within* a disrupted dural border cell layer. Some authors have suggested that the term 'subdural compartment' should replace 'subdural space' in order to more accurately reflect the anatomy of this structure (Mack *et al.* 2009; Squier and Mack 2009).

2.7 Animal models of traumatic brain injury

The forces resulting in brain injury can be divided very broadly into contact and accelerative forces, and to some extent experimental models of brain injury have been designed to separately replicate these two types of forces. The earliest researchers in the field of animal models of brain injury were Denny-Brown and Russell, who developed a primate model in the 1940's (Denny-Brown and Russell 1941). Initially they compared damage occurring following impact to restrained and unrestrained heads, thus attempting to separate contact and inertia damage. Because it is almost impossible

to completely prevent inertial movement of the brain however, both of these models fit into the category of impact-acceleration. Impact-acceleration models have been developed and used in primates, rats, sheep, cats and rabbits (Finnie and Blumbergs 2002). In direct impact models, inertial forces are minimised by preventing head motion, thus maximising the contribution of contact forces to the resultant injury. These models have demonstrated that contact forces tend to produce focal lesions such as contusions, subarachnoid haemorrhage and skull fractures, as well as varying degrees of axonal injury (Finnie and Blumbergs 2002). Within this impact group of animal models are those developed to assess the effects of impact in humane slaughter of livestock (Finnie 1995; Finnie *et al*. 2000; Finnie *et al*. 2001; Finnie *et al*. 2002; Finnie *et al*. 2003).

In contrast, the effects of inertial forces alone are demonstrated by using models that involve application of accelerative forces without impact. Such inertial acceleration models have been developed in primates (Ommaya and Gennarelli 1974; Gennarelli *et al*. 1982; Graham *et al*. 1982), pigs (Smith *et al*. 2000; Raghupathi and Margulies 2002; Raghupathi 2004; Duhaime 2006; Ibrahim *et al*. 2010) and lambs (Finnie *et al*. 2006, 2010). Inertia models have been able to consistently replicate diffuse brain damage such as concussion, coma and diffuse axonal injury, and to identify differences in the susceptibility of animals to angular and translational acceleration (Finnie and Blumbergs 2002). Several models have been developed specifically to investigate shaken baby syndrome, and have demonstrated a number of lesions believed to be distinctive of this syndrome in human infants, including subdural and retinal haemorrhages and axonal injury (Raghupathi and Margulies 2002; Raghupathi 2004; Finnie *et al*. 2010; Ibrahim *et al*. 2010).

A third group of experimental models is that of direct brain deformation, which can involve either injection of a pulse of fluid directly onto the exposed brain or dura (fluid percussion) (Li *et al*. 1998) or mechanical application of a piston (controlled cortical impact or rigid indentation) (Smith 2011). This type of model maximises contact forces,

and tends to result primarily in focal brain damage rather than diffuse lesions. While most direct brain deformation models utilise rats, models have also been developed in cats and ferrets (Finnie and Blumbergs 2002).

2.8 Extrapolating data from human and experimental animal traumatic brain injury

Although experimental models have been central to an understanding of traumatic brain injury in humans, there are both biomechanical and biological differences between experimental and human traumatic brain injury. In experimental animal models biomechanical parameters such as peak acceleration, impact duration and angle of force are controlled and can be applied in a uniform and repeatable manner. This is obviously not the case in human head injury, where relationships between mechanism of injury and clinicopathological outcomes must often be derived from reconstructions of accidents (Ommaya *et al*. 2002). While thresholds for certain types of injury can be determined accurately in experimental models, these thresholds are not necessarily directly applicable to humans due to anatomical and physiological differences between species. Finnie *et al*. (2001) identifed a number of key distinctions between animal models and human patients, including brain size, brain mass:head mass ratios, craniospinal angle (the angle between the axis of the brain and the spinal cord), complexity of gyral structure, and degree of protection of the brain by other head structures (e.g. temporal muscles, frontal sinuses). Finnie and Blumbergs (2002) also noted that currently there is no single experimental animal model that accurately reproduces all aspects of traumatic brain injury in human beings.

Similar problems occur when attempting to extrapolate information from human traumatic brain injury and experimental animal traumatic brain injury to 'naturally' occurring head injury in veterinary practice. The nature and magnitude of forces encountered in experimental brain injury are unlikely to be the same as those

experienced by clinical veterinary cases. By definition, experimental animal models have been designed to replicate human injury, and may not be relevant to the veterinary situation.

Even when the mechanism of injury in a veterinary case is known (for example a dog hit by a car or a cat falling from a height) it is difficult to predict how brain injury may develop. Shearing forces and inertial loads are affected by brain mass, with smaller brains requiring greater angular acceleration to cause injury (Holbourn 1945; Ommaya *et al*. 2002), so inertial damage may be less common in small animal species. The craniospinal angle is almost linear in quadrupeds, decreasing the likelihood of rotational acceleration of the head (Finnie and Blumbergs 2002), therefore possibly decreasing the likelihood of diffuse axonal injury. Similarly, internal structures such as the tentorium cerebelli can affect development and propagation of shear forces (Finnie and Blumbergs 2002), so if diffuse axonal injury does develop in animals, its distribution may well differ from that seen in humans.

2.9 Non-experimental traumatic brain injury in animals

In his 1964 paper on central nervous system injuries in animals, Palmer noted that 'little attention has been paid to the various forms of brain damage' in the veterinary literature (Palmer 1964). Little had changed by 1982, when Palmer published a review of concussion (Palmer 1982). Today, a literature search for studies of non-experimental brain injury in animals produces mostly individual case reports or reviews of diagnostic and therapeutic aspects. A few larger retrospective studies of cases of equine, canine and feline traumatic brain injury have been published, although none include detailed gross and histopathological findings (Little *et al*. 1985; Dewey *et al*. 1993; Feige *et al*. 2000; Feary *et al*. 2007).

In horses, most traumatic brain injuries are of the impact type, and tend to involve either the poll or the frontal area (Feary 2007). Skull fractures are common in these cases (Feige *et al*. 2000; Feary *et al*. 2007), including those of the basilar bones in young horses that fall backwards after rearing up. The outcome of injury is variable, but is particularly poor with basilar fractures (Little *et al*. 1985; Feary 2007). Dewey *et al*. (1993) found intracranial haemorrhage in nine cats and 14 dogs that had sustained a variety of types of head trauma. Ten of their cases had subdural haemorrhage, which is in keeping with Palmer's statement that subdural haemorrhage is 'one of the most common types of injury'(Palmer 1964).

No published studies have specifically examined the possibility of axonal injury as a contributing factor to morbidity or mortality of veterinary cases of traumatic brain injury. Palmer (1964) described a cat that lost consciousness for three hours after being hit by a car. This was followed by an apparent clinical improvement, but after three days the cat deteriorated and died. At necropsy there was severe axonal degeneration in the pons, tegmentum, and cerebellar peduncles. Although this could have been diffuse traumatic axonal injury, similar changes could have resulted from vascular damage. Zachary (2007) states that diffuse axonal injury has been detected in animals, but this statement is not referenced, and may in fact refer to experimental animal cases. In contrast, Summers *et al*. (1995) stated that diffuse axonal injury 'appears not to have been recognised in spontaneous trauma cases in animals'.

Similarly, there have been very few published reports in the veterinary literature concerning shaking as a possible cause of brain injury. One paper described ocular examination of two kittens and one rabbit that had been shaken by a dog. The authors did not find any retinal or optic nerve sheath haemorrhages, and the brains were not examined (Serbanescu *et al*. 2008). Another publication described subdural haemorrhage and encephalopathy in a neonatal puppy. The authors attributed the haemorrhage to inertial injury sustained while being 'swung' as a means of resuscitation immediately

following parturition (Grundy *et al*. 2009).

The dearth of data on the pathology of traumatic brain injury in animals necessitated a detailed necropsy investigation in NZ sea lion pups before any conclusions could be made concerning the importance or origin of brain injury in this population, and this is set out in Chapter Three.

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Chapter three

Preliminary investigations of traumatic brain injury in NZ sea lion pups

3.1 Introduction

In human medicine, shaken baby syndrome is the main differential diagnosis whenever subdural haemorrhage is found in the intracranial cavity of an infant (Kemp 2002; Donohoe 2003; Cohen *et al.* 2010). The co-existence of anecdotal reports of subadult male NZ sea lions picking up and shaking pups, and necropsy reports describing intracranial subdural haemorrhage suggests that a 'shaken puppy syndrome' could exist in NZ sea lions. Before determining whether shaking and subdural haemorrhage were firstly related, and secondly important, one would first need to have a better appreciation of the potential mechanisms of injury that exist for NZ sea lion pups, and to more clearly define the nature, severity and frequency of traumatic brain injury-like

lesions at necropsy.

Several published reports describe attacks on NZ sea lion pups. Marlow (1975) observed the Sandy Bay colony for a total of 400 hours in the summer breeding season of 1971/72, and over this period observed four attacks on pups. In each case these attacks were carried out by females, who were seen to lift and throw pups 1-2m. During a visit to the NZ sea lion breeding colony on Dundas Island in the summer of 1999, Wilkinson and Childerhouse (2000) saw one live pup being seized by a subadult male, then carried out to sea before being shaken violently from side to side and eaten. Nine episodes of males shaking and eating dead pups were also observed. Chilvers *et al.* (2005) state that male sea lions at Sandy Bay kill pups by 'biting, shaking, crushing and suffocation', although they do not discuss how this was determined.

The NZ sea lion research team visits Enderby Island during the summer sea lion breeding period each year, from early December to late February. The primary purpose of these visits is to collect population and foraging data, but since 1998 necropsies have also been conducted. Castinel *et al.* (2007) published necropsy findings from NZ sea lion pups examined at Enderby Island between 1998 and 2005. Trauma was interpreted to be the major cause of pup mortality, being responsible for 21 – 42 % of pup deaths each season, and occurring in two peaks. The first peak was in the month following the median birth date and was attributed to crushing of pups during fights between mature males, resulting in signs of suffocation, "cranial haemorrhage" and "thoracic haemorrhage".

The second peak of traumatic death occurred in the later part of the season, and was attributed to subadult males. This latter part of the season is when shaking attacks by subadult males would be more likely to occur, as during this time aggression in males decreases markedly and by the end of January the larger territorial males have moved away from the rookery area, and subadult males can move into the harem unchallenged (Cawthorn 1993).

Necropsy records from Enderby Island have been archived since the 1998/99 breeding season. Analysis of these records would likely provide information about the importance of traumatic brain injury if they were conducted in conjunction with an appreciation of the types of traumatic interactions sustained by pups, and the types of lesions likely to result from various interactions. Lesions of traumatic brain injury are well described in the human literature, but much less so in the veterinary literature. Static loading, which results from the gradual application of force, is the biomechanical mechanism that would result from crushing of a pup by an adult male, or from a strong sustained bite to the head of a pup. In children over 12 months of age head crushing results in skull fractures, contusions, parenchymal lacerations and epidural, subdural and subarachnoid haemorrhages (Duhaime *et al.* 1995; Prasad *et al.* 1999; Case 2008). However, in very young infants, flexible cranial bones and unfused sutures mean that the skull is likely to deform without fracture under static loading (Margulies and Thibault 2000). The only report of crushing found in the veterinary literature described subarachnoid haemorrhages in the absence of skull fractures in two young kittens (Dewey *et al.* 1993).

In contrast, shaking generates shear forces, which cause lesions such as subdural haemorrhages, diffuse vascular injury, and diffuse axonal injury. In human infants, violent shaking is believed to cause subdural, subarachnoid and retinal haemorrhages, while impact tends to cause focal injuries such as skull fractures and contusions. Skull fractures are also a key feature of contact injury in animals (Little *et al.* 1985; Dewey *et al.* 1993; Feige *et al.* 2000; Platt *et al.* 2002; Feary *et al.* 2007). In general, impact with a small object results in a focal displaced fracture, while larger objects cause linear or multiple fractures (Halliday 1999). Single displaced fractures can be defined as 'depressed', when the inner table of the skull is displaced into the brain parenchyma, or 'pond' fractures, when a shallow depression results (Saukko and Knight 2004; Blumbergs *et al.* 2008).

By reviewing the archived necropsy reports for descriptions of lesions such as those

identified above, typical patterns of injury might begin to emerge. These patterns could then be compared with potential mechanisms of injury in order to detect relationships between the two.

3.2 Chapter aim

The aim of this chapter was to gain an insight into the nature of attacks on pups and the possible mechanisms of traumatic brain injury that may result. In the first part of the study presented below, potential mechanisms of brain injury are identified through observations made by the author and through reports obtained from sea lion biologists. Following this there is a brief review of the archived necropsy reports of pups found dead on Enderby Island in the breeding seasons from 1997/98 to 2006/07, in which the nature and pattern of lesions consistent with traumatic brain injury are described. These findings are then discussed in the context of evidence for a role of traumatic brain injury in the deaths of Enderby Island pups.

3.3 Materials and methods

3.3.1 Mechanisms of injury

Direct observations

From 12th to 30th January 2008, periodic observations of the rookery were conducted by the author from various view-points along the sward edge of the breeding site at Sandy Bay (Figures 3.1 and 3.2). These observations were sporadic and opportunistic, as they had to be timed around other responsibilities associated with the sea lion research project. Several 1 - 2 hour observations were made from Windy hut and the eastern end of Sandy Bay, and a digital video camera (Sony Handicam HDR SR5) was used to record some of the interactions that occurred during those periods. Observations from more distant sites were aided by binoculars (Quest Field 7.5; 7 x 21), and numerous

Figure 3.1. The sea lion breeding environment at Sandy Bay, Enderby Island, January 2008. Sea lions aggregate along the sand during the early breeding season. Most females remain on the sand with their pups until late February, while sexually mature males move away by mid January. Subadult males inhabit the edges of the breeding colony, including the grass sward. The main observation point, Windy Hut, is indicated by a black arrowhead.

Figure 3.2. A NZ sea lion breeding harem at Sandy Bay, Enderby Island. Females form a group (harem) guarded by a dominant mature male. Pups gather together in groups at the edges of and amongst the harems. Subadult males (black arrowheads) keep some distance from the harem. Note the dry sand substrate, with occasional rocks towards the grass sward.

shorter observation periods (10 – 20 minutes) were conducted while undertaking beach surveys, during which times image capture was not possible.

Descriptions of previous attacks

Five experienced sea lion team scientists were contacted by email and asked to describe any attacks on pups that they had observed during their time on Enderby Island. All had been to the island for at least three breeding seasons. No attempt was made to quantify the attacks; the reports requested were descriptive only.

3.3.2 Nature and prevalence of gross lesions of traumatic brain injury

Archived necropsy records

Since the 1998/99 breeding season all dead pups detected at Sandy Bay during the period the colony is observed (generally early December until late February) have been recovered for necropsy. Electronic records of the necropsy findings are held at Massey University, Palmerston North, New Zealand. The necropsy examinations were conducted by a range of individuals with varying degrees of pathology training, from ecology postgraduates with limited experience as prosectors, to veterinarians specialising in pathology. There is no documentation describing the protocols used each season.

Electronic copies of the necropsy reports for each season from 1998/99 to 2006/07 were reviewed. In these records, pups were individually identified using a unique code designed to signify the location, breeding season, and species. For example, E03/04-02Ph:

E = (Enderby Island)

03/04 = (breeding season)

-02 = (second necropsy of that season)

Ph = (*Phocarctos hookeri*)

For each season, the total number of necropsy examinations was noted, and the records of gross necropsy findings for each pup were screened for phrases describing lesions that could be associated with injury to the brain. Such descriptions included:

- bruising to the soft tissues of the head or neck;
- severe damage to the neck structures, including muscle tearing and cervical vertebral dislocation;
- fractures of the skull (neurocranium, maxillae, orbits, facial bones, mandibles) or cervical vertebrae;
- 'bruising' of the surface of the brain (either contusions or subarachnoid haemorrhage); and
- haemorrhage involving the meninges of the brain or spinal cord.

These descriptions were interpreted as indicators of possible traumatic brain injury ('traumatic brain injury-like lesions'). Details were entered into an Excel spreadsheet for all pups with necropsy descriptions corresponding to bruising of the head or neck, severe neck injury, skull fractures, contusions, subarachnoid haemorrhage, subdural haemorrhage or epidural haemorrhage.

3.4 Results

3.4.1 Mechanisms of traumatic brain injury

Several types of injurious interactions were identified. First, some pups were accidentally crushed by adult males during mating and territorial fighting in the early part of the breeding season. Secondly, hungry pups that attempted to suckle from a female that was not their mother would either be bitten and thrown by the female, or struck on the head by the female's teeth. Finally, some pups were grabbed around the neck or

back by a subadult male, who would then shake them several times and throw them. Occasionally a subadult male would bite a pup around the head or body, then lift and fling it several meters without shaking it. This behaviour occurred predominantly in the later part of the season, when dominant males had begun to move away from the harem and subadult males could more easily access pups. Figure 3.3 shows a pup being shaken by a subadult male. Excerpts from observer reports are presented in Appendix 1.

Figure 3.3. NZ sea lion pup being shaken by a subadult male. This interaction took place in late January 2008, and was observed by the author. The pup was picked up multiple times, held either by the neck or the back, and shaken 3-4 times before being thrown or dropped. After about 10 minutes the female in the background moved towards the pair, making threat-like barks. The subadult male then dropped the pup, which lay still for several minutes before moving off apparently unharmed. This pup was a tagged individual that was still alive at the time the research team left the island. (Photograph courtesy of Louise Chilvers, NZ Department of Conservation.)

In biomechanical terms, these behaviours can be divided into three mechanisms of injury: inertia loading (shaking); impact loading (throwing or striking); and static loading (crushing by biting or trampling).

3.4.2 Review of necropsy records 1998/99 to 2006/07

A total of 592 pup necropsy records were reviewed. Gross lesions consistent with traumatic brain injury were described in all seasons, and were present in 192 pups (see Table 3.1). The terminology used varied between prosectors and between seasons, but an interpretation of the nature of the lesions described could usually be made. Because no records were available of the necropsy protocol followed each season, it was not possible to determine whether the absence of reports of a certain lesion reflected a lack of examination of the relevant area or a true absence of the lesion. This was particularly true of cervical spinal cord meningeal haemorrhage (abbreviated to 'spinal haemorrhage' below), which was not described in any pups in three seasons, probably reflecting under-reporting. Summary data is presented in Appendix 2.

	98-99	99-00	00-01	01-02	02-03	03-04	04-05	05-06	06-07	Totals
Head/neck bruises	7	5	7	17	34	10	7	15	14	116
Severe neck injury	1	2	2	0	7	1	1	1	2	17
Fracture	2	0	0	1	3	7	1	3	9	26
SAH/contusion	1	1	0	1	6	7	0	0	2	18
SDH brain	1	2	4	14	29	9	3	6	17	85
Spinal haemorrhage	0	0	3	0	20	0	2	0	1	26
Total with TBI-like lesions	7	6	8	37	58	17	8	19	32	192
Total necropsies	32	47	52	104	112	69	53	55	68	592

Table 3.1. Summary of recorded gross lesions from pup necropsies conducted between 1998/99 and 2006/07. (Note: many pups had more than one type of traumatic brain injury-like lesion.) SAH = subarachnoid haemorrhage; SDH = intracranial subdural haemorrhage; Spinal haem = spinal cord meningeal haemorrhage; TBI = traumatic brain injury.

The percentage of pups with lesions of traumatic brain injury varied from 13% in 1999/2000 to 52% in 2002/03. Head bruising was the most commonly recorded lesion overall, with 116/592 pups (19.6%) affected. Subdural haemorrhages within the cranial vault were the next most frequently reported lesion, and were present in 85/592 (14.4%) of pups necropsied. The temporal distribution of traumatic brain injury lesions is shown in Figure 3.4.

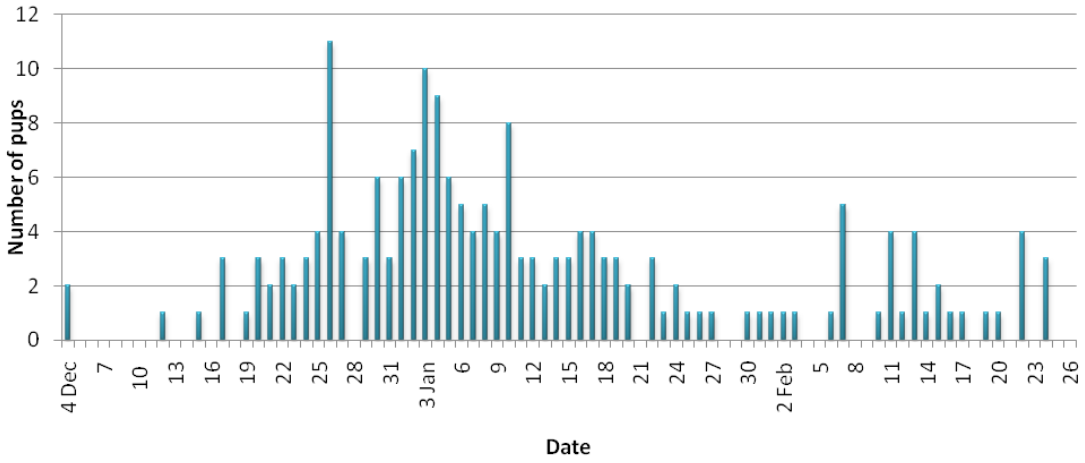

Figure 3.4. The cumulative total number of pups (1998/99 – 2006/07) with gross lesions consistent with traumatic brain injury, demonstrating two peaks of traumatic brain injury-like lesions. The largest peak occurred in the first part of the season, and was separated from a second, smaller peak by a low-prevalence phase extending from 25th January to 7th February.

Two observed incidents of fatal crushing were reported, both of which occurred early in the season. At necropsy examination one of these pups (E05/06-36Ph) had an intracranial subdural haemorrhage but no skull fractures, while the second (E03/04-17Ph) had traumatic separation along the cranial sutures and a transverse fracture through the base of the skull, with aspiration of blood into the airways.

Figure 3.5 shows the prevalence of selected early season and late season lesions for live-born pups, concentrating on those lesions identified as being consistent with crushing, impact or shaking. A total of 26 pups had skull fractures, all but one of which occurred in the early part of the season. Eight of these pups had single depressed or pond

fractures (consistent with a tooth strike), and 13 had multiple linear fractures, often along cranial suture lines.

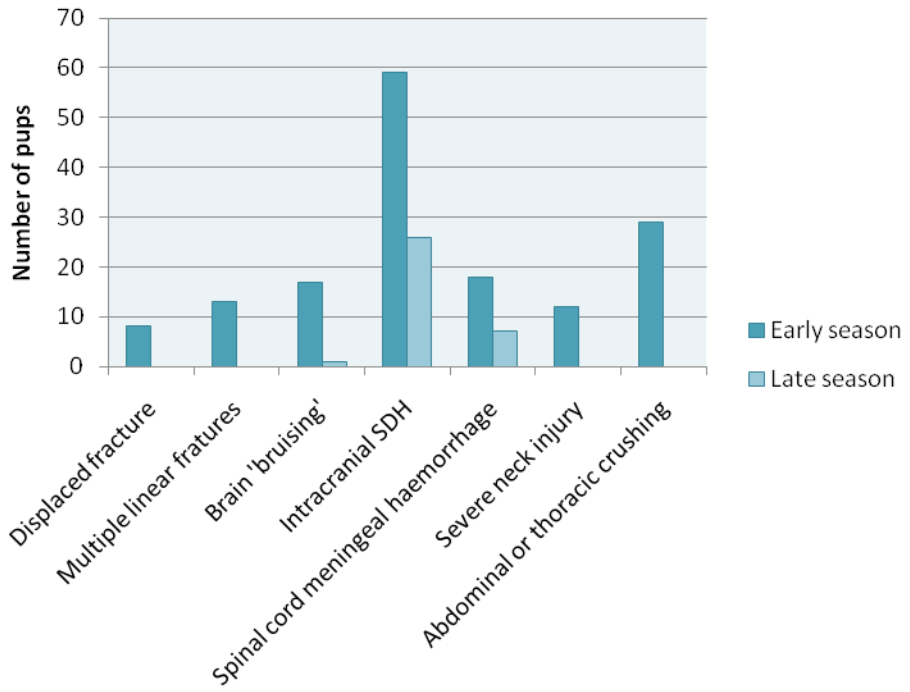

Figure 3.5. Frequency of selected traumatic brain injury-like lesions in live-born early and late-season pups, for years 1998/99 to 2006/07. The lesions depicted here are indications of one or more of the mechanisms identified as potential causes of traumatic brain injury in NZ sea lion pups (tooth strike impact, substrate impact, crushing by biting or trampling, and shaking).

3.5 Discussion

The two peaks of traumatic injury noted by Castinel *et al.* (2007) are reflected in the temporal analysis conducted in this chapter (see Figure 3.4), which found two peaks of apparent brain injury. The observations conducted by the author and the reports received from sea lion biologists familiar with the Enderby Island colony confirm crushing, shaking and impact as mechanisms of injury. These observations also support temporal injury patterns, with crushing predominant in the first part of the season, and shaking/impact injury being more common in the late season. Analysis of the types of lesions described in the necropsy records also showed that most crushing occurred early in the season, as evidenced by rupture of abdominal and thoracic organs,

and multiple linear skull fractures.

Shaking and throwing of pups was the major injury mechanism identified in the later part of the season. Impulse loading due to shaking alone would cause tissue injury via generation of shear forces, producing lesions such as subdural haemorrhages, diffuse vascular injury and diffuse axonal injury. Only the first of these three lesions would be evident at gross necropsy examination; demonstration of the latter lesions would require microscopy of well preserved tissues. Most late season traumatic brain injuries in these sea lion pups involved intracranial subdural haemorrhages and spinal cord meningeal haemorrhages, consistent with an interpretation that shaking was the predominant mechanism of injury at this time. Unexpectedly however, both of these lesions were actually more frequent in the early season. Some of these early season subdural haemorrhages could have been birth-associated. Subdural haemorrhages have been found to occur following non-traumatic deliveries of human babies (Whitby *et al.* 2004) and in neonatal foals, lambs and calves, where they persist for at least 7 days post partum (Haughey 1975; Haughey and Jones 1976; Haughey 1980). Thus subdural haemorrhages present in a pup of less than a few weeks of age would not necessarily be associated with post-parturient trauma.

Lesions suggestive of shaken baby syndrome include retinal, subdural and sub-arachnoid haemorrhages (Caffey 1974; Duhaime *et al.* 1998), as well as cervical spinal cord damage (Geddes and Whitwell 2003). A combination of subdural haemorrhage and severe neck injury occurred in only one pup, and no retinal haemorrhages were recorded, although it is unclear whether ocular examination was a routine part of the necropsy protocol. In general, the lack of a consistent necropsy protocol incorporating examination of the eyes and cervical spinal cord made retrospective analysis of archived records an unreliable method for assessing shaking injury.

Impact injuries are represented in this study population by skull fractures and brain 'bruising'. Soft tissue bruising of the head was very common, but is a non-specific

change which could occur with biting, crushing or impact. Conversely, the nature of the substrate (particularly soft sand) and the thick skin and hair coat of sea lions mean that bruising would not necessarily occur in every case of impact. Overall, 37/592 (6%) pups had skull fractures and/or brain 'bruising' that could have occurred due to impact.

It is not possible to determine the clinical significance of brain injury based solely on the gross lesions present. With impact injuries for example, the human literature shows that the presence of a skull fracture in itself is not conclusive evidence of a significant brain injury, as skull fractures frequently occur in both adults and children without any signs of neurological dysfunction (Saukko and Knight 2004; Blumbergs *et al.* 2008). Conversely, fatal impact injuries commonly occur without skull fractures (Ryan *et al.* 1994; Saukko and Knight 2004; Blumbergs *et al.* 2008). Assessing the likely clinical consequences of brain injury in NZ sea lion pups would require more thorough investigations, including examination of brain tissues using histological techniques.

3.6 Summary

The results of the studies described in this chapter demonstrate that traumatic brain injury-like lesions were present in a considerable proportion of pups that died during the 1998/99 to 2006/07 breeding seasons. Two peaks of these lesions were identified, correlating with distinct mechanisms of damage occurring in the early (crushing) and late (shaking and throwing) parts of the season. Shaking attacks on pups were observed, but follow up of outcome for individual pups was not possible. Intracranial subdural haemorrhages and haemorrhages around the spinal cord were common, but although these are suggestive of shaking injury, existing data did not consistently provide sufficient detail to rule out other possible causes, or to determine the clinical significance of any brain injury.

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Chapter four

The role of traumatic brain injury in NZ sea lion pups, 2007/08

4.1 Introduction

In Chapter Three, an analysis of archived necropsy records showed that gross lesions consistent with head and/or brain injury occurred in a number of pups each season. Prominent amongst these lesions were subdural haemorrhages, which were found in 2-25% of pups each season. In human neonates subdural haemorrhages are considered to be caused by shaking, and in this context are often accompanied by retinal and subarachnoid haemorrhages or damage to the cervical spinal cord. Details of examination of eyes, brain and spinal cord were not always included in the pup necropsy records (Chapter Three), making it difficult to determine if a similar

constellation of signs occurred in these pups. Accordingly, this chapter describes a series of investigations of pups found dead in the 2007/08 breeding season using a standardised necropsy protocol, with the following aims:

1. To determine the cause of death of each pup;
2. To characterise the nature and origin of traumatic brain injury-like lesions; and
3. To determine the relative importance of traumatic brain injury in pup mortality.

Annual NZ sea lion research visits to Enderby Island are divided into two phases, with one group of scientists stationed on the island from early December, and a second group taking over in early January. In the 2007/08 season the author was part of the second group, and was able to conduct necropsies during this time. In the first part of the season there was no veterinarian in the team, so the possibility of removing and freezing the heads of the pups that died during this time was considered during the planning phase of this study. The heads could then be thawed and examined by the author during the second part of the season. In previous studies the author had found several consistent changes resembling trauma in the bodies of adult NZ sea lions that had been frozen before necropsy examination (Roe 2010). Similar changes had earlier been attributed to trauma (Duignan and Jones 2007). In order to investigate this phenomenon further, an experiment was designed to determine whether frozen-thawed bodies could reliably be used to assess gross and histological lesions associated with trauma. This experiment is presented in Section 4.2 below. Section 4.3 describes the gross lesions found in pups from the 2007/08 season, focussing particularly on those with traumatic brain injury-like lesions. The cause of death of each pup is ascertained, and concurrent syndromes that could be associated with morbidity are identified. Lesions known to be associated with shaking in human patients, including subdural, subarachnoid and retinal haemorrhages, are characterised more closely, and possible mechanisms of traumatic brain injury are discussed based on patterns of injury and a temporal analysis of lesion frequency.

4.2 Investigation of freeze-thaw artefact

4.2.1 Materials and methods

Ten otherwise healthy male fur seals were sourced from animals incidentally captured during commercial fishing operations in Cook Strait, New Zealand, between 26th June 2008 and 2nd September 2009. These seals were retrieved dead from nets and packed in two layers of polythene, placed on ice and transported to Massey University, Palmerston North. On arrival, they were allocated alternately into one of two groups: 'frozen' or 'non-frozen'. Those in the frozen group were stored in a -20°C freezer for 4 – 8 weeks, then removed and placed in dorsal recumbency for thawing and necropsy. Animals in the non-frozen group were necropsied within 12 hours of receipt.

Necropsy examinations were conducted according to a standard protocol. Bodies were weighed and examined for external evidence of wounds or other lesions, before being skinned to reveal subcutaneous tissues. Areas of apparent bruising were mapped on line drawings. The body cavities were then opened and examined. Where present, abdominal cavity fluid was removed and measured, and the nature of the fluid recorded. Internal organs and vessels were examined, and the head was then removed by disarticulating the atlanto-occipital joint and severing all soft tissue attachments. The head was sectioned longitudinally using a band saw and each half of the brain was removed and inspected for gross lesions. The two halves were then placed into 4 litres of 10% neutral buffered formalin. Brains were fixed for at least two weeks prior to a second gross examination, at which time lesions were photographed and recorded.

Sections of formalin-fixed brain, kidney and subcutaneous tissue were processed into paraffin for examination under a light microscope. Paraffin-embedded 4-µm thick sections were stained with haematoxylin and eosin (H&E) and with Martius Scarlet Blue trichrome (MSB).

The effects of autolysis alone were assessed using an unfrozen adult male fur seal that died due to severe blunt-force trauma to the head. This animal had a post mortem interval of seven days (five days in situ at ambient temperature (7 – 14°C) and two days in transit on ice). In order to evaluate frozen tissue known to have been bruised ante-mortem, the head of this fur seal was frozen for two weeks after necropsy, and then thawed for 24 hours. Pieces of bruised tissue were fixed in 10% neutral buffered formalin and then processed for histology as described above.

The differences in 'lesion' frequency between the groups were analysed using Fisher's exact test.

4.2.2 Results

Ten bycaught male fur seals were received between 1st July 2008 and 4th September 2009. Death was attributed to drowning in all cases, and all animals were in good body condition with no evidence of concurrent disease.

Gross pathology

Details of relevant gross findings are summarised in Table 4.1. None of the non-frozen animals had soft tissue bruising, but each of the five frozen animals had lesions resembling bruises (dark red, glistening to gelatinous foci) within the blubber or muscle along the ventral midline (Figure 4.1A), hereafter referred to as 'pseudo-bruising', in the axillary soft tissues or over the shoulders. In these areas the superficial blubber was invariably normal in colour, with discolouration and gelatinous change confined either to the deep blubber and superficial muscle layer or to the deeper muscle layers only, with normal overlying superficial musculature. No animals had associated damage to the skin or pelage. In addition to these lesions, two frozen animals had small symmetrical bruise-like lesions of the deeper layers of muscle of the dorsal neck without involvement of superficial tissues. One of these animals had several millilitres of unclotted, dark red fluid beneath the muscle fascia in the apparently bruised area.

Case No.	Frozen?	Days in transit	Days frozen	Days thawed	Weight (kg)	'Bruised' sternum	'Bruised' shoulders	Abdominal fluid (ml)	'Bruised' renal capsule	'Bruised' reproductive tract ligaments	Head 'bruises'	Focal brain 'contusion'	Mottled brain discolouration	Blood from nares	Ocular 'haemorrhage'
2008-1	-	5	0	0	105	-	-	-	-	-	-	-	-	-	-
2009-2	-	2	0	0	80	-	-	-	-	-	-	-	-	-	-
2009-4	-	3	0	0	54	-	-	-	-	-	-	-	-	-	-
2009-8	-	3	0	0	75	-	-	-	-	-	-	-	-	-	-
2009-9	-	2	0	0	55	-	-	-	-	-	-	-	-	-	-
2008-2	+	3	25	3	80	+	+	100	+	+	-	+	-	-	-
2009-1	+	5	36	4	52	+	+	10	+	+	neck/throat	-	+	+	+
2009-3	+	3	24	4	55	-	+	180	+	+	+	+	+	+	-
2009-5	+	4	52	4	120	+	+	260	+	+	-	-	-	-	+
2009-7	+	3	44	4	65	+	+	380	+	+	+	+	+	+	+

Table 4.1. Comparison of gross lesions from fur seals that were chilled (light shading) or frozen and thawed (darker shading) prior to necropsy examination.

One fur seal from the frozen group (2009-1) had severe haemorrhage of the muscles and blubber of the right side of the neck, extending from the inner surface of the skin down to the level of the cervical vertebrae. The superficial and deep musculature of this region was crushed and torn. There was no damage to the outer skin surface.

All five frozen fur seals, but no non-frozen ones, had thick, dark red, unclotted fluid in the abdominal cavity. The volume of intra-abdominal fluid varied from approximately 10 ml to 380 ml. In all cases intra-abdominal organs and vessels were intact. Smaller, non-quantified volumes of similar fluid were found in the thoracic cavity (n = 3) and pericardial sac (n = 2) of several of the frozen animals. The intra-abdominal fluid was red/brown and hazy with low cellularity (up to 10.4 x 10^9 nucleated cells per litre). Cytological examination of centrifuged precipitate showed sheets of non-reactive mesothelial cells and occasional macrophages. Extensive haemolysis prevented accurate assessment of specific gravity, PCV or protein concentration.

All five frozen animals had focal areas of dark red discolouration of the external surface of the kidneys, which was most prominent along the lumbar surfaces and at the poles (Figure 4.1B). In four cases this change was bilateral. Removal of the capsule from each kidney showed that this discolouration corresponded to dark red gelatinous thickening of the renal capsule, and that the underlying kidney was unaffected. All five frozen animals also had dark red discolouration of the vas deferens of one (n = 1) or both (n = 4) testes (Figure 4.1C). In bilateral cases, the affected tissue was much darker on one side than the other. Two animals had several millilitres of dark red fluid within the tunica vaginalis. Segments of intestine were discoloured dark red in all frozen animals but in no non-frozen animals.

All previously frozen brains had slightly increased opacity of the meninges, with bulging or flattening of gyri and loss of clarity of the superficial vasculature. Discolouration of the surface of the brain was apparent only in frozen animals, each of which showed a mottled pattern of red/brown staining within sulci and adjacent to meningeal vessels.

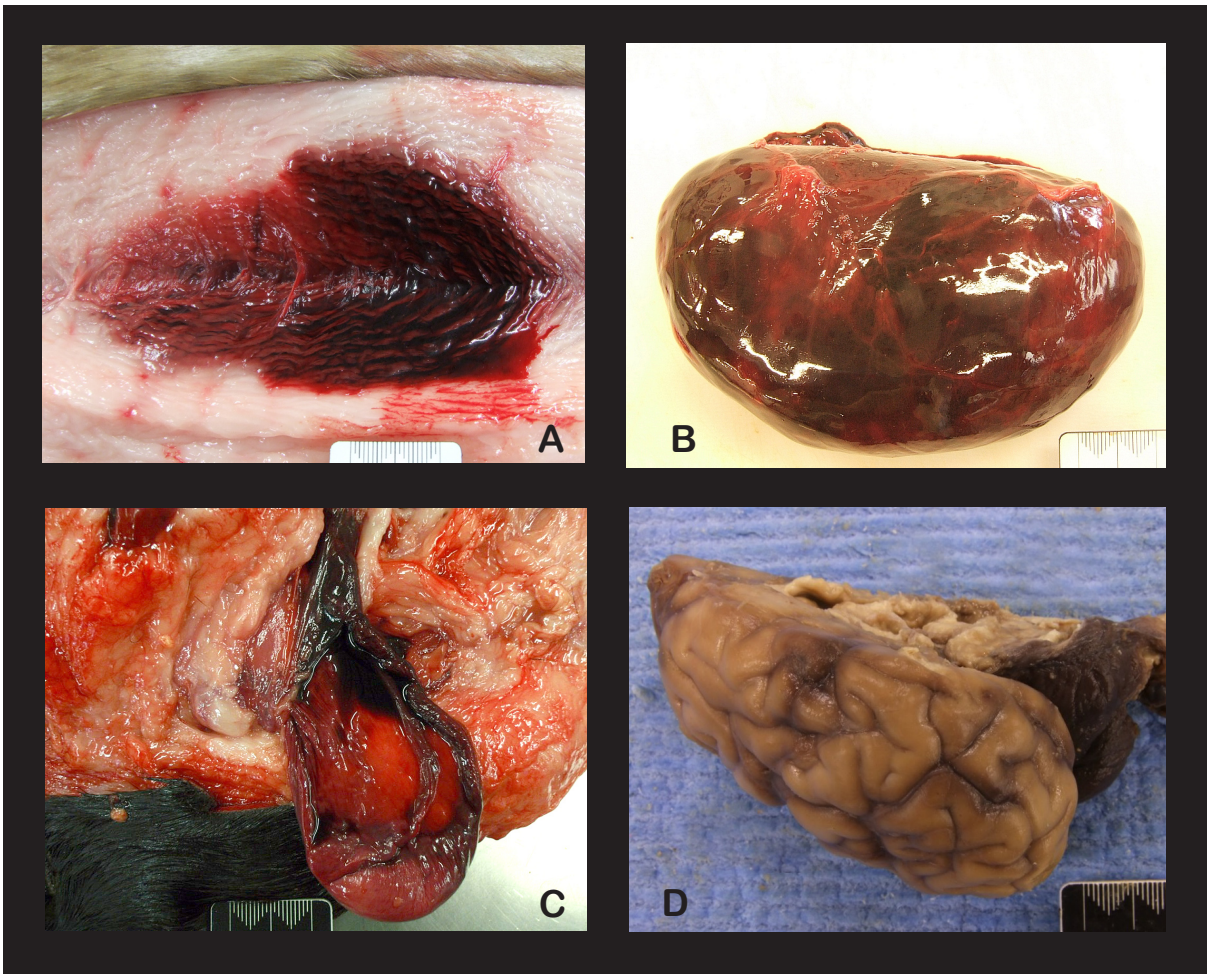


Figure 4.1. Gross lesions present in pinnipeds that have been frozen and thawed before necropsy examination. **A**. Pseudo-bruise in the ventral blubber. The superficial blubber and skin are unaffected. **B.** Lumbar surface of the kidney with capsule intact. Note the discolouration of the capsule by dark red gelatinous material. **C**. Dark red fluid distends the tunica vaginalis of one testis. **D**. Dark red discolouration of the entire cerebellum, with mottled discolouration of the leptomeninges of the cerebral cortex along the margins of meningeal vessels.

In addition, three frozen brains had larger focal areas of red/brown to black staining which resembled contusions, one on the left occipital lobe, one on the dorsal aspect of the parietal lobe, and the third involving the entire left lobe of the cerebellum (Figure 4.3). No gross lesions were noted on the brains removed from non-frozen animals. Meningeal vessels were congested in three of the non-frozen fur seals, with the congestion being unilateral in two of these.

Other lesions found only in the frozen group were blood-tinged fluid in the anterior ocular chamber (n = 3) and haemorrhage from the nares (n = 3).

There was a significantly higher prevalence of dark red intra-abdominal fluid, and of bruising of the sternum, shoulders and axillae in frozen fur seals ($p = 0.004$), but there was no difference between groups for focal brain 'contusions' ($p = 0.08$), ocular 'haemorrhage' ($p = 0.08$) or 'bleeding' from the nares ($p = 0.08$).

The autolysed control animal had transverse bands of pink discolouration of the blubber at the level of the shoulders and caudal part of the ribs affecting the ventral and left lateral body wall. This seal had been killed by multiple impacts to the head, causing skull fractures, contusions, and extensive muscle bruising. It also had a focal, 20 x 20 mm area of dark red gelatinous material within the fascia and superficial part of the right deltoid muscle, with no corresponding discolouration of the blubber or skin. There was no detectable fluid in body cavities. The poles and lumbar surfaces of both renal capsules were darkened, but no gelatinous thickening was present, and the testes were normal in colour. There was segmental reddening of the serosal surfaces of the intestines.

Histopathology

The microscopic appearance of pseudo-contusions is shown in Figures 4.2 - 4.5. Within sections of the focal dark lesions from frozen-thawed brains there was increased eosinophilia of the superficial cortex, with rows of variably sized, brightly eosinophilic globules within the grey matter. There was a general loss of cellular detail, and numerous 'cracks' (narrow clear spaces 20 – 40μm wide and up to 5mm long) were present throughout the parenchyma. Within the cortex and subarachnoid space, intact and lysed erythrocytes were present both within and outside blood vessels, and blood vessel walls were often indistinct. The subarachnoid space in some areas contained granular to globular eosinophilic deposits. MSB stains showed scattered individual intact erythrocytes both within and surrounding blood vessels.

Pseudo-bruising of the blubber and renal capsule are depicted in Figure 4.6 - 4.9. Sections of subcutaneous pseudo-bruises showed brightly eosinophilic granular material (interpreted as fluid) dissecting along connective tissue layers within the blubber.(Figure 4.6). Underlying muscle was not affected. Examination of sections of a frozen antemortem bruise on the head of the autolysed control animal showed sheets of lysed erythrocytes within the connective tissue between muscle bundles (Figure 4.7). In some areas there were moderate numbers of entrapped leucocytes. Sections of kidney from a frozen fur seal showed distension of the space between the two capsular layers (Figure 4.8) by variable amounts of eosinophilic fluid admixed with lysed and, less frequently, intact erythrocytes (Figure 4.9).

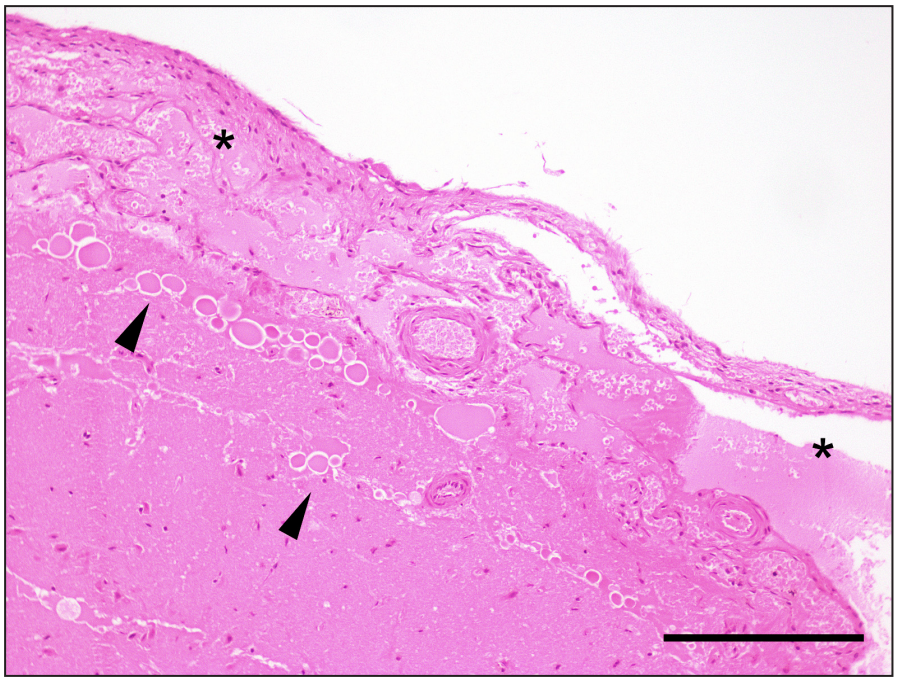


Figure 4.2 A pseudo-contusion of the cerebral cortex from a frozen-thawed fur seal. Note the expansion of the subarachnoid space by eosinophilic fluid (asterisks). The superficial cortex contains rows of brightly eosinophilic globules (arrowheads). Bar = 400 µm. (H&E)

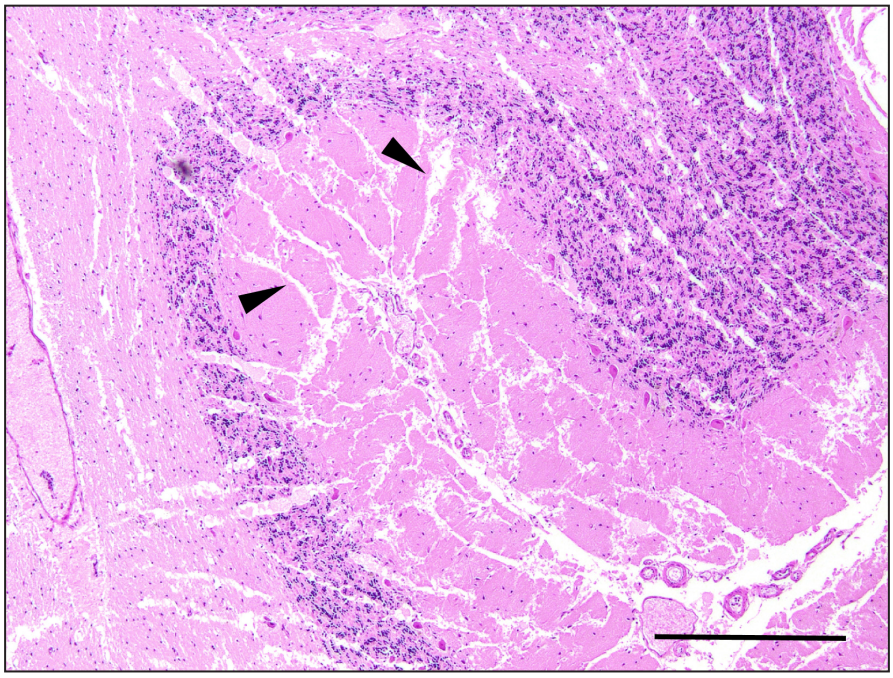


Figure 4.3. A section of cerebellum from a frozen-thawed fur seal, showing 'cracks' in the parenchyma (arrowheads): clear clefts of dirsupted tissue up to 5 mm long. Bar = 400 µm. (H&E)

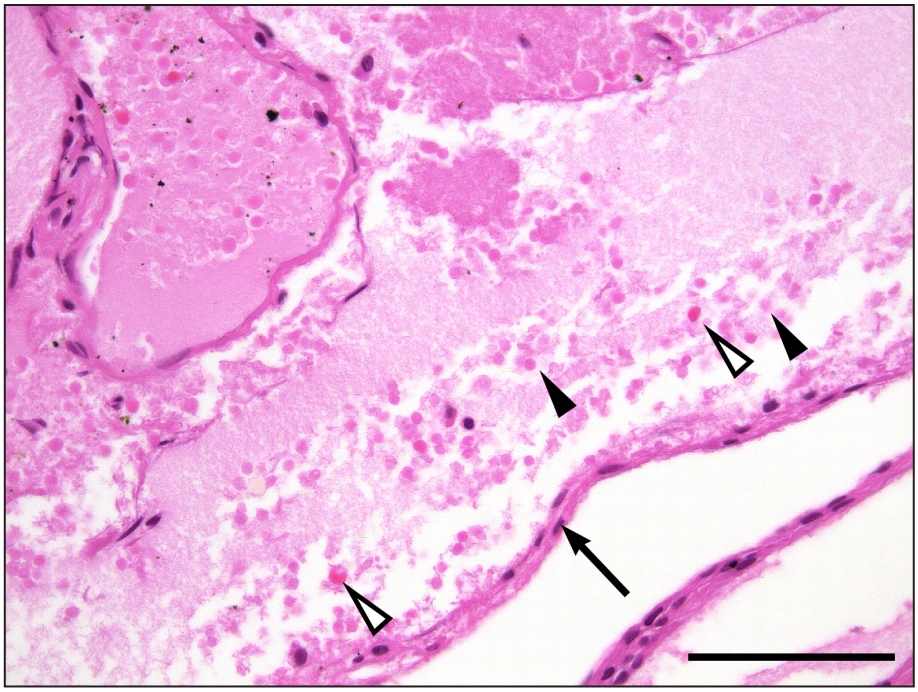


Figure 4.4. Higher power magnification of a cerebral pseudo-contusion from a frozen-thawed fur seal. The arachnoid mater is indicated by an arrow. Note that there are free erythrocytes within the subarachnoid space, including both lysed (black arrowhead) and intact (white arrowheads) forms. Bar = 200 μm, (H&E)

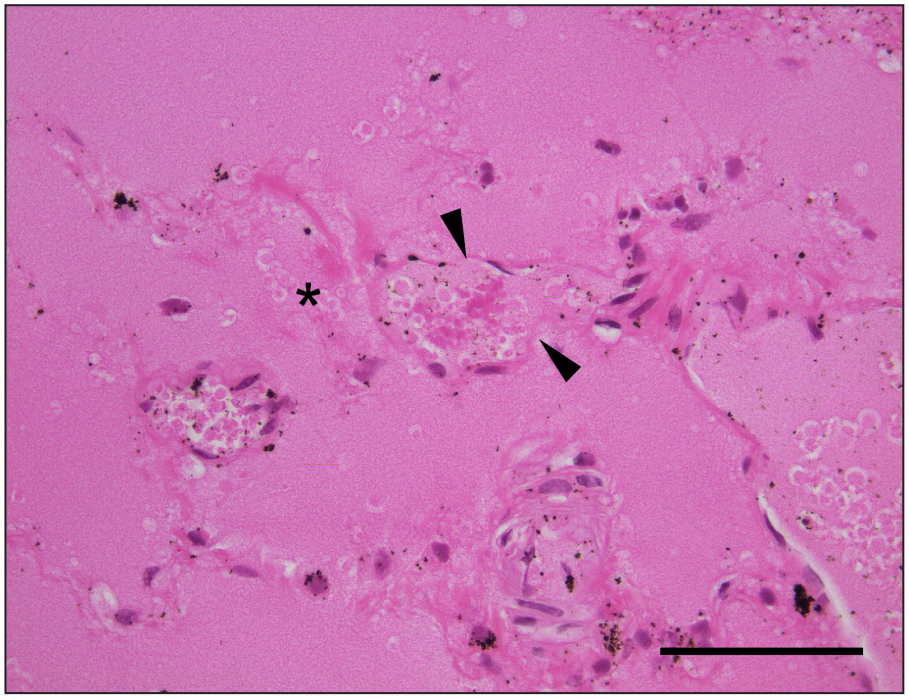


Figure 4.5. High power view of subarachnoid space in an area of pseudo-contusion. Note the disrupted blood vessel wall (arrrowheads), with extravascular accumulation of erythrocytes (asterisk), most of which are lysed. Bar = 200 μm, (H&E)

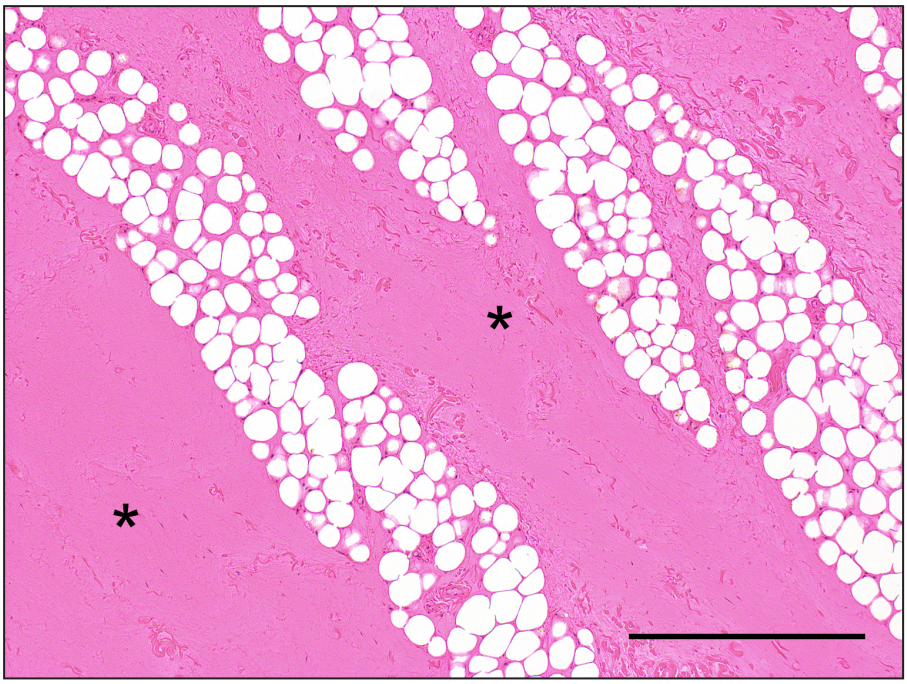


Figure 4.6. A pseudo-bruise from the ventrum of a frozen fur seal, showing eosinophilic fluid (asterisks) in the connective tissue of the blubber. Bar = 400 µm. (H&E)

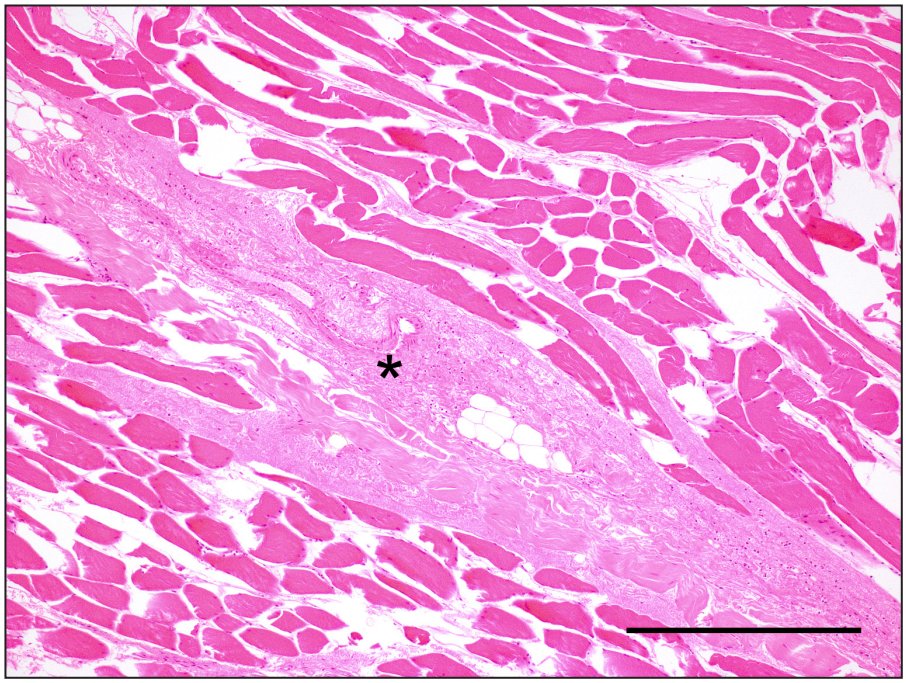


Figure 4.7. An ante mortem bruise from a fur seal that had been frozen and thawed. Brightly eosinophilic material containing erythrocytes, mostly lysed, in intermysial connective tissue (asterisk). Bar = 400 µm. (H&E)

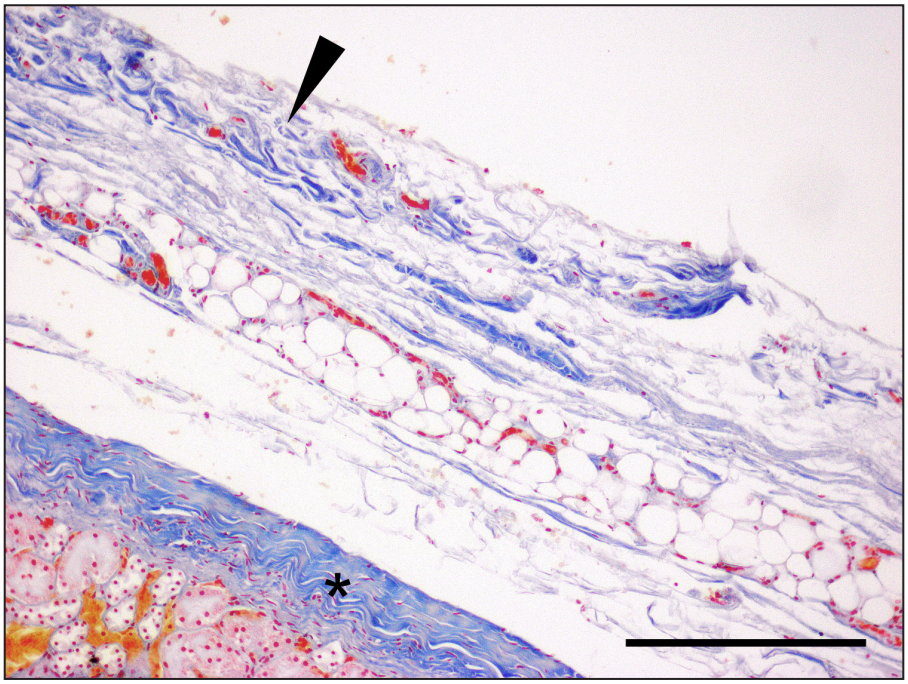


Figure 4.8. Capsular surface of the kidney from a non-frozen fur seal, showing two layers of collagen. The inner layer (asterisk) is closely adherent to the cortical parenchyma, and is separated from the outer layer (arrowhead) by loose connective tissue containing adipocytes and blood vessels. Bar = 200 µm. (MSB)

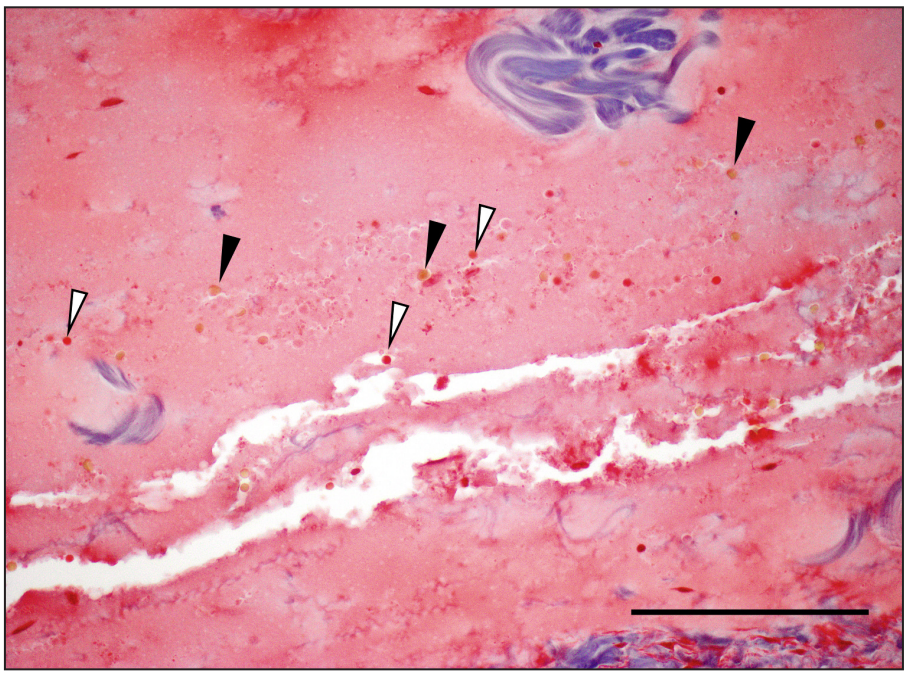


Figure 4.9. A pseudo-bruise from the kidney of a frozen fur seal. There is expansion of the subcapsular tissue by eosinophilic fluid admixed with lysed (black arrowheads) and intact (white arrowheads) erythrocytes. Bar = 200 µm. (MSB).

4.2.3 Discussion

Despite extensive research into the mechanisms of freeze-thaw damage at the cellular level, there are few publications that describe the gross changes that occur due to freezing. Two papers describe changes interpreted as freeze artefact: dark red fluid found in the body cavities of a frozen human infant (Tabata et al 2000), and blood-tinged discharge from the nares of a frozen rat (Micozzi 1986). Both of these changes were also present in frozen fur seals in this trial, along with several other changes resembling traumatic lesions. While the small sample size means that for some of these changes the association with freezing did not reach statistical significance, the results of this experiment indicate that accumulations of intra-abdominal fluid resembling unclotted blood, pseudo-bruising of subcutaneous tissues, apparent haemorrhage into the renal capsule and brain pseudocontusions are artefacts created during the freezing and thawing process.

Freezing damages cells by a combination of fluid shifts and mechanical damage to cytoplasmic membranes (Mazur 1970; Pegg and Diaper 1988; Ishiguro and Rubinsky 1994; Scott et al 2005). As tissue temperatures decrease, extracellular water begins to freeze, increasing the solute concentration of the unfrozen fraction of extracellular fluid and resulting in osmotically-induced cellular dehydration as intracellular fluid moves out of cells. On thawing, net flow of fluid back into cells can cause membrane rupture, as can direct membrane damage inflicted by ice crystals. The overall effect of these processes is cell shrinkage, tissue fragmentation, accumulation of extracellular fluid, and cell lysis (Baraibar and Schoning 1985, 1986; Schafer and Kaufmann 1999). The dark red fluid in the body cavities of thawed fur seals, therefore, is likely to be partly composed of accumulated extracellular fluid tinged with haemoglobin from lysed red blood cells.

The role of autolysis in development of freeze-thaw artefact also warrants consideration. While the aim of freezing a body before post mortem is to limit the effects of autolysis,

several authors have noted that autolysis is actually accelerated in the external tissues of frozen bodies (Micozzi 1986; Zugibe and Costello 1993). Autolysis could have occurred in the frozen fur seals in the current study at several stages: during their time in the net after drowning; on board the fishing vessel post-capture; during transit (2-5 days); in the time taken to reach freezing point; and during thawing (3-5 days). Recognised autolytic changes include haemoglobin staining of tissues, which begins within 2-3 days of death (Saukko and Knight 2004), and would contribute to the red discolouration of soft tissues that was observed in the blubber, renal capsule and in the brain. The autolysed control fur seal in this trial, which had a death-to-post mortem interval of seven days, also had blubber discolouration and staining of the renal capsule, providing additional support for the theory that autolysis contributes to some freeze-thaw artefacts.

Post mortem autolytic changes have been extensively studied in human forensic medicine. Passive accumulation of blood under the effect of gravity (hypostasis or post mortem lividity) causes reddening of soft tissues that can be misinterpreted as bruising (Saukko and Knight 2004). In theory, microscopic examination of an ante-mortem bruise should demonstrate the presence of extravasated erythrocytes outside blood vessels, whereas in hypostasis erythrocytes should be contained within congested vessels (Saukko and Knight 2004; Pollanen et al 2009). The distinction is not always so clear-cut in practice, as extravasation of blood has also been shown to occur after death, i.e. pseudo-bruises, particularly within areas of hypostasis (Vanezis 2001; Saukko and Knight 2004; Bockholdt et al 2005; Pollanen et al 2009). In a study using human cadavers, Pollanen *et. al.* (2009) were able to induce pseudo-bruises that were indistinguishable from true bruises on microscopic examination. The authors concluded that lesions resembling soft tissue injury can occur after death, and that these probably develop as a result of leakage of red blood cells from autolysing congested venous plexuses as hypostatic pressure progressively increases. They proposed that these changes would be particularly likely to occur in tissues with an extensive system of thin-walled

vascular channels that are surrounded by loose connective tissue (Pollanen et al 2009). Blood leaking from autolysed vessels would contribute to the fluid accumulations in body cavities, including the tunica vaginalis and the anterior ocular chamber.

Hypostatic congestion and autolysis, along with haemoglobin staining, may also explain the staining of the renal capsule in frozen NZ fur seals. This capsule comprises an outer, well vascularised layer of loose connective tissue and fat and an inner layer closely adherent to the cortical parenchyma (Stewardson et al 1999). Thawing would cause the fluid volume within intracapsular veins to expand, increasing intravascular pressure and forcing red cells out through the autolysing blood vessel walls. The loose connective tissue present in the renal capsule and the potential space between capsule layers create a site in which extravasated red cells and haemoglobin-stained extracellular fluid can accumulate, with more fluid accumulating around the dependent kidney.

A similar process could have caused the changes resembling brain contusions in three frozen fur seals. Macroscopic examination of the superficial vasculature in the brains of two non-frozen fur seals showed marked asymmetrical congestion of meningeal vessels over the cerebellum and the caudal occipital lobes, likely reflecting hypostatic congestion that developed during transit of the bodies. In an animal that was then frozen and thawed, autolytic damage of the vessel walls and escape of erythrocytes, coupled with haemoglobin staining around these congested vessels, would explain the development of unilateral pseudo-contusions. The specific location of these focal lesions would depend on the position of the body in the early hours after death.

The pattern of subcutaneous pseudo-bruising that was consistently present in the ventrum, axillae and shoulders of the frozen fur seals in this trial is more difficult to explain. Since no attempt was made to standardise body position during transport and freezing it is unlikely that the ventral aspects of the body were consistently in a dependent position. Although the dermis and hypodermis of fur seals has been shown to contain numerous thin-walled arteriovenous anastomoses (Bryden and Molyneux

1978), all five fur seals were thawed in dorsal recumbency, making it improbable that there was a gradual, gravity-dependent increase in the intravascular pressure of ventral subcutaneous vessels during thawing.

The severe bruising and muscle damage present in the neck of one frozen fur seal was interpreted as a true ante mortem lesion. The cause of this damage was not established, but the involvement of significant damage to muscle, in conjunction with haemorrhage in affected muscle, overlying blubber and skin made this lesion distinct from the pseudo-bruising seen in the ventrum, axillae and shoulder regions of other frozen animals.

With respect to freezing NZ sea lion pups for later thawing and post mortem examination, there are several modifications of the protocol used for the adult fur seals that could decrease artefact formation. Firstly, careful and consistent positioning of pup bodies during freezing and thawing might decrease the creation of asymmetrical artefacts, although it seems likely that some pseudo-contusions may still have occurred. These could both be difficult to definitively distinguish from true contusions, and could also obscure true lesions such as a small subarachnoid haemorrhages or contusions. Secondly, autolytic changes may be less prominent than in sea lion pups as compared with the fur seals in this trial, since transport of the pup bodies would not be required and the smaller body size of the pups means that they would freeze and thaw more rapidly, which in turn would reduce autolysis.

Despite these considerations it is unlikely that all artefacts could be prevented, and accurate diagnosis of traumatic lesions was integral to this study of traumatic brain injury. Examination of tissues using histological techniques can help distinguish between lesions and artefacts in some circumstances. In human forensic medicine the presence of inflammation or haemosiderin in haemorrhagic tissue confirms that bruising occurred before death (Grellner and Madea 2007). Studies in animals indicate that there is considerable variation in tissue responses between species; aging using histological

criteria is not possible in very recent bruising (Thornton and Jolly 1986; Vanezis 2001). Hence these criteria cannot differentiate between peri-mortem bruising and post-mortem freeze artefact. In addition, although microscopy can identify haemoglobin staining as a cause of putrefactive discolouration or hypostatic congestion, evaluation of pseudo-bruises in non-frozen human cases shows extravasation of erythrocytes in a manner indistinguishable from true bruising (Prinsloo and Gordon 1951; Pollanen et al 2009), making routine histochemistry of little use in differentiating these from ante-mortem bruises. Similar findings were noted on examination of bruise-like changes in NZ fur seal brains, renal capsules and subcutaneous tissues.

Tabata and Morita (1997) used immunohistochemistry to demonstrate that glycophorin A, an erythrocyte membrane marker, was present outside blood vessels in ante-mortem bleeding, indicating haemorrhage rather than haemoglobin staining as a cause of discolouration in autolysed human tissues. In pseudo-bruises, where red cells escape from autolysed blood vessels, this technique will not add anything to routine histochemical evaluations. More recently, immunohistochemical detection of adhesion molecules and cytokines has shown promise in evaluation of peracute lesions (Grellner and Madea 2007), but these techniques are not yet validated for non-human species, and their utility in frozen tissues is uncertain.

In summary, freezing and thawing of NZ sea lion pup bodies would be likely to create artefacts that would be difficult or impossible to distinguish from true traumatic lesions. This study thus shows conclusively that storage of frozen bodies on site at Enderby Island for later examination by an experienced veterinary pathologist was not justified.

4.3 Gross, histological and microbiological analysis of tissues from 2007/08

4.3.1 Introduction

This section presents detailed findings of necropsy examinations carried out on all pups found dead at Enderby Island during the 2007/08 field season. Early season pups were examined by a pinniped biologist trained by the author in necropsy technique. Necropsy examinations in the later part of the season were conducted by the author (from 12th – 31st January 2008) or by a pinniped biologist (from 1st – 10th February).

4.3.2 Materials and methods

Identification of lesions

Details of the necropsy and sample collection methods are in Appendix 3. Briefly, in addition to standard examination of body systems, the brains and eyes were removed and examined for gross lesions, the cranial vault and cervical vertebral region were evaluated for evidence of fractures and of subdural or epidural haemorrhage, and the cranial part of the cervical spinal cord was removed and examined. In the second half of the season these tissues were also collected into formalin. All lesions were photographed using a digital camera (Olympus model 1030SW). In particular, lesions that could be caused by any one of the potential mechanisms of traumatic brain injury identified in Chapter Three (i.e. shaking, impact or crushing) were noted. These included subdural haemorrhage, subarachnoid haemorrhage/contusion, haemorrhage around the cervical spinal cord (abbreviated to 'spinal haemorrhage'), skull fracture, bruising of the soft tissues of the head and neck, vertebral fracture or dislocation, and puncture wounds. Data were recorded onto field data sheets. A data sheet template is shown in Appendix 4. The season was divided into weeks as described in Chapter Three.

After immersion fixation in 10% buffered formalin for at least seven days, fixed tissues

and formalin were transferred from plastic buckets into double-layered zip-lock plastic bags for transport by boat back to the New Zealand mainland. Tissues were freighted overland from Port Chalmers to Massey University. There the brains, spinal cord sections, eyes and dura were removed from formalin, examined and photographed. Eyes were sectioned in the transverse plane at the level of the ora serrata, and the posterior and anterior calottes were examined for gross lesions. The posterior calotte was photographed from both the anterior and posterior views (Figure 4.10).

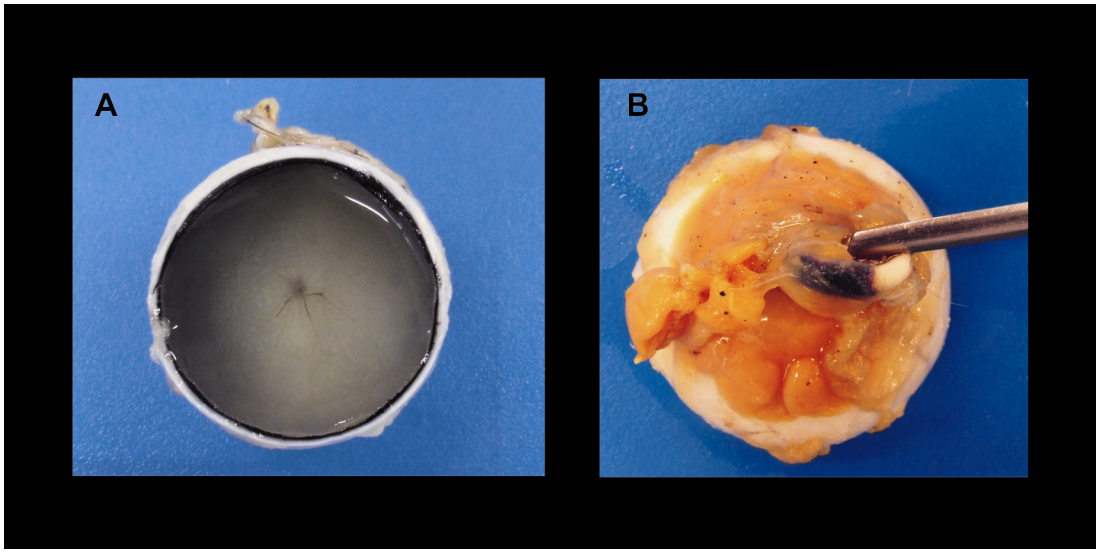


Figure 4.10. Posterior calotte of an eye from a NZ sea lion pup (E07/08-34Ph). **A**. Retinal surface, showing optic disc (centrally) and retinal vessels. **B.** Optic nerve surface. Note haemorrhage into optic nerve sheath.

Each brain was examined for evidence of superficial haemorrhage (reflecting either subarachnoid haemorrhages or contusions), subarachnoid exudate, and swelling of gyri. The brain was incised at areas of superficial haemorrhage to evaluate the underlying parenchyma.

Examination of representative tissues using histological techniques was conducted in order to confirm gross diagnoses. Sections of brain were collected from sites of superficial haemorrhage to enable characterisation of these lesions as either contusions or subarachnoid haemorrhages. Sections of cerebral cortex and cerebellum from each pup were examined in order to diagnose meningitis, and sections of optic nerve and

eye were also prepared (see Appendix 4 for details of eye trimming method). All tissues were embedded in paraffin, processed routinely for histology, and stained with haematoxylin and eosin (H&E).

Microbiological investigations were conducted by an experienced microbiology technician. Tissue samples were incised using a sterilised scalpel and a sterile swab was used to swab the cut surface. The swab was inoculated onto both sheep blood agar and MacConkey agar (Fort Richard Laboratories, Otahuhu, Auckland, New Zealand). Samples that had been collected on swabs were inoculated directly onto the plates. The blood agar plates were incubated in 5% CO_2 and the MacConkey plates in aerobic conditions at 37°C. All plates were examined after 24 hours incubation and those that did not have any growth were incubated for a further 24 hours and re-examined. Plates that showed mixed growth and did not have a predominant organism were considered contaminated and were discarded. If a single colony type was seen or if there was a mixed growth but with an obviously predominant organism, a single colony was sub cultured onto blood agar for further identification.

Identification began with a gram stain. Gram negative bacilli were identified using the Microbact™ system 12A (Oxoid New Zealand Ltd, Mairangi Bay, Auckland, New Zealand). In cases where the Microbact identification result was not clear, the API 20E® identification kit (Biomerieux, Ellerslie, Auckland, New Zealand) was used to confirm the identification. Gram positive organisms were identified using a catalase test as either streptococci (negative) or staphylococci (positive). Any beta haemolytic streptococci were identified using the API 20 Strep identification kit. A coagulase test using coagulase plasma (rabbit plasma + EDTA, Oxoid New Zealand Ltd) was performed on all staphylococci to identify them as either coagulase positive staphylococci or coagulase negative staphylococci. They were not identified further.

All organisms were stored in 10% glycerol at -80°C for any future analysis.

Determining cause of death

Each pup was assigned to a 'cause of death' category (see Table 4.2) based on gross, histological and microbiological findings. Categories used were: starvation; trauma; bacterial infection; hookworm infection; stillbirth; congenital defect; and undetermined (e.g. due to scavenging or autolysis). Stillborn pups were diagnosed on the basis of observation of a non-live birth, and/or presence of non-aerated lungs, a fresh umbilical stump and abundant meconium in the colon. Hookworm mortalities were diagnosed when pups had large burdens of hookworms present in the intestinal tract in conjunction with haemorrhagic enteritis and pale tissues. Emaciated pups were characterised by atrophy of blubber and muscle mass with prominent ribs and dorsal spinous processes once the skin had been removed. Bacterial infections were diagnosed by the presence of abscesses, or the presence of fibrinous or suppurative lesions within tissues or involving serosal surfaces.

In cases where several conditions occurred concurrently, the pup was assigned to the category which was the most likely cause of death, and the co-existing conditions were noted.

4.3.3 Results

The first science team arrived on Enderby Island on 8th December 2007. Although formalin had been left on the island the previous year, this could not be located when they arrived. A supply of formalin was despatched with the second science team, but this meant that no fixed tissues were able to be collected from necropsies performed in the first half of the season. Necropsies were conducted on 55 pups between 9th December 2007 and 10th February 2010. Full examinations, including collection of tissues for histological and microbiological analysis, were possible for 36 of these pups. Details of findings are included in Appendix 5.

Cause of death

Table 4.2 summarises the main necropsy findings. Bacterial infection was the most common cause of death (n = 27 (49%)). Lesions included pleuritis, suppurative arthritis, meningitis, osteomyelitis, subcutaneous abscesses or cellulitis, lymphadenitis, omphalophlebitis, and peritonitis.

Diagnosis	Cause of death	Concurrent syndrome
Bacterial infection	27	4
Trauma	14	3
Hookworm infection	4	6
Undetermined	4	-
Starvation	3	1
Stillbirth	3	-
Congenital defect	0	1

Table 4.2. Cause of death and concurrent syndromes diagnosed in pups found dead in the 2007/08 breeding season.

Pure growths of *Klebsiella pneumoniae* were cultured from 20 pups, and a further six pups had heavy growths of *K. pneumoniae* in conjunction with mixed organisms or with *Streptococcus* spp. Other significant organisms cultured as pure or heavy growths were *Streptococcus dysgalactiae* subsp. *equisimilis* (n = 2), *E. coli* (n = 4), and *Salmonella* spp. (n = 2).

Trauma was the second most common cause of death, affecting 14 pups (25%). Traumatic lesions included head bruises, bite wounds, skull fractures, bruising of the body wall, rupture of stomach or liver, intra-thoracic or pulmonary haemorrhage, and intra-abdominal haemorrhage. Several pups had aspirated sand (n = 3) or milk (n = 3). In most of these cases the injuries were consistent with blunt trauma, although cases of 'sharp' trauma due to penetrating bite wounds (n = 5) were also represented. Three of these bite trauma cases had concurrent blunt trauma lesions. Death was attributed to hookworm infection in four cases (7%). Less severe hookworm infection was present in

six further pups, but in each of these cases death was attributed to infection.

Three pups were stillborn. All of these had varying degrees of skin and hair sloughing, along with friable organs and increased body cavity fluid, indicating death some time before parturition. Two stillborn pups had multiple fractures along the suture lines of the skull, with apparent bruising of the head and underlying brain. The third had no evidence of bruising, and the skull sutures were fused.

No diagnosis could be made in four cases. In three of these there was marked scavenging by birds, resulting in near total removal of thoracic and abdominal organs. In the fourth case, which occurred in the first part of the season, there were no gross lesions and tissues were not collected for further investigation.

Traumatic brain injury lesions

36 of the 55 pups examined (65%) had gross lesions that were indicators of possible traumatic brain injury. As noted in previous years (see Chapter Three), there were two peaks of traumatic brain injury lesions, with eight pups affected in week four of the observation period (30th January to 5th February), and 11 in week nine (2nd – 8th February) (Figure 4.11). Spinal haemorrhage and intracranial subdural haemorrhage had a less pronounced first peak, instead showing an increase in prevalence over time, with peak occurrence in the last full week of the visit.

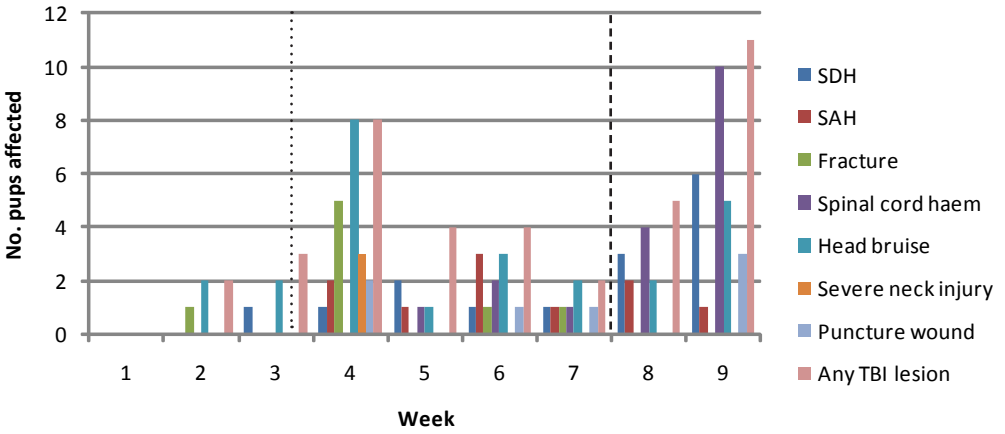


Figure 4.11. Frequency of lesions of traumatic brain injury (TBI) over time. SDH = subdural haemorrhage, SAH = subarachnoid haemorrhage. Dotted line in week 3 indicates mean pupping date; dashed line at end of week 7 indicates date at which most adult males have left the harem.

Figure 4.12 depicts the frequency of various traumatic brain injury lesions. The most frequent lesion was bruising of the head, which was present in 25/55 (45%) of necropsied pups. Examples of traumatic brain injury-like lesions, including head bruising, are shown in Figure 4.13. The majority of bruises (13/23 (57%)) were over the dorsal aspect of the neurocranium, but bruising was also noted over the nasal bones as well as lateral aspects of the parietal, temporal and occipital areas. In six cases the bruises were less than 20mm in diameter (likely bite wounds), but in the remainder they were more extensive. Six pups with head bruises had bite wounds in the overlying skin, but in the remainder there was no cutaneous damage.

Spinal haemorrhage was the second most frequent lesion, occurring in 18/55 (33%) of pups. Fourteen of these cases (78%) occurred after 25th January ('late' season). Examination of the fixed spinal cords showed that 16 pups had haemorrhage under the dura mater and two pups (E07/08-44Ph and 45Ph) had epidural haemorrhage.

Fifteen cases of intracranial subdural haemorrhage were found, with six occurring before January 25th ('early' season), and nine in the later part of the season. Figure 4.8 shows the frequency of subdural haemorrhages during the observation period. Subdural haemorrhages consisted of a thin (2-4mm) layer of blood, usually clotted (14/15; 93%),

coating the inner aspect of the dura once the brain had been removed from the cranium. In individual pups, subdural blood films were present in the cerebral or cerebellar compartments, or both. In seven pups the haemorrhage was bilateral. One pup had unilateral haemorrhage in the left cerebellar compartment, which extended into the inter-hemispheric fissure, three had haemorrhage in one cerebellar compartment only, and in four the location of the subdural haemorrhage was not recorded.

Ten pups had haemorrhage of the brain surface at gross necropsy examination. Eight of these occurred in the second half of the season; hence fixed tissues were available for further assessment. While examination of the cut surfaces did not show parenchymal haemorrhage or discolouration suggestive of contusion, microscopic examination confirmed contusion in five cases (E07/08-21, -22, -23, -29 and -34Ph) and subarachnoid haemorrhage in the remaining three (E07/08-30, -37 and -42Ph).

Eight pups (15%) had skull fractures, all of which were in the first half of the season. Two of these were stillborn pups with multiple fractures along suture lines (Figure 4.11) in conjunction with bruising of the soft tissues of the head. In one of these stillborn pups the surface of the brain appeared to be bruised. Of the live-born pups with skull fractures, one had a fracture at the nasal sutures, with bruising of the underlying brain parenchyma. Two pups had single displaced fractures associated with bite wounds. One of these had a cerebral abscess underneath the fracture fragment, and mixed species of bacteria were cultured from this lesion. Of the three remaining cases of skull fracture, one was a single fracture of the zygomatic arch, one was a single fracture 'near the left orbit', and the third pup had multiple skull fractures but had sustained extensive scavenging of the brain and other soft tissues of the head, which prevented accurate interpretation of other lesions.

Three pups had severe neck injuries, comprising bruising and tearing of the neck muscles.

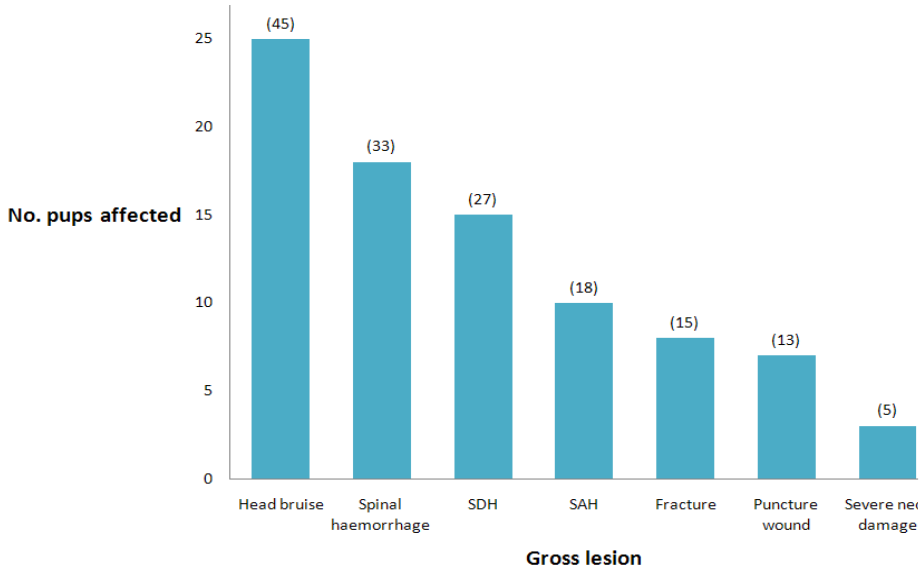


Figure 4.12. Gross lesions seen in pups necropsied at Sandy Bay, Enderby Island in 2007/08 breeding season, which could indicate traumatic brain injury. Figures in brackets at the top of each bar are the percentage of pups affected.

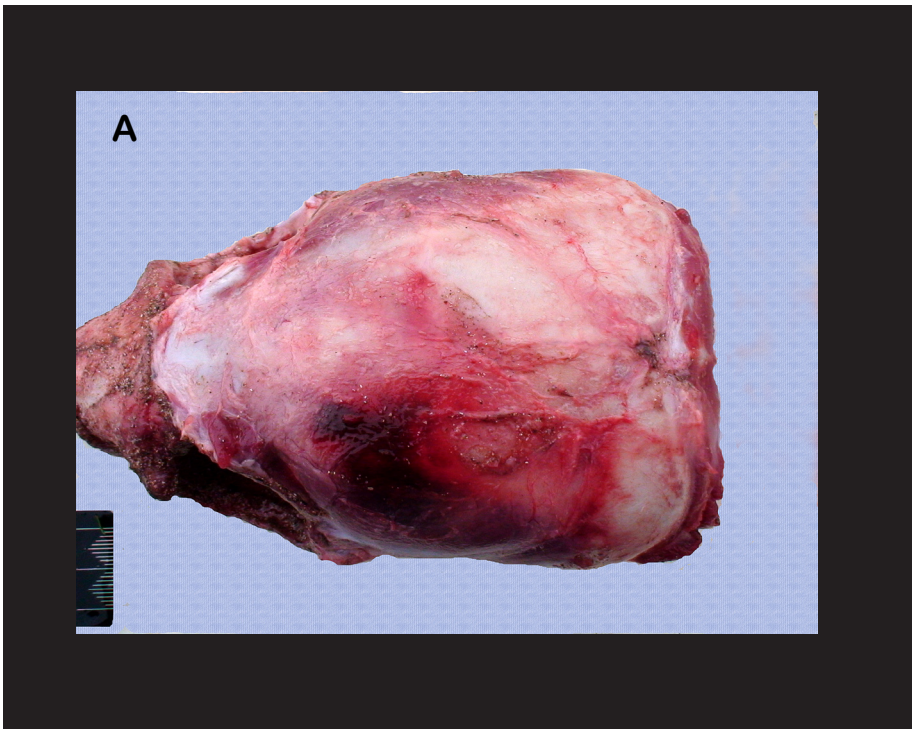
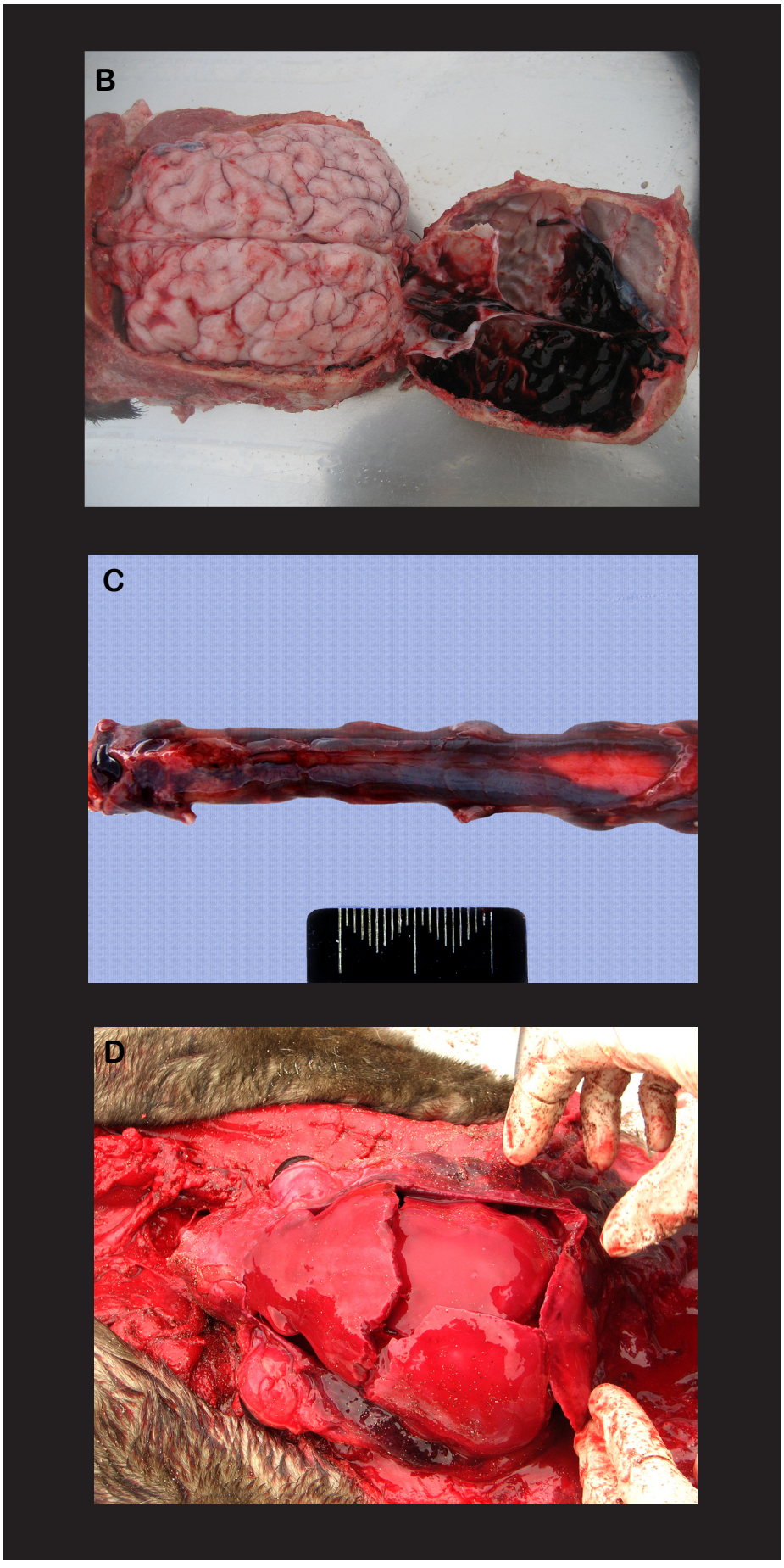


Figure 4.13. (This page and overleaf). Gross lesions consistent with traumatic brain injury (traumatic brain injury-like lesions) found in pups dying in the 2007/08 breeding season. **A.** Head bruising. The skin has been removed to show bruising of the subcutaneeous tissues over the dorsal aspect of the neurocranium in an impact type injury. **B.** Subdural haemorrhage in a pup with meningitis. The dura mater is attached to the resected skull bone, and is covered with a layer of clotted blood (right). Image courtesy of Kerri Morgan, Massey University. **C.** Spinal cord haemorrhage. There is a layer of clotted blood beneath the cervical spinal dura mater. **D.** Multiple fractures along the suture lines in a stillborn pup.


B
C
D

In one pup there was also a vertebral body fracture. All three also had severe bruising to the head, and two had concurrent skull fractures (one with multiple fractures of the neurocranium and mandible, and one with a bite wound involving the neurocranium). These three cases were found between the 2nd and 5th January. None of the pups with severe neck injuries had concurrent subdural, spinal or retinal haemorrhages.

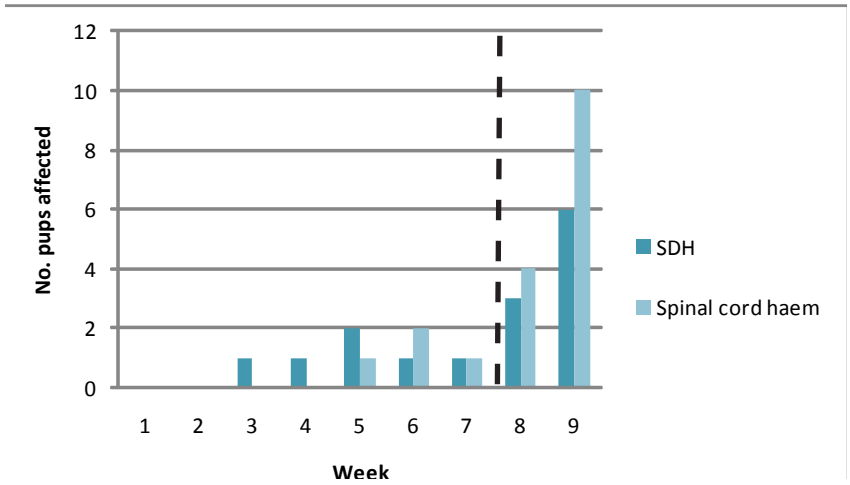


Figure 4.14. Number of pups with intracranial subdural haemorrhage or spinal sub-meningeal haemorrhage over time. The dashed line indicates the date at which most adult males have left the harem.

Several brain lesions that were not identified in the retrospective analysis were also present, including haemorrhage of the cerebellar vermis (nine cases) and cloudy meninges (nine cases).

Twelve pups had eye lesions. Eight had multiple haemorrhages of the sclera without any other eye lesions; one had bilateral retrobulbar haemorrhages as a sole eye lesion; one had scleral haemorrhages plus haemorrhage into the optic sheath; one had retrobulbar haemorrhage with optic sheath haemorrhage; and one had scleral and retinal haemorrhages. The latter pup was the only one with grossly visible retinal haemorrhages. All eye lesions occurred after week four of the season.

Microscopic examination of brain tissue was possible for 36 pups, and showed meningitis in 16 of these (44%).

4.3.4 Discussion

General comments

The results of the study described in this section indicate that lesions consistent with traumatic brain injury were common in the 2007/08 breeding season, with 65% of all pups necropsied having at least one traumatic brain injury lesion. Consistent with the findings detailed in Chapter Three, two peaks of traumatic brain injury lesions were noted; the first in week four (30th December – 5th January) and the second in week nine (2nd – 8th February). The possibility of these two peaks being due to two different modes of inflicted damage was discussed in Chapter Three, but there were deficiencies in the archived data that obscured identification of patterns of injury. The more detailed investigations carried out in 2007/08 added substantial information about the nature of traumatic brain injury lesions.

The types of lesions found were similar to those described previously, with some differences in frequency. As in previous years, head bruising and subdural haemorrhage were amongst the most frequently seen lesions. However in the 2007/08 season spinal haemorrhage occurred in 33% of pups (compared with 4.4% of all pups necropsied between 1999 and 2007), making this the second most common lesion. Although it is possible that this reflects a true increase in prevalence, it is more likely to be due to under-reporting in the past, as in many years necropsy reports do not describe any spinal cord or cervical vertebral findings. Macroscopic focal lesions of the brain itself were limited to superficial contusions in five pups, each with little parenchymal involvement evident histologically.

In Chapter Three, three biomechanical mechanisms were identified as possible causes of traumatic brain injury in pups, namely static loading (crushing due to either bites or trampling), impact loading (throwing or striking of pups), and impulse loading (shaking). These mechanisms are evaluated separately below.

Mechanisms of traumatic brain injury

A total of ten pups had sustained blunt crushing injuries. Of these, two (E07/08-03Ph and -09Ph) were stillborn pups that had multiple fractures along the suture lines of the skull. Although these two pups also had bruising of the soft tissues over the dorsum of the skull, the degree of autolysis of the bodies indicated that both were likely to have died *in utero*. Crushing of the head occurred after death, either during parturition (i.e. during transit through the pelvis) or shortly thereafter (e.g. due to trampling of the body by adults).

Six live-born pups died due to trampling-type crushing, and had lesions such as aspiration of sand or rupture of abdominal organs. Two of these crushed pups (E07/08-08Ph and -33Ph) had no concurrent lesions of traumatic brain injury, while one (E07/08-06Ph) had extensive bruising of the soft tissues of the head and neck, one (E07/08-02Ph) had a minor bruise on the head, one (E07/08-17Ph) had bilateral retrobulbar haemorrhage and one (E07/08-05Ph) had subdural haemorrhage. One further pup (E07/08-12Ph) died due to systemic bacterial infection but also had crushing lesions (bruising of head, neck, thorax and abdomen and ingestion of sand). None of these pups had skull fractures, and all were found on or before the 26th January (beginning of week 8). This is consistent with accidental trampling by adult males during territorial fighting, which decreases markedly from the third week of January onward (i.e. from the middle of week 7).

Three early season pups (E07/08-04Ph, -13Ph, and -14Ph) had severe bite wounds around the head and/or neck in addition to crushing of the thorax or abdomen. One of these (E07/08-13Ph) also had multiple skull fractures. This pattern of injury does not conform to either of the proposed early trauma mechanisms, (i.e. trampling or tooth-strike), instead being more consistent with a sustained and directed attack, as is more common with the subadult attacks in the second part of the season. All three of these pups were found on the grass sward rather than within the harem; these were the

only dead pups found on the sward in the first half of the season. It is likely that these pups were attacked by peripheral males, with the crushing lesions being due to bites or to attempted coitus rather than accidental trampling. Such attacks would be more likely to occur on the fringes of the colony at this time of year, firstly because immature males tend to be found outside the harem, and secondly since pups that move away from the harem would be less protected.

Skull fractures, contusions and soft tissue bruising were found in a number of pups in 2007/08, but it was not always possible to determine whether these were due to impact or crushing. Two pups (E07/08-16Ph and -30Ph) had focal displaced fractures of the neurocranium, with adjacent subcutaneous bruising and a single penetrating tooth mark in the overlying soft tissue. These were interpreted as strike-type impact injuries.

A central hypothesis of this thesis is that shaking of pups by subadult males during the latter part of the breeding season is responsible for pup morbidity or mortality. The second peak of traumatic brain injury lesions occurring late in the observation period (week 9) appears, at least on the surface, to be consistent with attacks by subadult males during this part of the season. Severe neck injury, subdural haemorrhage, spinal cord damage, and bruising or bite wounds around the neck or back (where pups are picked up by subadult males), are indicators that a pup may have been shaken. In the 2007/08 breeding season, three cases (E07/08-13Ph, -14Ph, and -16Ph) of severe neck injury were observed, all during the first part of the season (between 2nd and 6th January), well before the proposed peak period of subadult male attacks. Two of these were discussed earlier, and were found outside the harem with biting and crushing lesions. The third pup had severe neck muscle damage and a fractured cervical vertebra, along with an open skull fracture and meningitis. None of these three had subdural or retinal haemorrhages.

In human paediatric medicine, subdural haemorrhage is considered to be a strong

indicator of shaking, and in many cases is the basis of a diagnosis of inflicted brain injury (Donohoe 2003; Geddes et al 2003). Twenty-two pups in this study had subarachnoid and/or subdural haemorrhages. Fatal cases of shaken baby syndrome typically have a triad of lesions involving encephalopathy, subdural haemorrhages and retinal haemorrhages. Some also have intracranial subarachnoid haemorrhages and/or epidural haemorrhages around the cervical spinal cord (Geddes et al 2001a; Geddes et al 2001b; Leestma 2005; Gerber and Coffman 2007). None of the 2007/08 sea lion pups had both subdural haemorrhage and retinal haemorrhages, but other combinations of lesions consistent with shaken baby syndrome were seen: (a) subdural haemorrhage with subarachnoid haemorrhage and spinal haemorrhage (n = 3), and (b) subdural haemorrhage with spinal haemorrhage (n = 8) (see Table 4.3). While on the surface this would appear to support the hypothesis that these pups have sustained inertial brain injuries, subdural haemorrhage is not pathognomonic for trauma. Meningitis, for example, can lead to subdural haemorrhage (Kemp 2002) and was present in eight of the 11 subdural haemorrhage cases (74%) that had tissues available for analysis by histological techniques. The relationship between meningitis and subdural haemorrhage is detailed in Chapter Five.

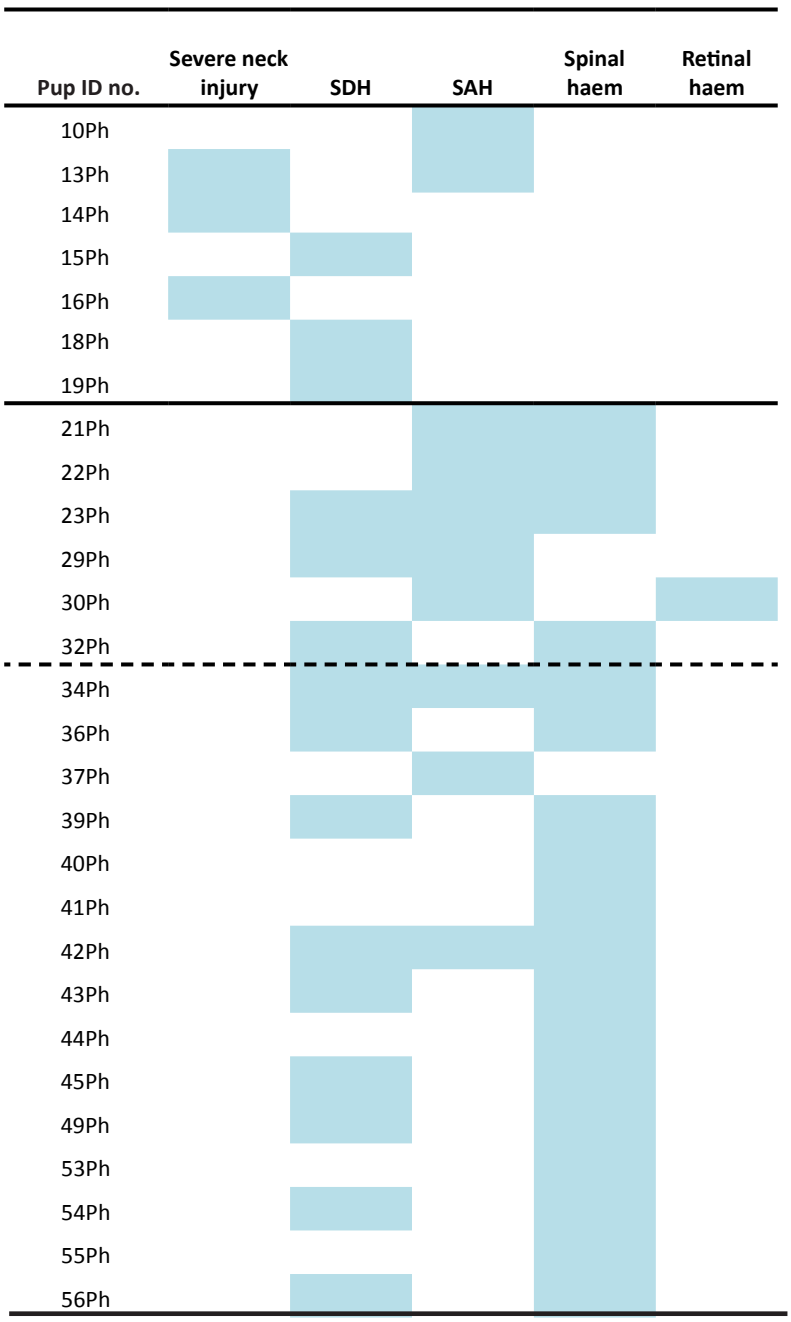


Table 4.3. Pups with lesions indicative of inertia injury. The presence of a lesion is indicated by blue shading. The solid line indicates the division between cases that did not have (above) and did have (below) tissues collected for microscopic examination.The dashed line indicates the division between early season (above) and late season (below) pup deaths. (ID no. = identification number; SAH = subarachnoid; SDH = subdural; haem = haemorrhage)

Importance of brain injury in NZ sea lion morbidity and mortality

The data presented in this chapter show that three pups (5%) died due to traumatic brain injury in the 2007/08 season. The role of traumatic brain injury as a contributing cause of mortality in the remaining pups is more difficult to determine, but the fact that traumatic brain injury-like lesions were present in 65% of all pups examined suggests that this role may be significant. Taking into account skull fractures, brain contusions, severe neck injury and extensive head bruising, a total of 19 pups (35%) could have had clinically significant brain injury due to trauma, although, as discussed above, the true clinical effect is impossible to ascertain.

In archived necropsy reports, the presence of subdural haemorrhage was often taken to indicate death due to traumatic brain injury. The findings of this chapter suggest that in many of these cases meningitis may actually be the cause of these haemorrhages, and that diagnosing the cause of death solely on the presence of these gross lesions is misleading. The high prevalence of meningitis in the Enderby Island pups also means that an appreciable amount of non-traumatic brain injury is occurring in this population, for example due to inflammatory parenchymal damage and to diffuse injury resulting from brain swelling. Specialised histological techniques aimed at detecting white matter damage would be useful in quantifying brain injury due to trauma and due to these other causes.

4.4 Summary

The results of the freeze-thaw trial (presented above) indicate that freezing and thawing causes artefacts that mimic ante-mortem haemorrhage and bruising, which, if wrongly interpreted, could easily lead to over-diagnosis of trauma. Because it is not possible to reliably distinguish freezing artefacts from true traumatic lesions using currently available histological and immunohistochemical methods, and because some of these artefacts could mask traumatic lesions, use of frozen sea lion pups for

analysis of traumatic brain injury is not warranted. Despite this, sufficient information to allow reliable interpretation of lesions was available from gross necropsy records made by the biologist conducting necropsy examinations under direction of the author in the first half of the season to enable diagnosis of cause of death for early season pup mortalities and classification of traumatic brain injury lesions.

Traumatic lesions were present in a large proportion of pups, and three pups died due to traumatic brain injury. The nature of traumatic brain injury-like lesions indicated that pups were injured by crushing, impact and shaking. Intracranial subdural haemorrhages and cervical spinal haemorrhages were common, but are not pathognomonic for shaking. Meningitis due to *K. pneumoniae* was present in a high proportion of pups and could be directly associated with subdural haemorrhages, as well as contributing to non-traumatic brain injury.

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Chapter five

The role of meningitis in subdural haemorrhage in NZ sea lion pups

5.1 Introduction

Subdural haemorrhages in human infants are considered indicative of a traumatic brain injury, either accidental or inflicted. In cases of inflicted injury the initiating event is rarely observed (Leestma 2005; Squier 2008), hence diagnosis is dependent on ruling out other possible causes. Similarly, in NZ sea lion pups, although shaking has been observed, a direct link between the shaking event and creation of recorded subdural haemorrhage in the shaken pup has not been established, so other causes of subdural haemorrhage must be investigated. The main non-traumatic causes of subdural haemorrhage in human neonates and infants are meningitis, congenital vascular abnormalities, and

coagulopathies (Kemp 2002). The high prevalence of subdural haemorrhages in NZ sea lion pups and the absence of compatible lesions mean that congenital vascular anomalies and congenital coagulopathies are unlikely. In contrast, meningitis was diagnosed in 16 pups from the 2007/08 season, and could be involved in some cases of subdural haemorrhage. In addition, birth-associated subdural haemorrhage has been shown to persist for at least 7 days in foals, calves and lambs (Haughey 1975; Haughey and Jones 1976; Haughey 1980), and could be responsible for recent haemorrhages in early season pups, and older haemorrhages in late season pups.

The traditional view of subdural haemorrhages is that most are due to rupture of the bridging veins that pass from the leptomeningeal surface through the subarachnoid space and dura mater, to empty into the intradural venous sinuses (Gennarelli and Thibault 1982; Blumbergs *et al.* 2008). While this could well be the cause of many traumatic subdural haemorrhages, an alternative origin is the *intradural* venous plexus. Haemorrhage from this source can dissect through the dural connective tissue and into the loose connective tissue layer that comprises the subdural compartment (Geddes *et al.* 2003; Cohen and Scheimberg 2009; Squier and Mack 2009). In order to investigate the possible origins of subdural haemorrhage in NZ sea lion pups, this chapter examines the histological structure of NZ sea lion dura mater and considers potential non-traumatic origins of subdural haemorrhage. Since meningitis is a reported cause of subdural haemorrhage, this chapter also aims to characterise meningitis, grossly, histologically and aetiologically, in NZ sea lion pups, and to examine the relationship between meningitis and subdural haemorrhages.

5.2 Materials and methods

Gross post mortem reports, frozen tissues, fixed tissues and digital images were available for 36 pups from the 2007/08 season. The seasonal distribution of systemic infection and meningitis was assessed using archived necropsy reports (2005/06 to

2006/07 and 2008/09 to 2009/10), or full investigations (2007/08). For years in which full investigation was not undertaken, diagnosis of meningitis was based on the presence of characteristic gross lesions identified in the 2007/08 pups, including cerebellar vermis haemorrhage, cloudy leptomeninges and purulent subarachnoid exudate.

Comparisons within and between years were made by dividing the breeding season into weeks. The starting point was taken as the 9th December, the earliest date for which data was available in all five breeding seasons included in this study.

Thorough histological examinations of brain, eye and cervical spinal cord sections were conducted. The left side of the brain was trimmed for histology to obtain blocks at the following levels (see Figure 5.1):

1. Rostral aspect of corpus callosum and frontal cortex;
2. Thalamus and internal capsule at level of rostral corpus callosum;
3. Mid corpus callosum and parietal cortex;
4. Temporal cortex and hippocampus;
5. Caudal aspect of corpus callosum (splenium);
6. Ventral temporal cortex (including hippocampus and parahippocampal gyrus) and midbrain;
7. Occipital lobe;
8. Pons at rostral colliculus; and
9. Cerebellum at level of confluence of peduncles.

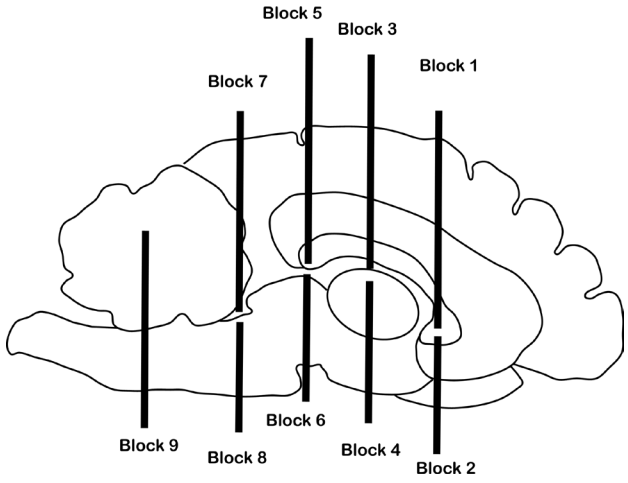

Figure 5.1. Trimming sites used for collection of tissues for the nine blocks used to assess histological lesions in the brain.

Two sections of spinal cord were collected, one from the level of C1, and a second from C4/C5. Sections from all available eyes (see trimming method in Appendix 4), and several sections of intracranial dura mater were also processed for histology. Brain, spinal cord, dura mater and eye sections were stained with H&E and all spinal cord and intracranial dura mater sections were also stained with Perl's stain and MSB stain. In addition, three sections of intracranial and spinal cord dura mater were processed immunohistochemically at MedLab, Palmerston North, New Zealand, using anti-Factor VIII antigen.

For the study comparing lesions present in pups with meningitis to those in pups without meningitis, seven sections of cerebrum were examined for each pup. Cerebellar lesions were assessed using section 9. Two transverse sections of spinal cord were used: one from the cranial end of the resected cord (C1) and one from the caudal end (C4/C5). The severity of the following lesions was scored semi-quantitatively on a scale of 0 – 3, with 0 reflecting an absence of the lesion in question, and three representing the most severe expression of the lesion. These lesions included the presence of intravascular and perivascular bacteria, thrombosis, congestion, vasculitis, numbers of intraluminal leukocytes, perivascular haemorrhage, ependymal cell vacuolation, ependymal

cell proliferation (formation of papillae projecting into the ventricle), superficial subependymal cellularity, and deep subependymal cellularity. Lesion scores were expressed as an average. Readings were repeated three times, and where scores were not in complete agreement, the score that was assigned in two out of three readings was recorded as the final score. In no case were three different scores allocated for any specific lesion. For lesions that were not able to be scored accurately due to wide variations between fields of view (e.g. vasculitis and perivascular cuffing) or due to variations in the specific anatomical location of tissue examined (e.g. sections of dura mater), lesions were noted as present or absent. All readings were made without knowledge of the cause of death. Details are included in Appendix 6.

Sections of intracranial dura mater from three pups were sent to MedLab, Palmerston North, for immunohistochemical staining using antibody to Factor VIII (DAKO-EPOS, rabbit anti-human Von Willebrand factor/factor VIII-related antigen; Sigma-Aldrich, Auckland, New Zealand).

In order to investigate the possible role of otitis as a route of infection of the meninges, computed tomography (CT) scans were conducted on skulls from 11 pups with *K. pneumoniae* infection from the 2008/09 season. For these pups, both halves of the skull were collected after being sectioned for brain removal. Each half was placed in a ziplock bag, which was then filled with 10% neutral buffered formalin, and sealed. At Massey University, skulls were removed from formalin, drained, placed into clean plastic bags to prevent operator exposure to formalin fumes, and scanned using a Phillips CT scanner.

In addition, the middle and inner ears from two 2007/08 pups with meningitis were sectioned transversely using a hand saw, and examined grossly for evidence of bone lysis and the presence of exudate.

Microbiological investigations were conducted as described in Chapter Four. In addition, for this chapter, for all *K. pneumoniae* colonies grown, viscosity was determined using the string test, as described by Fang *et al.* (2004). Briefly, an inoculation loop was touched to the colony surface and pulled vertically away. Formation of a viscous string greater than 5 mm in length was defined as a positive string test.

All organisms were stored in 10% glycerol at -80°C for future analysis.

Statistical methods.

For non-scored (i.e. present or absent) lesions, Fisher's exact test was used to compare prevalence of lesions in pups with meningitis versus those without meningitis. The Mann-Whitney test was used to compare severity of lesions that were evaluated semi-quantitatively. All analyses were conducted using Minitab© software.

5.3 Results

5.3.1 General and microbiological findings

Meningitis was diagnosed in 16 (10 females and 6 males) of the 36 pups necropsied in the second half of the 2007/08 season. From these, 13 brains grew either a pure or heavy growth of *K. pneumoniae.* One further brain sample (E07/08-53Ph) grew a light growth of *K. pneumoniae* in conjunction with mixed bacteria and a *Streptococcus* sp. and one (E07/08-37Ph) grew mixed bacteria and a *Streptococcus* sp. Ten of the pups with meningitis had tissues other than brain available for culture: all of these (including E07/08-37Ph) grew pure cultures of *K. pneumoniae.* All *K. pneumoniae* isolates formed glistening, mucoid colonies on agar, and were positive on string test.

Details of gross findings and culture results for pups with meningitis are presented in Table 5.1. Suppurative lesions were present in other organs of 10/16 (63%) of these pups, while in six pups there were no concurrent septic foci, although in three of these

the internal organs had been extensively scavenged by birds. Histological lesions suggestive of septicaemia were present in 11/16 (adrenalitis (n = 5), embolic pneumonia (n = 7), interstitial nephritis (n = 1), lymphadenitis (n = 5), and embolic hepatitis (n = 3)). The pattern of lesions suggested septicaemia and thus a haematogenous route of meningeal infection in 15/16 cases. The 16th case (E07/08-30Ph) had a penetrating bite wound with associated skull fracture, cerebral abscess and direct spread of infection to involve the meninges. Overall, bacterial infection was diagnosed in 29 pups in the 2007/08 breeding season, with increasing frequency of cases as the season progressed (see Figure 5.2).

No evidence of bone lysis or exudate was detected grossly (n = 2) or on CT scans (n = 11) of the middle or inner ear of pups with *K. pneumoniae* infection.

Pup ID	Suppurative lesions	Agent cultured	Tissues *K. pneumoniae* cultured from
23Ph	a/o arthritis	K	s/c tissue; joint
30Ph	cellulitis	M	x
31Ph		K	umb, LN
32Ph	a/o arthritis	K	joint
36Ph	polyarthritis	K	joint, liver, abdo
37Ph	polyarthritis; cellulitis; peritonitis	MS	joint, liver, abdo
38Ph	omphalophlebitis	K	umb, s/c tissue
39Ph		K	x
40Ph	polyarthritis; cellulitis; peritonitis	K	s/c tissue, abdo
41Ph	polyarthritis; cellulitis	K	joint
43Ph		K	x
49Ph	cellulitis	K	pericard, lung, joint
53Ph		MSK	x
54Ph	polyarthritis	K	joint
55Ph		K	x
56Ph		K	x

Table 5.1. Concurrent suppurative lesions and microbiological findings in pups with meningitis.
(K = *K. pneumoniae*; M = mixed bacteria; S = *Streptococcus* spp.; a/o arthritis = atlanto-occipital arthritis; s/c = subcutaneous; joint = joint fluid; pericard = pericardial fluid; abdo = abdominal cavity fluid)

umb = umbilicus; x = samples not collected)

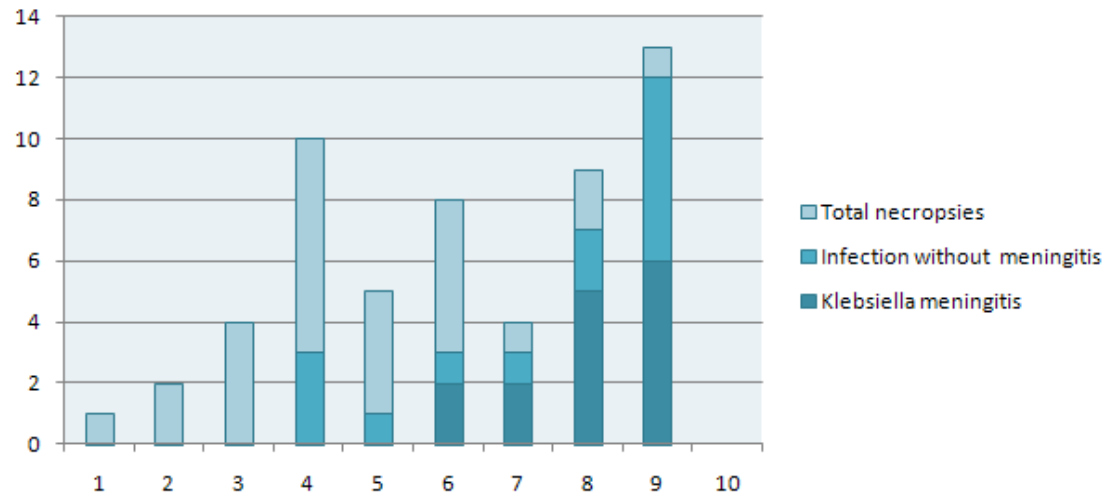

Figure 5.2. Cases of bacterial infection, including meningitis, in NZ sea lion pups in the 2007/08 season. Cases of bacterial infection without meningitis first occur in week 4, with the first cases of *K. pneumoniae* meningitis occurring in week 6. The frequency of infection increases over the rest of the season, with the majority of pups that were examined in the last three weeks of the season having systemic *K. pneumoniae* infection.

5.3.2 Gross lesions in pups with meningitis

Gross lesions frequently noted in the pups with meningitis were cloudy meninges (n = 11), swollen gyri (n = 12), haemorrhage of the cerebellar vermis (n = 10), intracranial subdural haemorrhage (n = 8) and haemorrhage beneath the cervical spinal cord dura (spinal sub-meningeal haemorrhage) (n = 12) (Figure 5.3). Cloudy meninges and haemorrhage of the vermis were specific to meningitis cases. In one pup (E07/08-30Ph), an obvious entry site for meningeal infection (penetrating bite wound with skull fracture) was evident. This pup had a cerebral abscess, which grew mixed bacteria, underlying the bite wound. In the remaining cases there was no evidence of direct extension from a focal lesion, and a *K. pneumoniae* infection was diagnosed in each. In one pup (E07/08-37Ph) *K. pneumoniae* was not cultured from the brain, but was present in pure culture of joint fluid, liver and peritoneal fluid, and gram negative rods were present in the

Pup ID	Skull fracture	Brain abscess	Intracranial SDH	SAH	Cloudy meninges	Swollen gyri	Spinal cord haem	Vermis haem	Retinal haem	Scleral haem
23Ph			+	+		+	+	+		
30Ph	+	+		+					+	+
31Ph										
32Ph			+		+	+	+	+		+
36Ph			+		+	+	+	+		+
37Ph				+						
38Ph										+
39Ph			+		+	+	+	+		+
40Ph					+	+	+	+		
41Ph					+	+	+	+		
43Ph			+		+	+	+			+
49Ph			+		+	+	+	+		
53Ph					+	+	+	+		
54Ph			+		+	+	+	+		
55Ph					+	+	+			
56Ph			+		+	+	+	+		

Table 5.2. Gross lesions present ('+') in the skull, brain, eyes and cervical spinal regions of pups with meningitis, 2007/08. (SAH = subarachnoid haemorrhage; SDH = subdural haemorrhage; haem = haemorrhage)

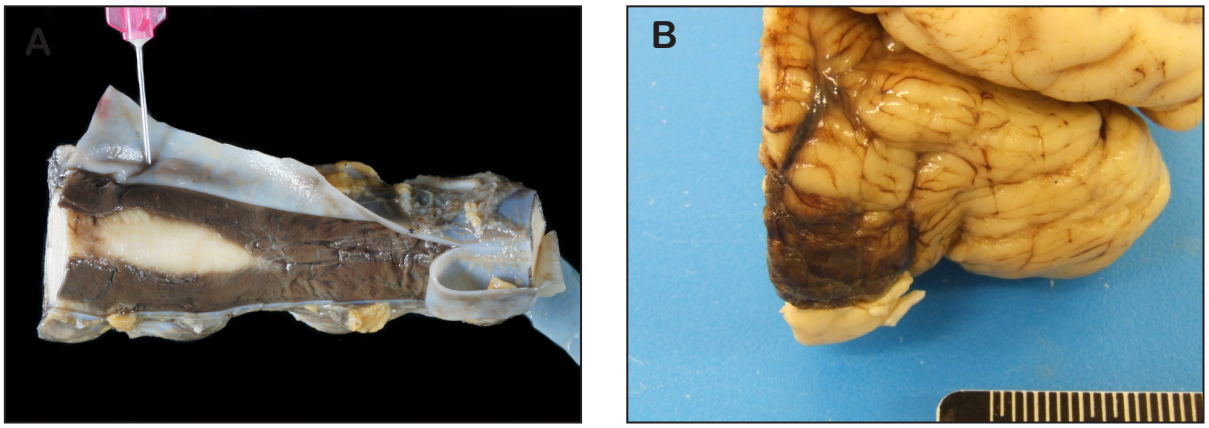

Figure 5.3. Gross lesions seen in pups with meningitis. **A.** Haemorrhage under the dura mater of the cervical spinal cord (spinal sub-meningeal haemorrhage). This lesion was also seen in pups that did not have meningitis. **B.** Haemorrhage of the cerebellar vermis, which occurred only in pups with meningitis.

subarachnoid exudate. In total, 12/15 (80%) of *K. pneumoniae* meningitis cases had either intracranial subdural haemorrhage or spinal sub-meningeal haemorrhage). Table 5.2 details gross lesions of the central nervous system in pups with meningitis.

5.3.3 Histopathology

The subarachnoid exudate varied from scant in one case (E07/08-38Ph), to 2 - 3 mm thick in the remainder. In four pups the exudate was most prominent over the dorsal surface of the cerebrum, in one it was thicker basally, and in the remainder the exudate was evenly distributed from dorsum to ventrum. In five cases the exudate became thicker caudally. In the cerebral cortices, the thickest accumulations of exudate were deep within the sulci. Parenchymal perivascular cuffs of inflammatory cells were a feature of all but two cases (E07/08-23Ph and -54Ph), and were confined to vessels in the superficial parenchyma of the cerebrum and occasionally also the cerebellum.

Macrophages and neutrophils were the predominant inflammatory cells, with occasional plasma cells and rare lymphocytes. Macrophages made up more than 70% of the cells within the subarachnoid space in nine cases, and approximately 50% in three cases. In the remaining four cases neutrophils were dominant (approximately 90% of the cellular exudate). In each case there were strands of fibrin admixed with the cellular exudate, along with variable numbers of intra- and extracellular gram negative bacterial rods. In addition, 11 pups (69%) had bacteria within subarachnoid vessels, and large perivascular proliferations of gram negative bacteria were common around subarachnoid and parenchymal vessels.

Inflammation of the choroid plexus and exudate within the ventricles were uncommon. A notable exception was the bite wound pup (E07/08-30Ph), which had abundant exudate between the fronds of the choroid plexus of the lateral and fourth ventricles, composed predominantly of degenerate and viable neutrophils with fewer macrophages and occasional lymphocytes. The choroidal plexus stroma was also inflamed, with large

numbers of macrophages and fewer plasma cells and neutrophils. In the remaining pups, intraventricular inflammation was restricted to a sparse exudate in the central canal in two cases (E07/08-41Ph and E07/08-49Ph), and three pups (E07/08-41Ph, E07/08-49Ph and E07/08-54Ph) had a sparse inflammatory infiltrate in the stroma of the choroid plexus of the lateral ventricles. More commonly, in pups with meningitis the subarachnoid exudate extended around the midbrain to the fornix, elevating the ependyma at the base of the choroid plexus but not extending into the stroma.

Encephalitis was present in 11 pups, and consisted of increased numbers of microglial cells with large, pale nuclei, infiltration by variable numbers of neutrophils and macrophages, and large spaces surrounding glial cells. In nine of these cases inflammation was diffuse and superficial, extending from adjacent subarachnoid accumulations of exudate, and involving either the cerebellum only (two cases) or the cerebellum and cerebral cortices. Two other pups had focal parenchymal inflammation in the brain: one (E07/08-23Ph) had a single small (approximately 50 μm diameter) area of fragmented neuropil and cellular infiltrate in the internal capsule, while the other (E07/08-30Ph) had a macroscopic cerebral abscess. Three pups had focal inflammation and gliosis in the parenchyma of the spinal cord (E07/08-31Ph, -37Ph and -40Ph).

Vasculitis was present in subarachnoid vessels (n = 12) and/or parenchymal vessels (n = 14) in all but two pups (E07/08-23Ph and -54Ph). Occasionally karyorrhectic nuclear debris was present within the wall of some veins, and very occasional veins had fibrinoid degeneration of the vascular wall. Small and medium-sized arterioles frequently had infiltration of the adventitia by neutrophils and histiocytes, but the media and intima were less severely affected. Occlusive thrombi were rare, consisting of fibrin clots alone, or fibrin clots with enmeshed platelets and leucocytes within small arterioles and venules. In some cases only veins were affected, with expansion of the vascular wall by neutrophils and macrophages. Vasculitis of parenchymal vessels was confined to grey matter, adjacent to accumulations of subarachnoid exudate. Within

the spinal cord, vasculitis was frequently present in the small vessels surrounding the central canal (n = 7), and was often pronounced in the subarachnoid space, where inflammation involved the full thickness of arteriolar walls, with karyorrhexis of endothelial nuclei and occasional occlusive thrombi.

Fourteen pups had exudate within the optic nerve sheath, which frequently extended out into surrounding peribulbar fat. Intraocular inflammation was present in nine cases, and comprised haemorrhage and inflammation at the base of the ciliary body (n = 5), low numbers of inflammatory cells in the stroma of the iris (n = 6), and a few leucocytes in the retina (n = 1). None had histological evidence of retinal haemorrhage.

Figures 5.4 to 5.13 show examples of the histological findings.

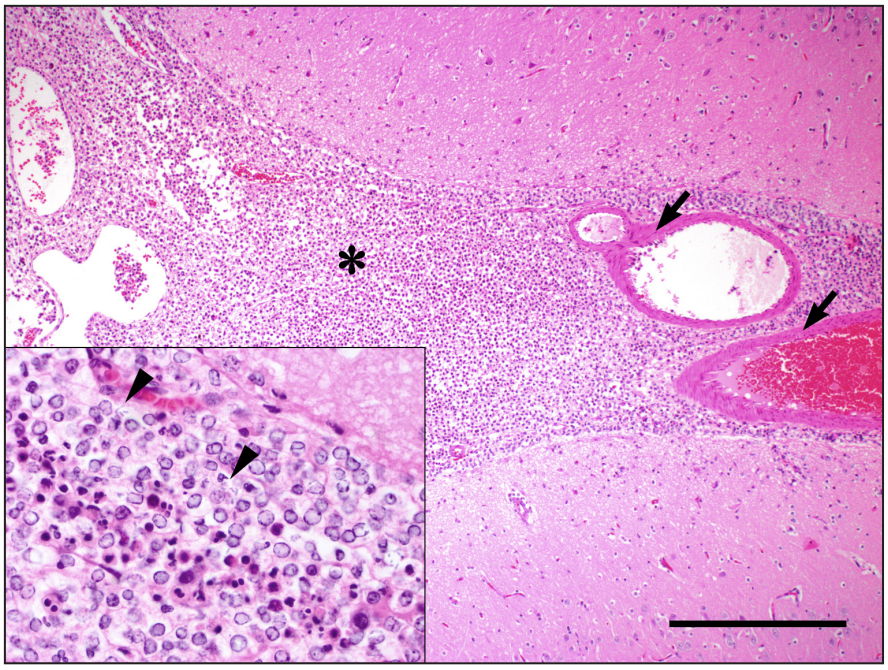

Figure 5.4. Exudate comprised predominantly of macrophages with fewer neutrophils expands the subarachnoid space (asterisk) of a pup with meningitis due to *Klebsiella pneumoniae*. Arteriolar walls (arrows) are spared in this case. The inset at lower left shows short bacterial rods (arrowheads), some of which are intracellular. Bar = 400 µm. (H&E)

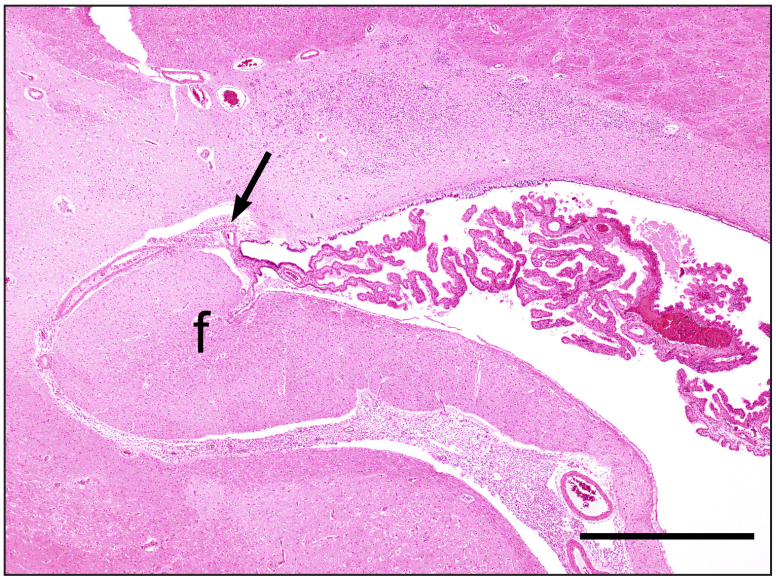

Figure 5.5. Lateral ventricle of a sea lion pup with meningitis due to *Klebsiella pneumoniae*. Inflammatory exudate within the subarachnoid space extends along the fornix (indicated by an 'f'), reaching the base of the choroid plexus (arrow). Bar = 1 mm. (H&E)

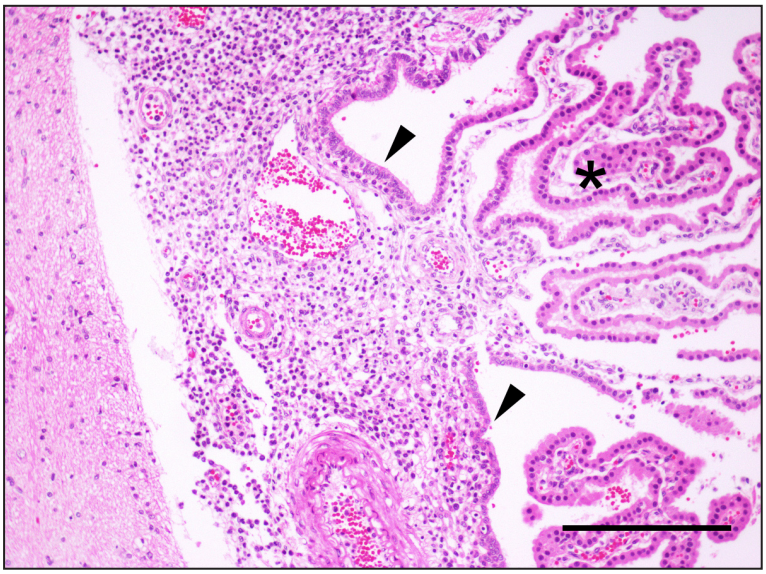

Figure 5.6. Higher magnification of the base of the choroid plexus in a pup with meningitis due to *Klebsiella pneumoniae.* The ependyma (indicated by arrowheads) is elevated from the underlying parenchyma by inflammatory exudate. This exudate does not extend into the stroma of the choroid plexus (marked by an asterisk). Bar = 200 μm. (H&E)

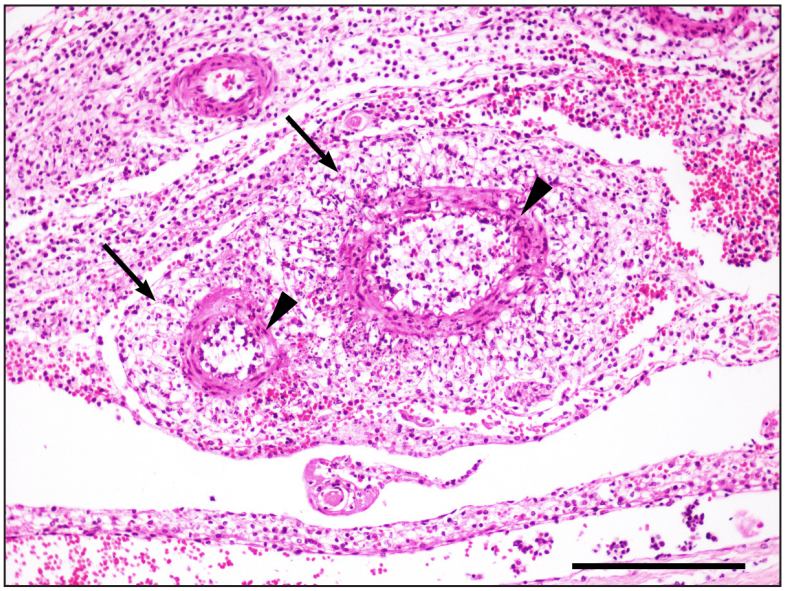

Figure 5.7. Arterioles within the spinal subarachnoid space in a pup with meningitis. The arteriolar media (arrowheads) is comparatively unaffected by inflammation, while the adventitia (arrows) is markedly expanded by histiocytes and neutrophils, with admixed necrotic nuclear debris. Bar = 200 µm. (H&E)

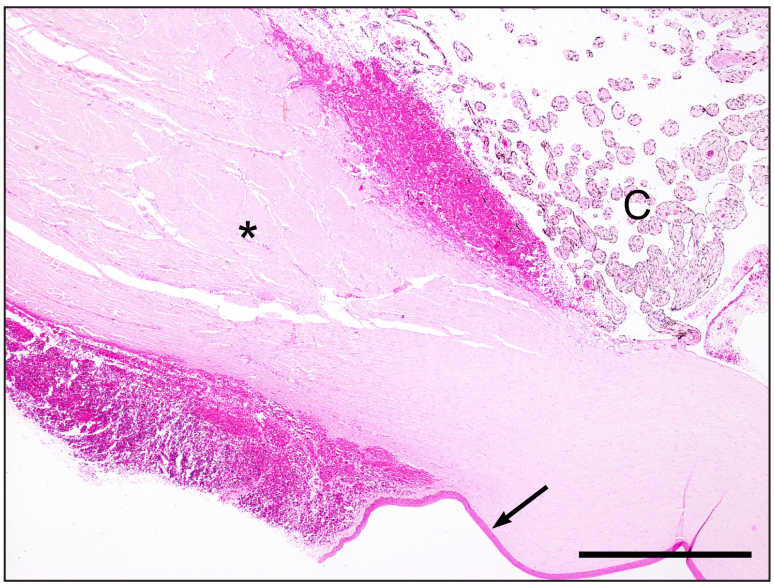

Figure 5. 8. An eye from a sea lion pup, showing the sclera (asterisk) at the level of the limbus. Note the haemorrhages at the inner and outer margins of the sclera. The corneal epithelium is indicated by an arrow, and the ciliary body by a 'c'. Bar = 200 µm. (H&E).

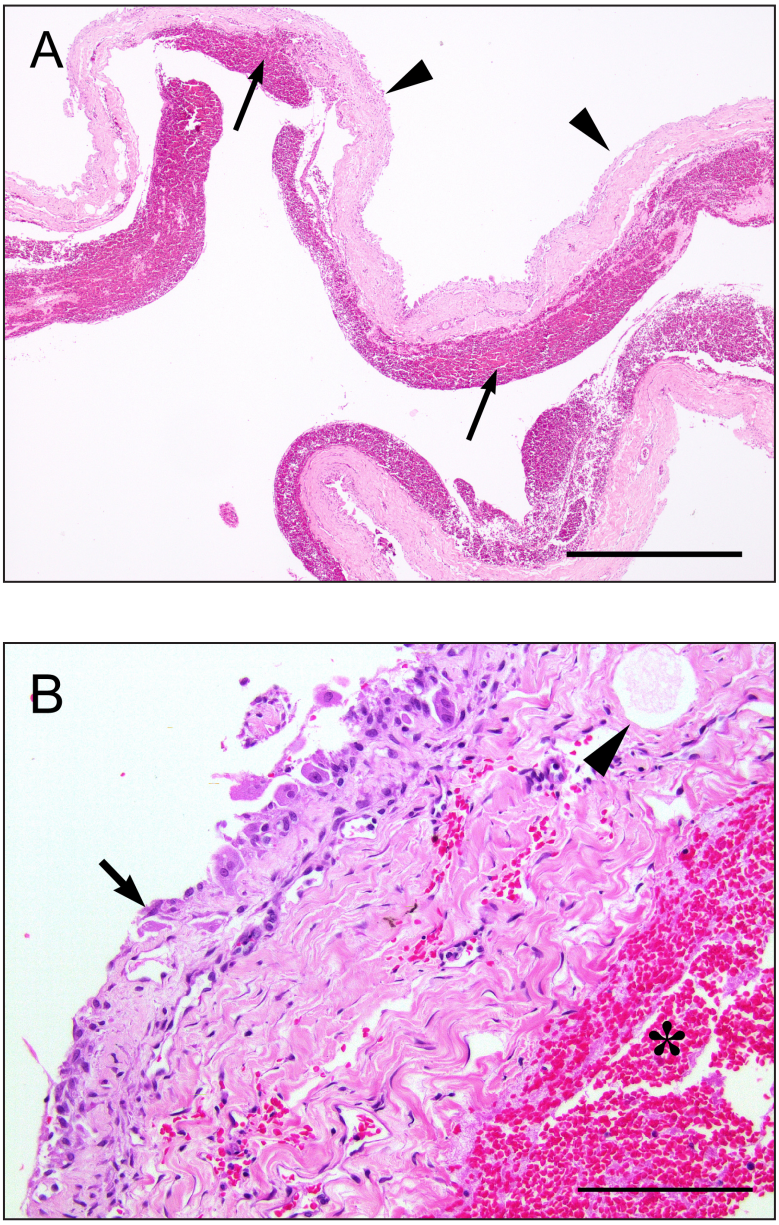

Figure 5.9. Histopathology of the intracranial dura mater.

A. Low power magnification of a section of intracranial dura mater from a pup with subdural haemorrhage. Arrowheads indicate the outer surface of the dura (adjacent to the inner aspect of the cranium). Haemorrhage (arrows) expands the subdural compartment. Bar = 1 mm. (H&E)

B. Higher power magnification of the dura mater, showing the layer of cuboidal epithelial cells that line the outer surface (arrow). Haemorrhage is present within the subdural compartment (asterisk). A rounded, unlined channel (arrowhead) contains wispy eosinophilic material. Bar = 100 μm. (H&E)

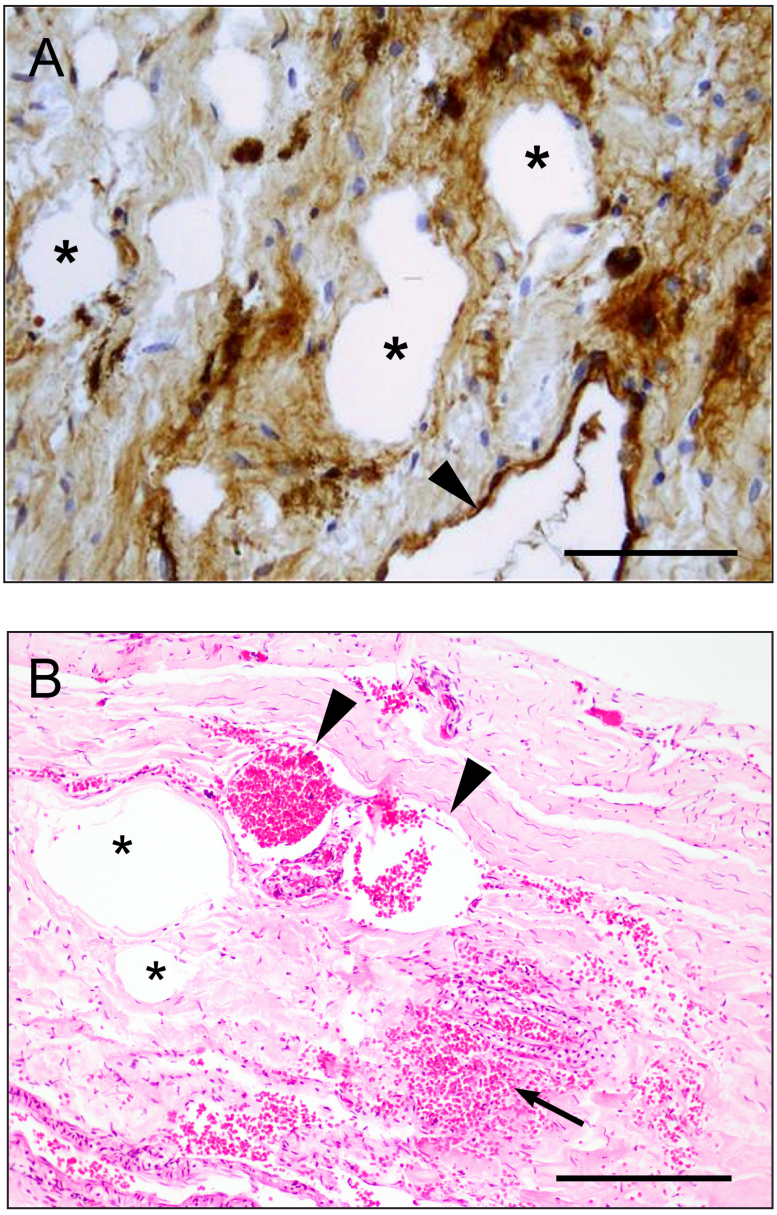

Figure 5.10. Details of intradural unlined channels.

A. Numerous rounded channels are present within the connective tissue of the intracranial dura mater, and are not lined by endothelial cells (asterisks). The arrowhead indicates an intradural vein, showing immunopositive staining consistent with an endothelial lining. Bar = 100 µm. (Anti-Factor VIII antigen immunohistochemistry)

B. In this section of intracranial dura mater there are several empty unlined channels (asterisks), along with several that contain haemorrhage (arrowheads). Note also the intradural haemorrhage (arrow). Bar = 200 µm. (H&E)

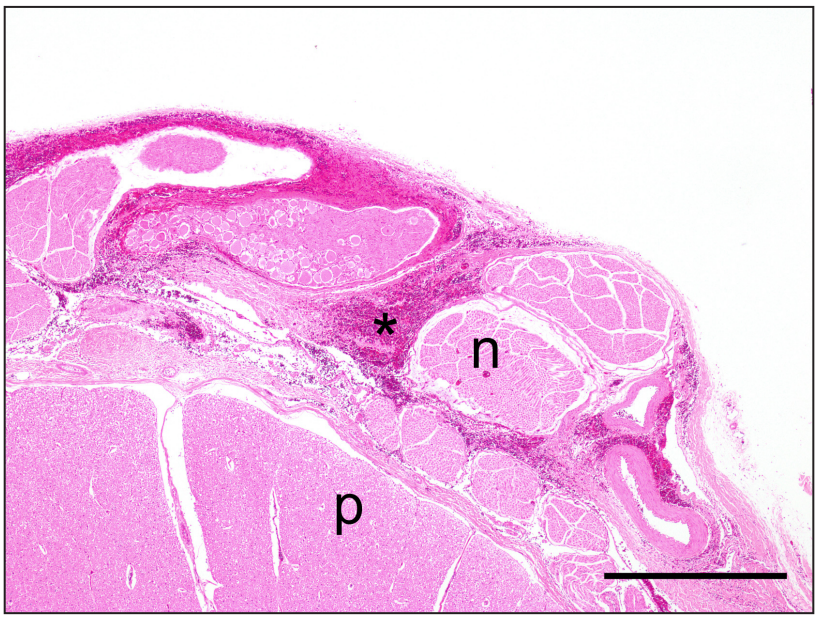

Figure 5.11. Spinal cord and meninges from a sea lion pup, showing intradural haemorrhage (asterisk). There is haemorrhage into the dural connective tissue that surrounds the spinal nerves ('n'). The letter 'p' indicates the spinal cord parenchyma. Bar = 400 µm. (H&E)

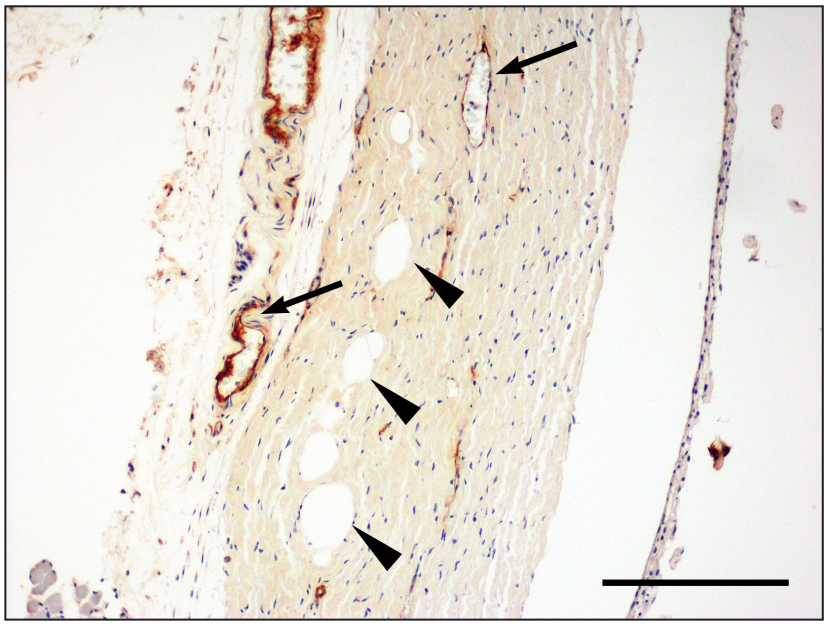

Figure 5.12. Spinal dura mater from a sea lion pup. Note the endothelial-lined vascular channels (arrows), in contrast to the rounded channels within the dura that are not lined by endothelial cells (arrowheads). Bar = 200 µm. (Anti-Factor VIII antigen immunohistochemistry)

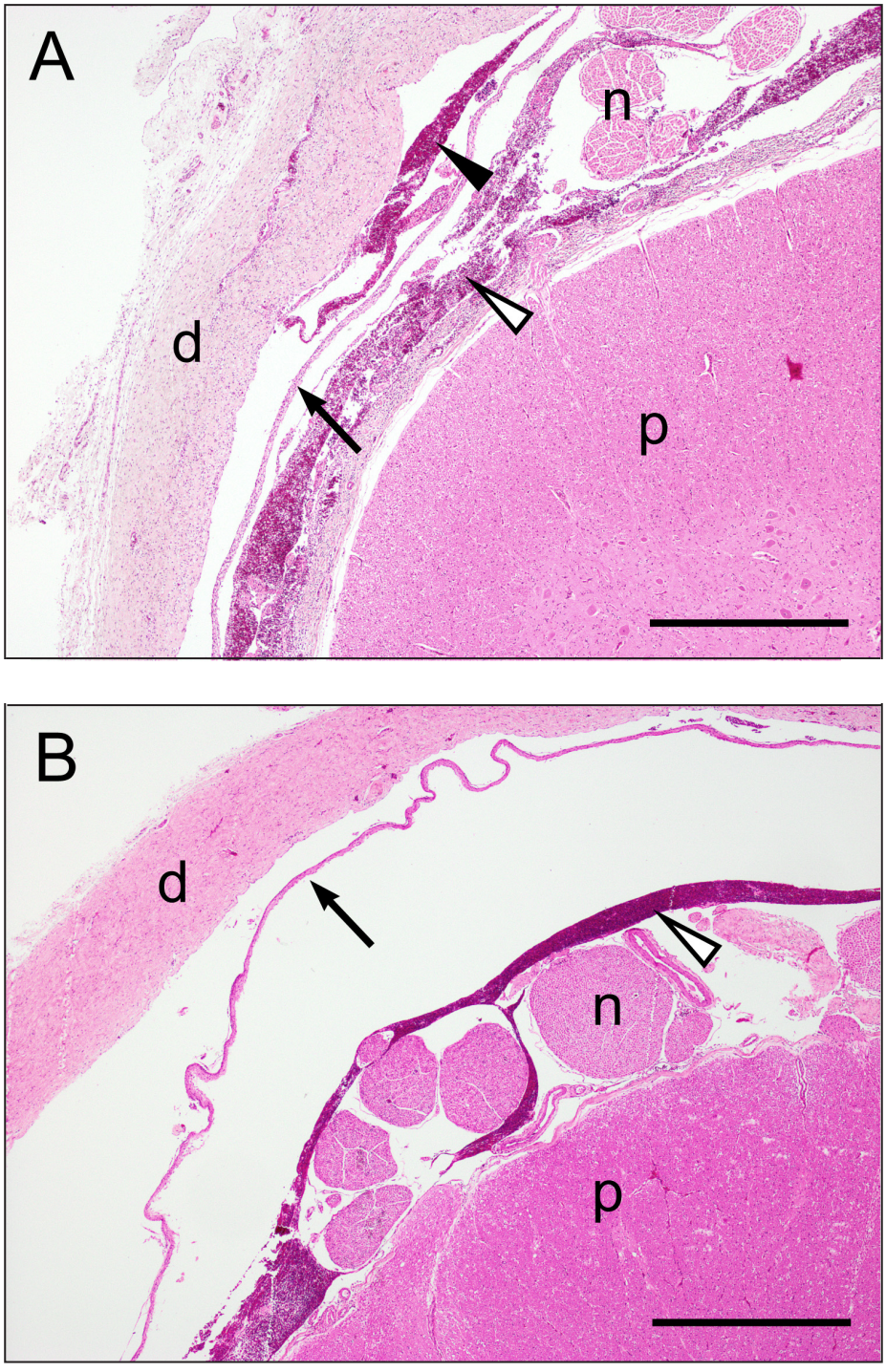

Figure 5.13. Anatomical location of sub-meningeal spinal cord haemorrhages. In both cases shown above, haemorrhage is located beneath the thick dura mater (d), but histological examination is able to localise the haemorrhage more precisely. The parietal layer of the arachnoid mater is indicated by an arrow. Bars = 1mm. (H&E)

A. In this pup, haemorrhage is both between the dura mater and the arachnoid mater (subdural; indicated by a black arrowhead) and under the parieral layer of the arachnoid mater (subarachnoid; white arrowhead).

B. This pup has subarachnoid haemorrhage only (white arrowhead). The arrow indicates the parietal layer of the arachnoid mater.

5.4 Comparison of lesions in pups with meningitis and without meningitis

For lesions that were scored, these scores were significantly higher for pups with meningitis compared with those without meningitis for intravascular leucocytes in the subarachnoid space ($p < 0.01$) and parenchyma ($p = 0.01$); thrombosis of choroid vessels ($p < 0.01$); vacuolation of ependymal cells ($p = 0.02$); and intravascular bacteria in parenchymal vessels ($p < 0.01$). Pups with meningitis had significantly less perivascular haemorrhage in the cerebral cortex ($p = 0.04$) and brainstem ($p = 0.02$) than pups without meningitis.

A number of lesions that were scored as either present or absent were only found in pups with meningitis, although this association only achieved statistical significance for vasculitis of the subarachnoid vessels ($p < 0.01$), exudate within the optic nerve sheath ($p < 0.01$), endophthalmitis ($p < 0.01$) and inflammation of the spinal cord dura mater ($p < 0.01$). In addition, pups with meningitis were significantly more likely to have spinal cord haemorrhage ($p < 0.01$). Haemorrhage of the ocular limbus, present in nine pups, was not associated with meningitis.

5.4.1 Frequency of meningitis in pups with subdural haemorrhage

Seventeen of 36 pups (47%) had either intracranial subdural haemorrhage or spinal sub-meningeal haemorrhage. The frequency of meningitis in these pups is shown in Figure 5.14. Ten pups had both intracranial subdural haemorrhage and spinal cord haemorrhage, six pups had spinal cord haemorrhage only and one pup had intracranial subdural haemorrhage only. Intracranial subdural haemorrhage ($p = 0.04$) and spinal cord haemorrhage ($p = 0.01$) were significantly more frequent in pups with meningitis.

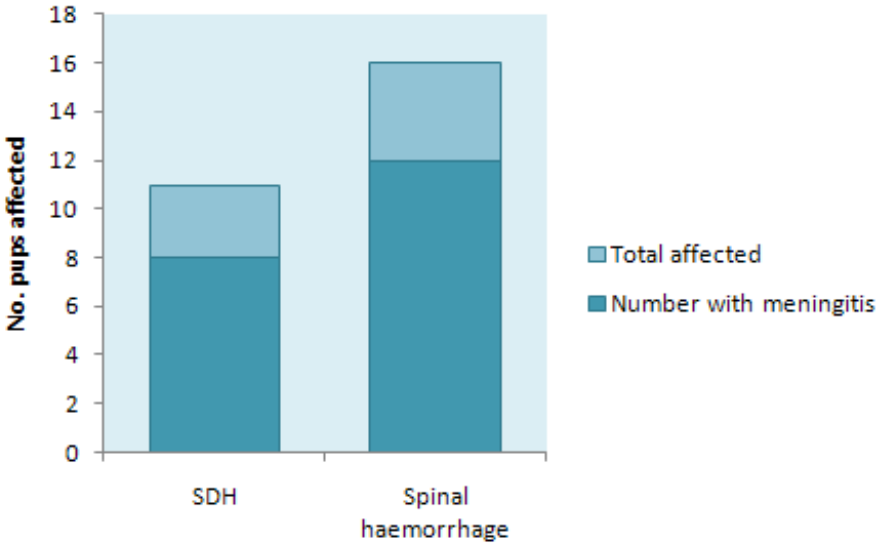

Figure 5.14. Association between meningitis and sub-meningeal haemorrhages in pups from the 2007/08 season. The majority of pups with intracranial subdural haemorrhage (73%) and spinal sub-meningeal haemorrhage (75%) had meningitis.

5.4.2 Intracranial dura mater and cervical spinal histology

Sections of intracranial dura mater and cervical spinal cord were available for 33 pups. The histological appearance of the dura mater was similar to human dura mater (Mack *et al.* 2009; Squier *et al.* 2009). The outer surface (adjacent to the cranium when *in situ*) had an adherent layer of cuboidal epithelial cells overlying a band of moderately dense collagen containing numerous small capillaries. Occasional osteoclasts and moderate numbers of hemosiderin-containing macrophages were present within this outer layer. Collagen density decreased toward the dura-arachnoid border. The deepest layers consisted of widely spaced mesenchymal cells separated by thin strips of collagen, comprising the dural border cell layer. Throughout the intermediate layers of dura were numerous small blood vessels as well as various numbers of rounded spaces which were either empty or contained a small amount of wispy eosinophilic material, occasionally with clusters of erythrocytes. These rounded spaces were often arranged in groups. Immunohistochemical staining for Factor VIII antigen confirmed that these channels did not have an endothelial lining.

Details of histological lesions of the intracranial dura mater are shown in Table 5.3. Twenty pups had haemorrhage within the layers of the dura mater. These intradural haemorrhages tended to coalesce within the dura mater, in some cases dissecting out into the subdural compartment There was no statistical association between the presence of intradural and subdural haemorrhages ($p = 0.26$). All haemorrhages were assessed as being less than 2 - 3 days old, based on the lack of clot dissolution and absence of haemosiderin, macrophages and fibrosis (Thornton and Jolly 1986; Geddes *et al*. 2003). In one case (E07/08-32Ph) the spinal subdural collection was a haemorrhagic exudate rather than pure haemorrhage and in two others the haemorrhage contained clusters of inflammatory cells, particularly macrophages (E07/08-41Ph and -55Ph).

Inflammation of the intracranial dura mater was present in five cases. In one pup (E07/08-36Ph) this comprised infiltration of the inner layer of the dura mater by numerous macrophages and neutrophils, bounded by a layer of haemorrhage adherent to the inner aspect of the dura. Pup E07/08-40Ph had a layer of fibrin and leucocytes (predominantly histiocytes) adherent to the deep border of the dura mater, in the absence of haemorrhage. Three pups (E07/08-39Ph, -41Ph and -45Ph) each had a focal area of inflammatory infiltrate in one section of dura mater. Only one of these five (E07/08-45Ph) did not have concurrent meningitis, although *K. pneumoniae* was cultured from the brain as well as from body tissues which had neutrophilic inflammation.

Table 5.3 shows microscopic details of spinal haemorrhages. Macroscopic examination of fixed spinal tissues indicated that haemorrhage was under the dura mater (i.e. sub-meningeal), but histologically the precise anatomical location of the haemorrhage was found to be between the dura mater and arachnoid mater (subdural) in eight pups, deep to the parietal layer of arachnoid mater (subarachnoid) in two pups, and both subarachnoid and subdural in six pups. Of major significance was the finding that

	Intracranial dura mater			Spinal cord meninges			
Pup ID	IDH	SDH	Dura mater inflammation	IDH	SDH	SAH	Dura mater inflammation
20Ph	o	o	o	o	o	o	o
21Ph	+				+	+	
22Ph	+						
23Ph	+	+		+	+		
24Ph	+						
25Ph	+			+			
26Ph				+			
27Ph	+			+			
28Ph	+						
29Ph	+			+			
30Ph	o	o	o	+			+
31Ph							+
32Ph		+			+	+	+
33Ph	o	o	o	o	o	o	o
34Ph	+	+			+	+	
35Ph				+			
36Ph	+	+	+	+	+	+	+
37Ph				+			+
38Ph	+			+			
39Ph	+	+	+	+	+		+
40Ph			+	+	+	+	
41Ph			+	+		+	+
42Ph	+	+		+		+	
43Ph		+		+	+	+	
44Ph	+						
45Ph	+	+	+				
48Ph	+			+			
49Ph	+	+			+		
50Ph				+			
51Ph							
52Ph	+						
53Ph					+		
54Ph	+	+		+	+		
55Ph					+		
56Ph				+	+		
57Ph	+			o	o	o	o

Table 5.3. Details of haemorrhagic and inflammatory meningeal lesions in the intracranial dura mater and cervical spinal tissues of pups from the 2007/08 breeding season. '+' indicates the presence of a lesion. IDH = intradural haemorrhage; SDH = subdural haemorrhage; SAH = subarachnoid haemorrhage; 'o' = sample not available. Pups with meningitis are shaded darker grey.

19 pups had *intradural* haemorrhage around the spinal nerve rootlets. In many pups erythrocytes could be seen dissecting through the dural connective tissue and into the subdural or subarachnoid spaces, but there was no statistical association between the presence of intradural haemorrhage and the presence of sub-meningeal haemorrhages: half of those with intradural haemorrhage had concurrent subarachnoid and/or subdural haemorrhages, and half did not. When the extent of intradural haemorrhage was taken into account, however, marked intradural haemorrhage (score 2) was strongly correlated with the presence of sub-meningeal haemorrhages ($p = 0.004$). Inflammation of the spinal dura ($n = 7$) and arachnoid mater ($n = 12$) was present only in pups with meningitis. Inflammation of the spinal dura mater ($p = 0.69$) or arachnoid mater ($p = 0.73$) was not statistically associated with spinal sub-meningeal haemorrhage.

5.4.3 Temporal pattern of meningitis deaths

Figure 5.15 shows the number of pups necropsied each week from 2005/06 to 2009/10, and the proportion with meningitis or other evidence of *K. pneumoniae* infection. From 2006/07 onwards, the number of pup deaths per week increased in the last few weeks of the season. Also, the proportion of dead pups with infectious disease, including meningitis, increased over this time, with the majority of pups necropsied toward the end of the season having *K. pneumoniae* infection.

5.5 Discussion

5.5.1 Cause of meningitis

In 15 of the 16 pups with meningitis, the causative agent was a highly viscous strain of *K. pneumoniae*. This bacterium is a ubiquitous, aerobic, gram negative bacillus that is common in the environment and is considered part of the normal human intestinal

flora (Procop and Tazelaar 2005). In human medicine, localised *K. pneumoniae* infections involving the lungs were typical in the past, but in the last few decades the pattern of disease associated with this agent has begun to change, with an increase in bacteraemia and invasive syndromes involving such lesions as meningitis, endophthalmitis and hepatic abscesses (Ko *et al.* 2002; Habib and Tambyah 2003; Keynan and Rubinstein 2007). This new pattern is associated with the emergence of a new, highly viscous (hypermucoviscous) phenotype (Lee *et al.* 2006; Chung *et al.* 2007; Keynan and Rubinstein 2007; Nadasy *et al.* 2007; Keynan and Rubinstein 2008). In addition to being regarded as an emerging human disease, hypermucoviscous *K. pneumoniae* infection has recently been reported in 27 California sea lions (Jang *et al.* 2010) and seven African green monkeys (Twenhafel *et al.* 2008). Although meningitis was not a feature in these recent hypermucoviscous phenotype-associated infections, meningitis due to other *K. pneumoniae* strains has previously been reported in non-human primates (Houser *et al.* 1970; Snyder *et al.* 1970; Fox and Rohovsky 1975; Tocidlowski 2006) and in one horse (Timoney *et al.* 1983).

5.5.2 Clinical syndrome

In the NZ sea lion pups in this study, *K. pneumoniae* meningitis was haematogenous in origin. Most, but not all, pups had other septic foci, particularly arthritis and cellulitis. In human medicine, environmentally-acquired hypermucoviscous *K. pneumoniae* meningitis occurs as part of an invasive syndrome involving bacteraemia, with or without primary liver abscesses (Ko *et al.* 2002; Habib and Tambyah 2003; Ma *et al.* 2005; Fang *et al.* 2007). The clinical disease in NZ sea lion pups differs from human cases of *K. pneumoniae* meningitis in several aspects. First, most authors report a strong male bias in human cases (Soscia *et al.* 1964; Tang *et al.* 1997; Habib and Tambyah 2003), whereas the male:female ratio in NZ sea lion pups was 6:9. Secondly, hepatic abscesses were not present in the sea lion pups. Thirdly, subdural haemorrhage is not a feature of hypermucoviscous *K. pneumoniae* meningitis in human patients. Finally, community-

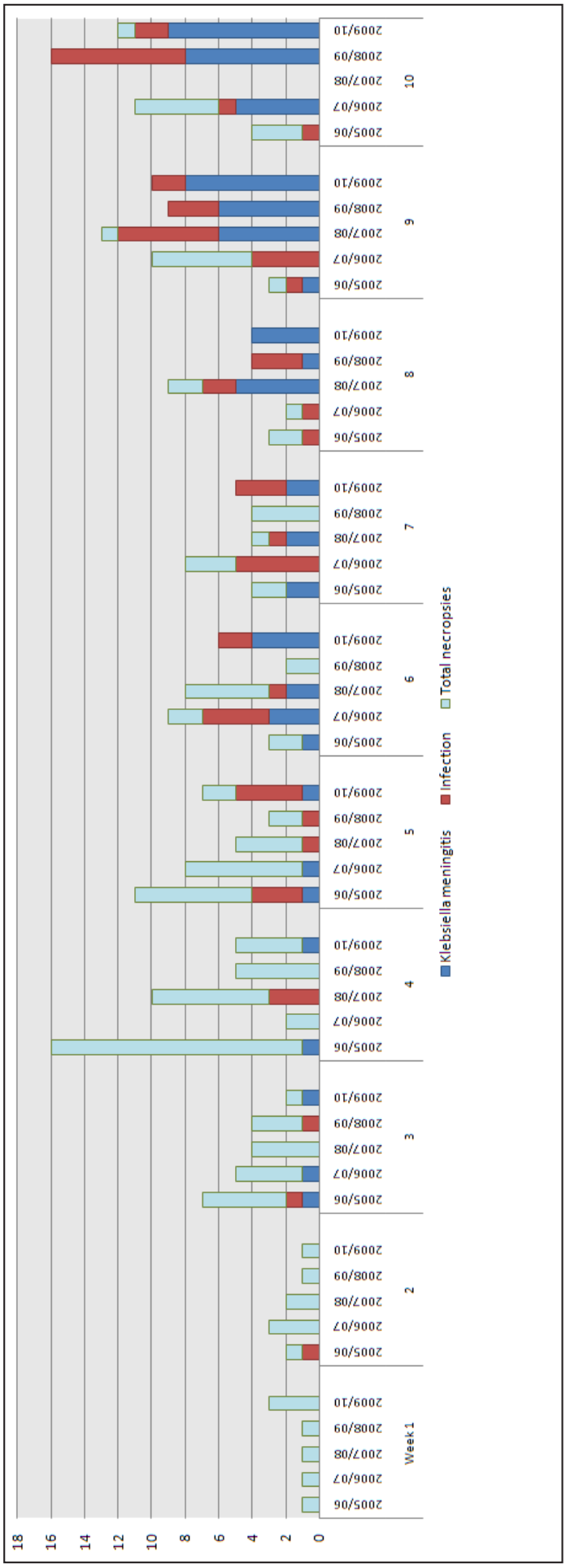

Figure 5.15. Prevalence of *K. pneumoniae* infection and meningitis in NZ sea lion pups necropsied at Sandy Bay, Enderby Island, for the 2005/06 to 2009/10 breeding seasons. Note that the number of fatal cases of *K. pneumoniae* infection increased during weeks 9 and 10 from 2006/07 onwards, and that from 2007/08 onwards the majority of pups that died during the last three weeks of the observation season had *K.pneumoniae* infection.

acquired *K. pneumoniae* meningitis is rare in human infants (Tang *et al.* 1997), although this may merely reflect a decreased chance of exposure.

Hypermucoviscous *K. pneumoniae* infection in California sea lions also appears to be quite distinct from that in NZ sea lion pups. Abscesses were the predominant feature in live-stranded California sea lions, none of which had evidence of bacteraemia or meningitis (Jang *et al.* 2010). Males were also over-represented in the affected group, with only one female in the case series of 27 animals. In addition, only one of the 27 cases occurred in a pup, although the age structure of the at-risk population was not described. While it has been suggested that distinct clinical syndromes of hypermucoviscous *K. pneumoniae* in human patients may correspond to specific bacterial genotypes (Ma *et al.* 2005; Fang *et al.* 2007; Yu *et al.* 2007), the pattern of lesions could also be affected by route of infection, or by host factors such as age and species.

5.5.3 Role of meningitis in subdural haemorrhage

While the pathological features of meningitis due to *K. pneumoniae* have been described in five non-human primates (Houser *et al.* 1970; Snyder *et al.* 1970; Fox and Rohovsky 1975; Tocidlowski 2006), and one horse (Timoney *et al.* 1983), subdural haemorrhage was not described in any of these. Only two human cases of *K. pneumoniae* meningitis with subdural haemorrhage were found in the English literature (Tancabelic and Haun 2004; Wong *et al.* 2006) and in each the meningeal clot was considered to have formed first, with subsequent infection.

The overall lack of subdural haemorrhage in *K. pneumoniae* meningitis in other species suggests that novel host factors may be involved in the NZ sea lion pup syndrome. There are several mechanisms by which subdural haemorrhage, shaking and meningitis could be linked. First, pups with meningitis would be less able to escape an attack than a healthy pup would be, resulting in an over-representation of pups with meningitis amongst those attacked by subadult males. Secondly, pups with meningitis may be

predisposed to bleeding when shaken, and therefore less likely to survive an attack. Thirdly, infection of a pre-existing subdural blood clot could result in meningitis. To determine whether shaking is in fact involved in the pathogenesis of subdural haemorrhages, further investigation is required.

5.5.4 Other causes of subdural haemorrhage

The differential diagnosis of subdural haemorrhage in human beings includes trauma, meningitis, coagulopathy and birth-associated haemorrhage (Kemp 2002). Aging of intracranial subdural haemorrhages and spinal meningeal haemorrhages showed that all clots in the sea lion pups were recent (less than 2-3 days), ruling out birth associated haemorrhage in late season pups, all of which would have been between one and seven weeks old. Acquired coagulopathies cannot be definitively ruled out, and gram negative sepsis with subsequent disseminated intravascular coagulation has been reported as a cause of subdural haemorrhage in neonates (Ng *et al.* 1998). Despite a large number of pups with sepsis in the 2007/08 season, only one pup (E07/08-31Ph) had petechial haemorrhages, in this case around the umbilicus and in lymph nodes. This pup did not have intracranial or spinal cord haemorrhage. It therefore is improbable that coagulopathy is a direct cause of spinal or intracranial haemorrhage.

Trauma is a possible primary or contributory cause for subdural haemorrhage in these pups. Three pups had subdural haemorrhage with no meningitis (E07/08-34Ph, -42Ph and -45Ph). Head trauma was the diagnosed cause of death in two of these (E07/08-34Ph and -42Ph). Rupture of bridging veins is the most widely-quoted cause of traumatic intracranial subdural haemorrhage, but is seldom definitively proven at necropsy (Mack *et al.* 2009). Examination of bridging vessels was not attempted in this study, and is technically difficult to undertake (Mack *et al.* 2009) particularly under field conditions with limited equipment. Thus the role of bridging vein rupture cannot be established for NZ sea lion pup subdural haemorrhage. In the published veterinary literature, subdural haemorrhage has been described in one neonatal puppy (<24 hours

old) (Grundy *et al.* 2009), reportedly due to deceleration forces generated during attempts to resuscitate the pup by swinging it. The authors did not demonstrate bridging vein rupture, nor did they consider the likelihood that this haemorrhage was a result of the birth process.

In addition to the mechanisms discussed above, the possibility has recently been raised that some subdural haemorrhages originate within the dura itself (Geddes *et al.* 2003; Mack *et al.* 2009; Squier *et al.* 2009; Squier and Mack 2009). This would certainly be consistent with the frequent presence of intradural haemorrhages in both the intracranial and spinal cord dura mater in NZ sea lion pups. While Croft and Reichard (2009) believe that intradural haemorrhages could be an artefact of removal of the dura mater, their sample groups were small, and analysis of their data shows that there is no statistically significant difference in frequency of intradural haemorrhage between the groups (Fisher's exact test; $p = 0.06$). In addition, Cohen *et al.* (2010) and Squier *et al.* (2009) found intradural haemorrhages in dura mater that was still attached to cranial bone. The pathogenesis of spinal and intradural haemorrhages is considered in more detail in Chapter Eight.

5.5.5 Importance of meningitis in morbidity and mortality

The findings of this chapter emphasise the importance of thorough gross, histological and microbiological analyses in definitively diagnosing cause of death in NZ sea lions. Six of the 15 pups with *K. pneumoniae* meningitis did not have gross lesions suggestive of bacterial disease, and the diagnosis would have been missed if the brain had not been examined grossly and histologically. In previous years, even when the brain was closely examined, death was attributed to trauma on the basis of subdural haemorrhage, whereas the majority of these pups are likely to have had *K. pneumoniae* meningitis. An accurate knowledge of the causes of mortality in this species is vital, particularly in view of the fact that pup production rates have decreased by more than 50% in the

past ten years (Chilvers and MacKenzie 2010), and that the species has recently been classified as critically endangered (Baker *et al.* 2010).

Although direct intervention is not currently a population management tool for the Enderby Island sea lion population, there may be a point at which this could be considered, should the decline in pup production continue or worsen. If subdural haemorrhage was attributed solely to attacks by subadult males, and specific males were thought to be habitual offenders, one possible management tool would be to remove these individuals from the site. However, the findings of this chapter indicate that the majority of pups with subdural haemorrhage have meningitis. Human literature indicates that the mortality rate in untreated *K. pneumoniae* meningitis is extremely high. Soscia *et al.* (1964) state rates of greater than 99%. Hence it seems unlikely that many, if any, affected pups would survive. Thus even if inflicted trauma was a factor in development of the haemorrhage, these pups would have been unlikely to survive the meningitis alone. Logically, if intervention was to be attempted, it would be better directed at preventing *K. pneumoniae* infection.

It also appears that the relative importance of *K. pneumoniae* infection may be increasing. Castinel *et al.* (2007) examined cumulative death rates from 2000/01 to 2003/04, and found that few deaths occurred in the last few weeks of the season. During this period, bacterial infections tended to peak in early-to-mid January, with very few deaths due to infection after mid February. In contrast, data from the current study indicate that there is now a second peak of mortality occurring late in the season, and that the majority of pups dying in the last few weeks have *K. pneumoniae* infection. In addition, *K. pneumoniae* deaths are still increasing at the time the science team leaves the island, so the exact number of pups dying due to this infection each year is unknown, but could be much higher than currently thought. The possibility that the activities of the science team itself might directly or indirectly increase prevalence of *K. pneumoniae* infection should also be considered. For example, the necropsy procedure could

result in increased environmental contamination, as well as increasing exposure of scavenging birds to infected tissues, with the birds then disseminating infectious bacteria. Alternatively, repeated disturbance of the NZ sea lion breeding colony could lower immunity and increase susceptibility to disease, while tagging of pups could provide a portal of entry for bacteria.

5.6 Summary

Microbiological and histological techniques were used to determine the possible origins of subdural haemorrhages in NZ sea lion pups that died in the later part of the 2007/08 breeding season. All subdural haemorrhages in these pups were recent, eliminating birth-associated haemorrhage as a cause. Intradural haemorrhages within the cranial and spinal dura mater were a likely source of subdural haemorrhage for many pups. Infection by a highly viscous strain of *K. pneumoniae* was common, and many of these pups had meningitis due to this bacterium. There was an association between subdural haemorrhage and meningitis, and the relative age of the lesions indicated that the haemorrhages occurred after the meningitis. Since subdural haemorrhages are not a feature of *K. pneumoniae* meningitis in other species, a novel factor must be involved in this syndrome in NZ sea lion pups. The possibility that shaking is this novel factor is considered in the following chapters.

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Chapter Six

β-amyloid precursor protein as a marker of axonal injury in NZ sea lion pups

6.1 Introduction

Diffuse axonal injury is defined as widespread damage to axons in white matter. It is recognised as an almost universal consequence of blunt head trauma, occurring in a spectrum of injuries from mild concussion through to severe fatal injuries (Gennarelli *et al.* 1982; Blumbergs *et al.* 1994; Gentleman *et al.* 1995; Oehmichen *et al.* 1998). In forensic medicine, the presence and distribution of axonal injury can indicate not only that blunt head trauma has occurred (Geddes *et al.* 2000; Reichard *et al.* 2005), but in some cases can help determine the mechanism of injury (Reichard *et al.* 2005; Dolinak and Reichard 2006).

While routine histochemical staining techniques cannot detect diffuse axonal injury unless a patient survives for 15 (silver stains) to 24 (H&E) hours (Oehmichen *et al.* 1998; Blumbergs *et al.* 2008), anti-β-amyloid precursor protein (βAPP) immunohistochemistry can demonstrate damaged axons in cases with survival times as short as 2 hours (Sherriff *et al.* 1994; Geddes *et al.* 2000; Reichard *et al.* 2005). Beta-APP is a membrane spanning polypeptide that is synthesised in the neuronal soma and transported by fast axonal transport. Disruption of transport, for example due to shearing of axons, causes accumulation of βAPP at the site of axonal injury. This is seen microscopically as anti-βAPP immunopositive axonal swellings or bulbs.

The first aim of this chapter was to determine whether anti-βAPP immunohistochemistry could be used for the definitive diagnosis of traumatic brain injury in NZ sea lion pups. Although this technique is extremely sensitive, it is not specific to trauma, as hypoglycaemia and hypoxia-ischaemia can also cause βAPP accumulation in axons (Dolinak *et al.* 2000a, 2000b; Geddes *et al.* 2000; Reichard *et al.* 2003a; Reichard *et al.* 2005; Blumbergs *et al.* 2008). In human cases, the role of hypoglycaemia and hypoxia-ischaemia can be assessed by reviewing the distribution of acidophilic neurons in the hippocampus, cerebral cortex and cerebellum (Dolinak *et al.* 2000b; Reichard *et al.* 2005), as well as by analysis of tissue fluid glucose levels. Aqueous humour glucose equilibrates rapidly with blood glucose, and the glucose concentrations of these two fluids are closely correlated (Cameron *et al.* 2001). This makes it possible, in theory at least, to use post mortem aqueous humour samples to identify hypoglycaemia. While post mortem glycolysis results in a progressive decrease in glucose levels that can result in spurious hypoglycaemia (Coe 1993), a normal or high glucose level can rule out ante mortem hypoglycaemia.

The second aim of this chapter was to determine whether anti-βAPP immunohistochemistry could be used to detect shaking injury in pups. Although a number of published studies have suggested that a diffuse pattern of axonal injury

occurs in shaken human infants (Duhaime *et al.* 1987; Vowles *et al.* 1987; Gleckman *et al.* 1999), more recent studies using anti-βAPP immunohistochemistry concluded that encephalopathy in these cases was more frequently due to vascular compromise associated with brain swelling than to the direct effects of trauma (Geddes and Whitwell 2003a; Reichard *et al.* 2003b; Dolinak and Reichard 2006; Finnie *et al.* 2010). Axonal injury was also frequently seen in the cervical spinal cord (Shannon *et al.* 1998; Geddes *et al.* 2001b; Reichard *et al.* 2003b; Dolinak and Reichard 2006) and optic nerve (Gleckman *et al.* 2000; Reichard *et al.* 2004) of affected infants, and axonal injury at these locations could also be an indicator of shaking in pups.

In order to determine whether anti-βAPP immunohistochemistry could detect the presence and identify the mechanism of traumatic brain injury in NZ sea lion pups, this chapter evaluates the amount and distribution of axonal injury in the brains, spinal cords, and optic nerves of pups from the 2007/08 season. Patterns of axonal injury are identified, and relationships between these patterns and other brain lesions, particularly intracranial subdural haemorrhage and meningitis, are examined. Ancillary methods are used to investigate the potential contribution of hypoglycaemia and hypoxia-ischaemia to axonal injury.

6.2 Materials and methods

6.2.1 Sample population

Brains, eyes and cervical spinal cords collected from pups that died in the second half of the 2007/08 field season (n = 36) were used in this study.

6.2.2 Examination of fixed brain and spinal cord

Following fixation for at least one month, both brain halves and the resected cervical spinal cord of each pup were photographed, and any gross lesions noted. The left

hemisphere was sectioned at 10 mm intervals and sections were examined for gross abnormalities. Particular attention was paid to the brainstem and corpus callosum, which show focal haemorrhage in human cases of Grade III diffuse axonal injury.

Nine brain tissue blocks, as described in Chapter Five, and two transverse sections of spinal cord (one taken at the level of C1 and one at C4 – C5) were prepared. One 4 μm section was cut from each block, embedded in paraffin, processed routinely for histology, and stained with H&E. A second 5 μm section was cut from each block for immunohistochemistry. These sections were mounted on positively charged glass slides and stored at room temperature before processing for immunohistochemistry. Mounted sections were rehydrated through a series of increasing concentrations of xylene using a Leica automatic tissue processor. Rehydrated sections were transferred from the processor directly into tap water before being subjected to heat-induced antigen retrieval. This involved placing slides into coplin jars filled with citrate buffer at pH 6.0. Coplin jars were then placed in a pressure cooker, and slides were held at pressure for 10 minutes and then cooled for 10 minutes under running tap water. Endogenous peroxidase activity was blocked by incubating slides in 0.3% H_2O_2 for 30 minutes at room temperature, followed by washing in 0.05M tris-buffered saline (TBS). Non-specific antibody was blocked using normal horse serum, and slides were then incubated for 30 minutes at room temperature in βAPP antibody (clone 22C11; Chemicon, Abacus ALS NZ, Auckland, New Zealand) diluted to 1:5,000 in TBS. Slides were then washed in TBS for 5 minutes. Antigen detection was achieved using horse anti-mouse/rabbit biotinylated secondary antibody and ABC reagent (Vectastain Universal Elite kit; Vector Laboratories) and the resulting complex visualised using 3-3'-diaminobenzidine diluted in TBS (Liquid DAB Substrate Chromogen System; DakoCytomation). Sections were then counterstained in haematoxylin and coverslipped. The presence of a brown chromogen reaction product indicated specific binding to βAPP antigen, described here as positive anti-βAPP immunoreactivity, indicating axonal injury.

Brain tissue from a sea lion pup (E07/08-30Ph) which had axonal swellings visible on H&E stain was submitted to a human immunohistochemistry laboratory that routinely uses βAPP antibody clone 22C11. Sections from this pup were processed with human control tissues, and showed positive immunoreactivity of axonal bulbs and swellings. This tissue was subsequently used as a positive control section in all runs performed as part of the current study. Isotype controls were run using an irrelevant antibody (anti-infectious bursal disease virus (IBDV) monoclonal antibody produced in mice; CSIRO/IBDV/17-82; TropBio Pty Ltd., Townsville, Queensland, Australia) to check specificity of primary antibody. Negative antibody controls were run by omitting primary antibody to check specificity of secondary antibody binding.

6.2.3 Examination of eyes

Gross examination of eyes was described in Chapter Five. Trimming was performed as follows. The posterior calotte was sectioned transversely, on either side of the optic nerve. The nerve was severed approximately 2 mm from the optic disc, sectioned longitudinally and each half placed cut side down in a cassette. The lens was removed from the anterior calotte using forceps, and the cornea and attached sclera sectioned transversely. Blocks were processed routinely for histological examination, with 4 μm sections being cut for H&E staining, and 5 μm sections for immunohistochemistry as above. For sections of the posterior calotte, sequential trimming was undertaken until the lamina cribrosa was visible.

6.2.4 Assessment of βAPP immunoreactivity

Brain and spinal cord sections were examined under the light microscope. Line drawings of each brain section were used to record the type and amount of immunoreactivity, with each tissue section being divided into a number of neuroanatomical regions. The amount of immunoreactivity present in each region was rated on a semi-quantitative scale similar to that described by Gentleman *et al.* (1995) and Reichard *et al.* (2003a).

Details of this scoring system are summarised in Table 6.1.

0	no positive staining
+ (mild)	low numbers of individual positively stained axons usually requiring a 40x objective for good visualisation
++ (moderate)	scattered clusters of positively stained axons, or moderate numbers of individual positively stained axons
+++ (marked)	extensive staining of large areas of white matter, visible using a 1.5x objective

Table 6.1 Key for semi-quantitative scoring of anti-βAPP immunoreactivity in brain sections.

Figure 6.1 (below) shows examples of moderate and marked anti–βAPP immunoreactivity.

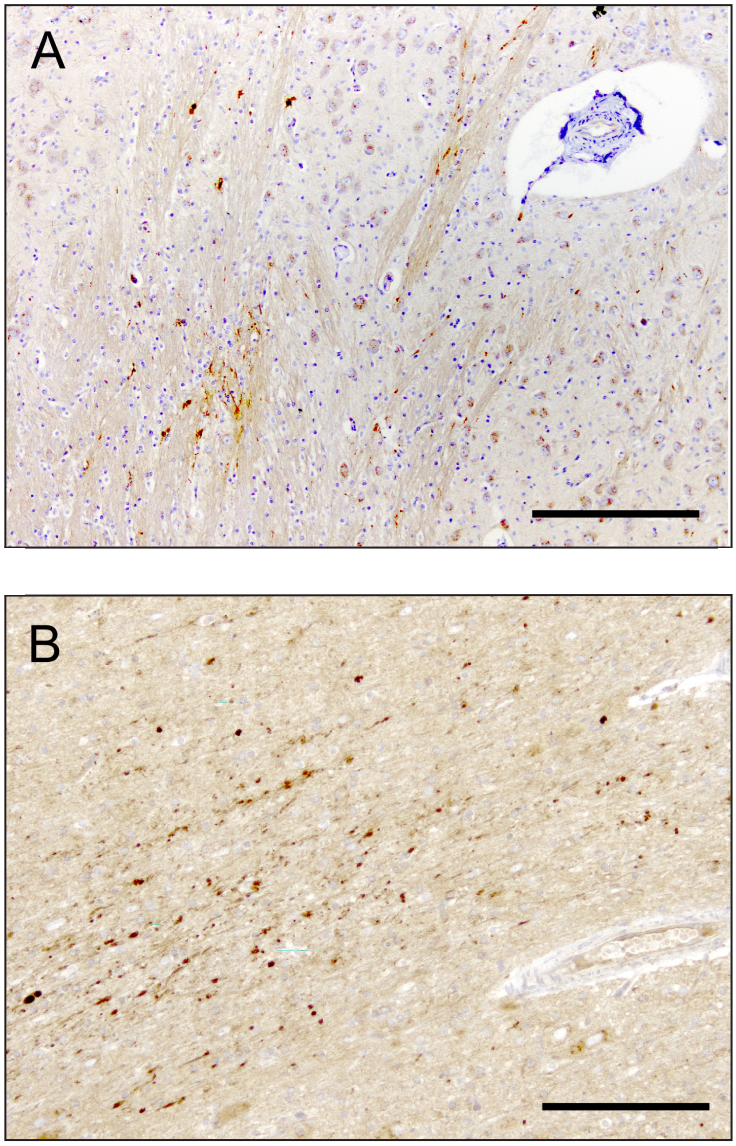


Figure 6.1. Examples of immunoreactivity scored as '++' (moderate; **A**) and '+++' (marked; **B**). Both images were captured using a 10x objective for comparison. Anti-βAPP immunohistochemistry. Bars = 100 μm.

Composite scores were assigned by adding the scores in each neuroanatomical region to give a total axonal injury score for each animal. Results for individual pups were translated to an electronic format using digitised line drawings created on Xara software. For each case, the distribution and amount of anti-βAPP immunoreactivity in each brain section was mapped using greyscale values as shown in Figure 6.2.

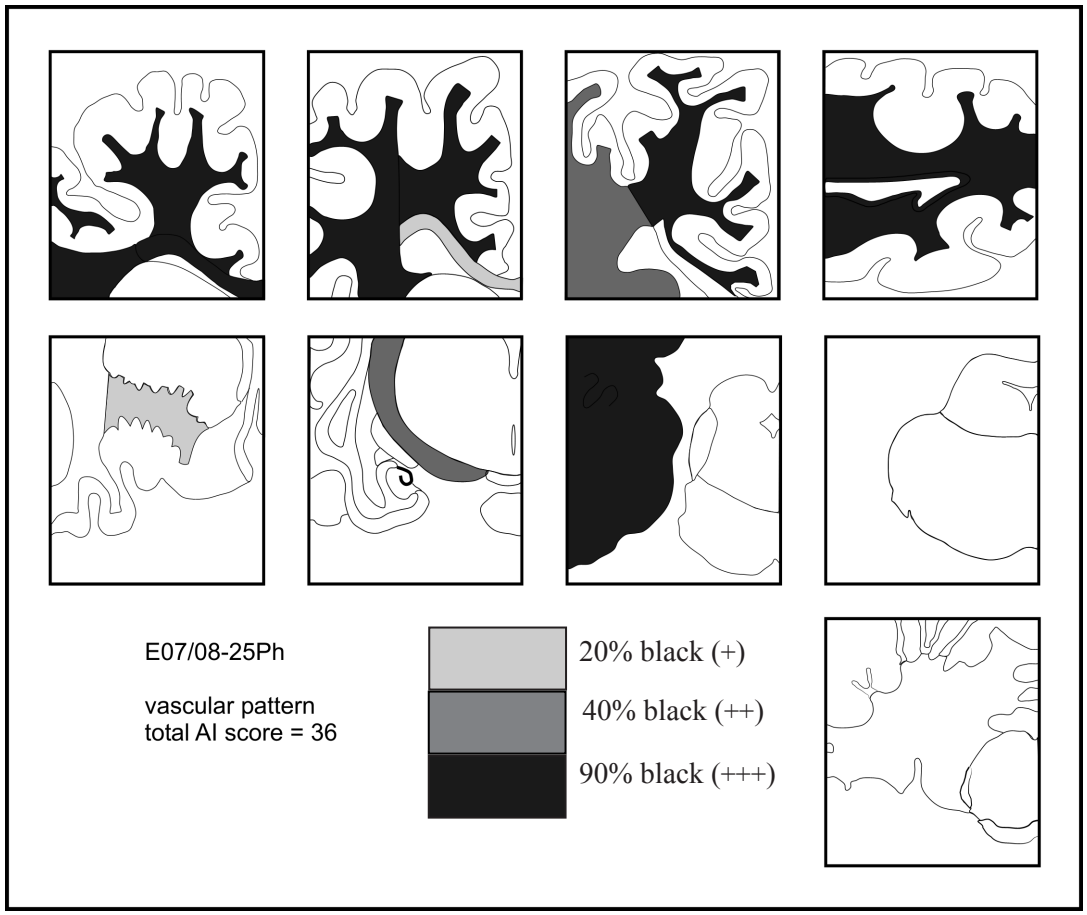


Figure 6.2. An example of mapping βAPP scores using greyscale values. Scores are indicated using increasingly dark shades of grey, as shown on the key in the middle of the lower panel.

Slides were examined and mapped prior to review of the gross necropsy findings and histological brain lesions associated with the case. Patterns of immunoreactivity defined by Reichard *et al.* (2003a) and Blumbergs *et al.* (2008) were used as a basis of classification. This method describes five patterns:

(i) Diffuse traumatic axonal injury

A history of trauma, in conjunction with scattered individual swellings/bulbs or

groups of swellings/bulbs in the corpus callosum, cerebral hemispheric white matter and brainstem, in a pattern that is not consistent with vascular axonal injury (see below).

(ii) Multifocal traumatic axonal injury

A history of trauma, in conjunction with scattered/groups of swellings or bulbs in the corpus callosum and hemispheric white matter, but not in the brainstem, in a pattern that is not consistent with vascular axonal injury.

(iii) Vascular axonal injury

A 'zig-zag' like pattern of immunoreactivity, often seen as clearly demarcated areas within white matter tracts, caused by compromise of the vascular supply due predominantly to increased intracranial pressure.

(iv) Metabolic axonal injury

Scattered immunopositive axons in the absence of a history of trauma, and not in a pattern of vascular axonal injury, probably due to hypoglycaemia or, more rarely, global hypoxia-ischaemia (Dolinak *et al.* 2000a, 2000b; Reichard *et al.* 2005).

(v) Penumbral axonal injury

Axonal immunoreactivity surrounding a focal lesion.

This classification method could not be directly applied to the sea lion pups as no history was available, thus excluding diffuse axonal injury and multifocal traumatic axonal injury from classification. Furthermore, because it was not possible to definitively exclude trauma, metabolic axonal injury could also not be diagnosed. Instead, a new category of 'multifocal' axonal injury was used to describe cases with widespread white matter immunoreactivity that was not distributed in a pattern suggestive of vascular axonal injury. A number of pups also had a periventricular pattern of immunoreactivity, which was included as a separate category. Thus the modified classification system used in this study was as follows:

(i) Vascular axonal injury: clusters of immunopositive axons arranged in a zig-zag like pattern, as confirmed by D. Blumbergs, *pers. comm.*

(ii) Multifocal axonal injury: scattered clusters or individual immunopositive axons arranged in a pattern not consistent with vascular axonal injury.

(iii) Penumbral axonal injury: immunopositive axons arranged around the margins of a focal lesion.

(iv) Periventricular axonal injury: immunopositive axons surrounding a ventricle.

Pups that had only a few scattered positive axons (scored as '+') in a few sections were classified as having 'minimal' overall immunoreactivity. 'Widespread' axonal injury was defined as the presence of moderate or marked immunoreactivity in a non-vascular pattern, in the cerebellar peduncles and brainstem, and at two or more levels of the corpus callosum and cerebral white matter, with a total axonal injury score of 20 or more.

Anti-βAPP immunoreactivity of neurons was evaluated manually using a light microscope. Cerebellar sections (block 9) were scanned using a 40x objective lens, and three sets of 100 Purkinje cells were counted using non-overlapping fields. Immunopositive Purkinje cells were defined as those with anti-βAPP positive granules occupying more than 50% of the neuronal soma, and were expressed as an average percentage of the three counts.

Neurons in the frontal cortex and brainstem were assessed for immunoreactivity of the neuronal soma, using a semi-quantitative scale: '0' (no immunoreactivity), '+' (less than 25% of neurons immunopositive), '++' (approximately half of neurons immunopositive) or '+++' (over 75% of neurons immunopositive).

A 4mm grid graticule was placed over sections of cortex and brainstem to evaluate neuronal soma immunoreactivity as described by Finnie *et al.* (2010). Grid squares

were assessed as positive if they contained one or more neurons with immunopositive granules occupying more than 50% of the soma.

Eye sections were evaluated for the presence, amount and location of anti-βAPP immunoreactivity, as well as the type of axonal profile.

6.2.5 Comparisons between groups of pups

The relative distribution of axonal injury was determined for pups with meningitis, and for pups with subdural haemorrhage. Pups that had both meningitis and subdural haemorrhage were excluded from this part of the analysis. Pups that had neither subdural haemorrhage nor meningitis were analysed as a control group.

To determine the relative distribution of axonal injury in each group, the average axonal injury score was calculated for each neuroanatomical region. The average score for each region was then weighted *pro rata*, so that the area with the highest score was assigned 100%. Values were rounded up to the nearest factor of 10. Results for each group were then mapped on a line drawing by shading each neuroanatomical area using greyscale values corresponding to the weighted average axonal injury score.

6.2.6 Review of H&E sections: evidence of hypoglycaemia or hypoxia-ischaemia

H&E stained sections of frontal cortex (block 1), occipital cortex (block 7), hippocampus (block 4) and cerebellum (block 9) were reviewed. The frequency of acidophilic neurons was noted and scored semi-quantitatively as follows: '+' = one affected neuron every few 10x fields; '++' = several affected neurons every 10x field; '+++' = many affected neurons in every 10x field. In addition, the location was noted as superficial, mid or deep layers for cortical neurons, and as CA1, CA3, CA4, subiculum or dentate gyrus for hippocampal neurons. The location of acidophilic neurons was compared with human neuropathological criteria, where hypoglycaemia typically causes necrosis of

superficial cortical, CA1 and dentate neurons, without involvement of basal nuclei or Purkinje cells, while ischaemia results in necrosis of Purkinje cells, neurons in the deep layers of the cerebellar cortex, and the CA1 and CA4 regions of the hippocampus.

6.2.7 Glucose assays

Aqueous humor samples were assayed for glucose concentrations on a Hitachi Modular P800 Chemistry Analyser using a photometric method.

6.2.8 Brain swelling

Brain weights were initially assessed on site, using kitchen scales. These scales failed in week eight, so no un-fixed brain weights were available for pups E07/08-40Ph to -57Ph. Therefore, to standardise weights, both halves of all fixed brains were weighed in the laboratory, using Mettler PJ6000 digital scales.

Since brain weight increases with age, and may differ between sexes, comparisons were made between brain weight and other parameters that might allow outliers (swollen brains) to be identified. Brain weight was compared with head girth, body length, girth at axilla, and girth at neck, and relationships between the paired parameters assessed using regression analysis.

To assess for parahippocampal gyrus necrosis, the right hippocampus was trimmed in for each pup. Left hippocampus sections had previously been trimmed. All were assessed grossly for evidence of necrosis; the left sides were also assessed histologically.

6.2.9 Statistical analysis

Statistical analyses were performed using Minitab© software. Average axonal injury scores for each neuroanatomical region and total axonal injury scores were compared for groups of pups using the Mann-Whitney test for non-parametric data.

6.3 Results

6.3.1 βAPP immunohistochemistry

Gross examination of transverse brain sections showed no focal parenchymal haemorrhagic lesions in the corpus callosum or brainstem.

Several normal structures were immunopositive for βAPP protein (Figure 6.3), including choroid plexus epithelial cells, satellite cells in spinal nerves, and retinal pigmented epithelial cells. The proportion of immunopositive Purkinje cell soma ranged from 62 to 94%. The frequency of Purkinje cell soma immunoreactivity was not statistically associated with subdural haemorrhage or meningitis. Neuronal soma immunoreactivity in the hippocampus, frontal cortex and brainstem nuclei was scored as +++ in all pups. Examination of sections of frontal cortex and brainstem using the graticule system (Finnie *et al.* 2010) showed that all grid squares containing neurons were positive, negating comparisons between individuals and groups.

Three main profiles of immunoreactive axons were present: round axonal profiles in variably swollen axons ('swellings); tear-shaped profiles ('bulbs'); and longitudinal sections of irregularly swollen axons ('varicosities'). In addition, immunoreactive lengths (segments of positively stained axon up to 100 μm long) were present in the brainstem of three pups (E07/08-23Ph, -42Ph and -55Ph). Examples of profile types are shown in Figure 6.4. Two or more profile types were often present in one region.

Total axonal injury scores ranged from 2 - 48, and eleven pups had a total axonal injury score of 20 or more. Table 6.2 shows details for each pup.

Periventricular, penumbral, multifocal and vascular patterns of axonal injury were recognised (Figure 6.5). Seven pups had a vascular pattern, and total axonal injury scores in this group ranged from 18 - 48. In these pups the distribution of axonal injury

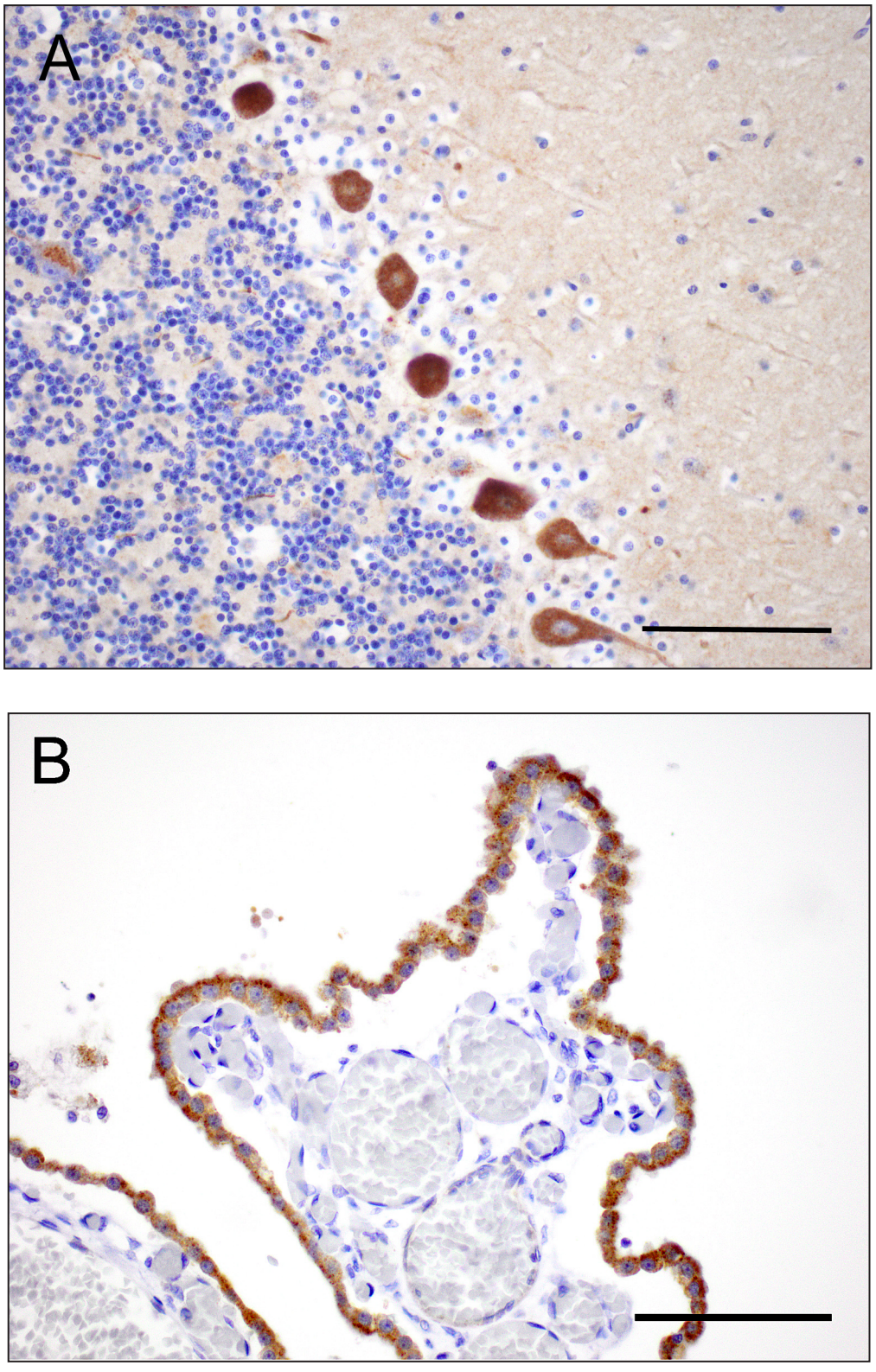


Figure 6.3: Normal structures showing immunoreactivity for βAPP in the brains of NZ sea lion pups. **A.** Cerebellar Purkinje cells. Bar = 100 μm. **B.** Choroid plexus epithelial cells. Bar = 200 μm.

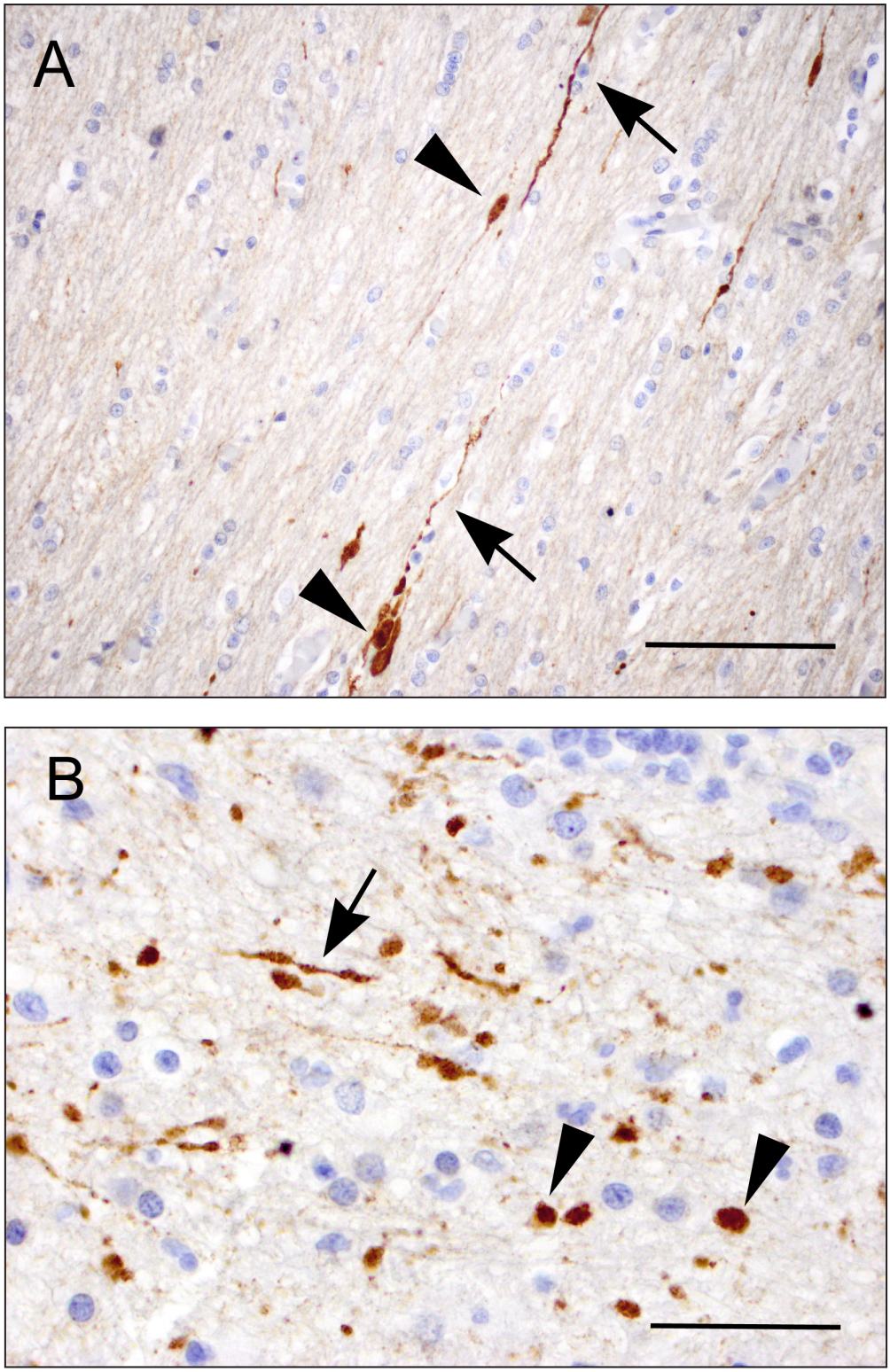


Figure 6.4. Immunoreactive profiles present within brain sections. Both sections have been stained with anti-βAPP antibody. **A.** This section depicts immunoreactive bulbs (arrowheads) and varicosities (arrows). Bar = 100 μm. **B.** In this section of brain there are both varicosities (arrows) and swellings (arrowheads). Bar = 50 μm.

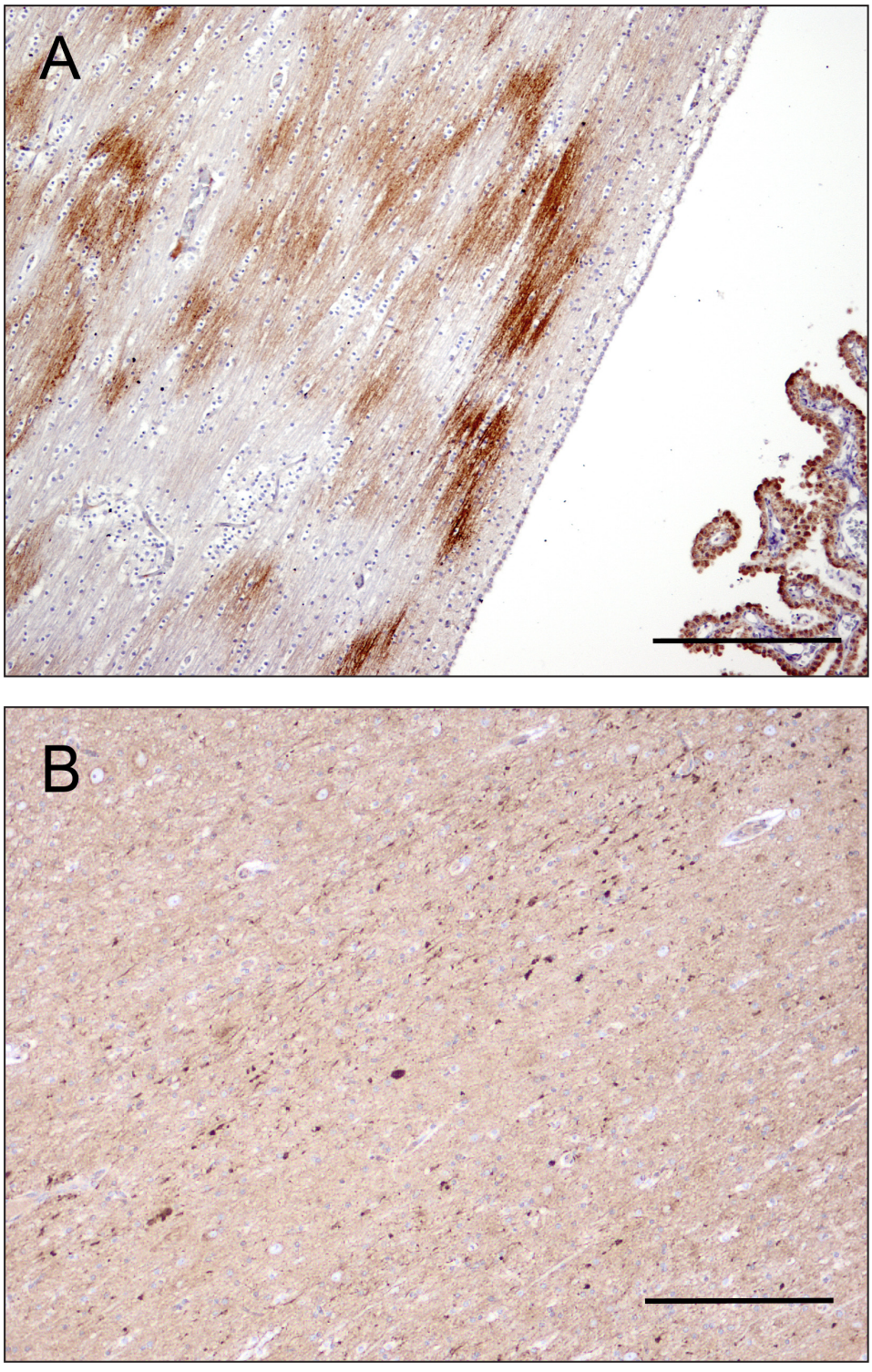
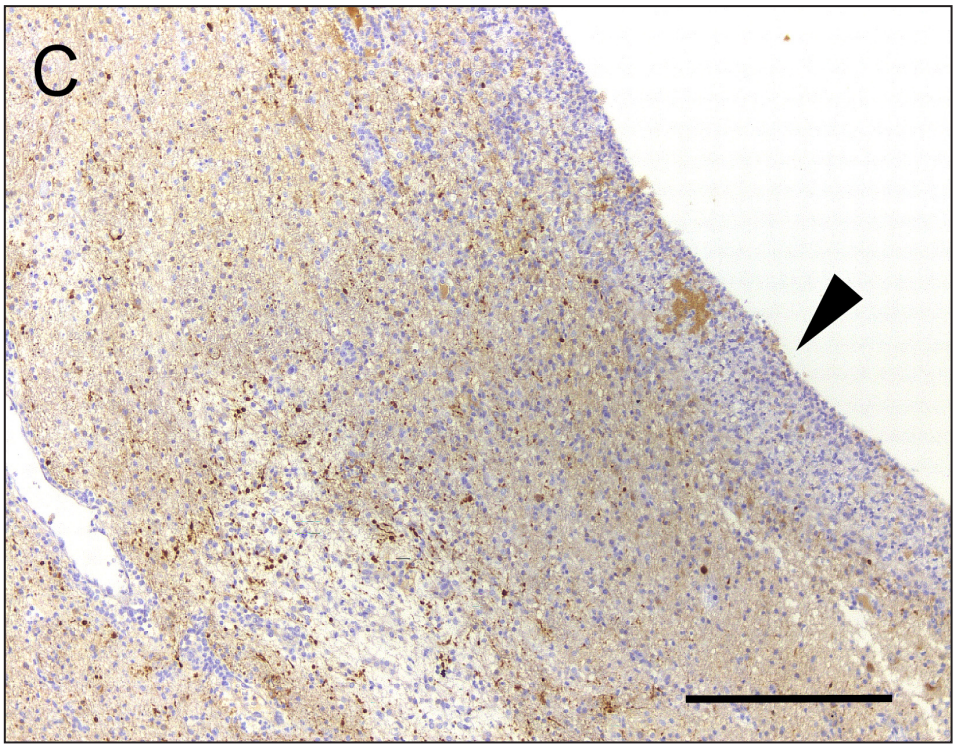


Figure 6.5. (This page and overleaf.) Patterns of axonal injury identified in NZ sea lion pups. **A.** Vascular axonal injury. This pattern is recognised by a 'zig-zag' distribution of immunoreactivity. Bar = 200 μm. **B.** Multifocal axonal injury. Individual swellings, bulbs and varicosities are seen scattered throughout the affected area. Bar = 100 μm.



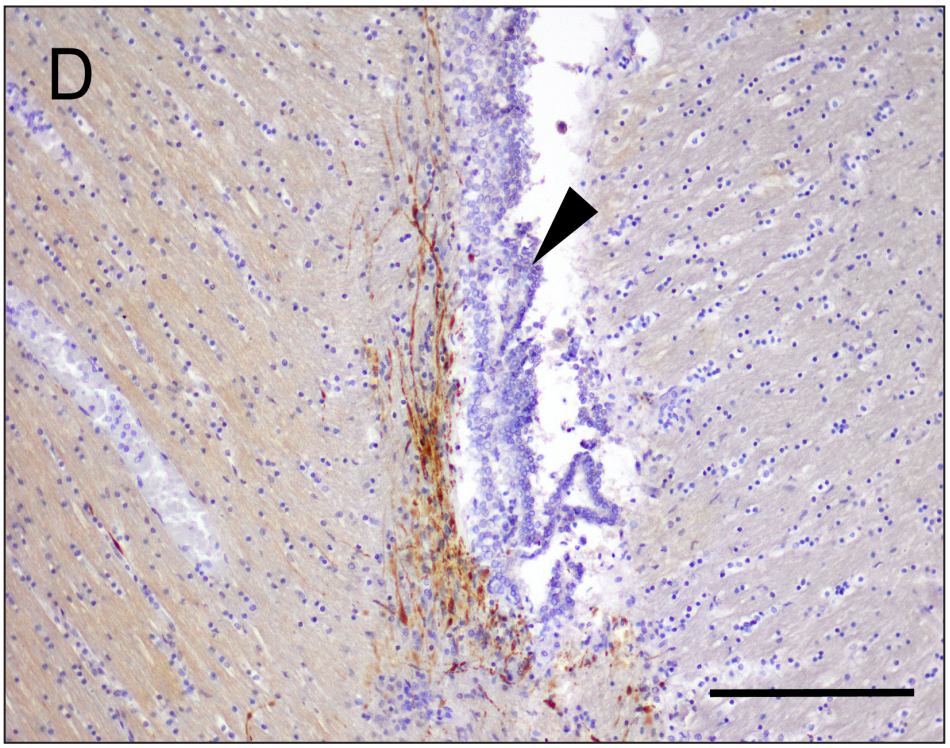


Figure 6.5 cont. **C.** Penumbral immunoreactivity in a sea lion pup with a focal cavitary lesion (abscess). Immunopositive swellings and bulbs are arranged around the abscess, the margin of which is indicated by an arrowhead. Bar = 200 µm. **D.** Periventricular immunoreactivity. Note scattered individual swellings and bulbs at the edge of the third ventricle (indicated by an arrowhead. Bar = 200 µm.

included the parasagittal white matter at all three levels of cortex examined (n = 7), the corpus callosum (n = 6), the brainstem (n = 2) and the cerebellar peduncles (n = 1). Vascular axonal injury was not associated with any specific cause of death.

Eight pups had a periventricular distribution of axonal injury, either alone (n = 2) or in conjunction with more widespread, multifocal immunoreactivity (n = 6). Penumbral axonal injury was present around a cerebral abscess in one pup (E07/08-30Ph), and around small foci of gliosis in two pups (E07/08-22Ph and -41Ph).

Fifteen pups had a multifocal pattern of axonal injury, with total axonal injury scores in this group ranging from 12 to 29. Based on the histological definition of diffuse axonal injury used by Dolinak and Reichard (2006), Geddes *et al.* (2001b) and Adams *et al.* (1989; 1991), (i.e. widespread damage to cerebral white matter including corpus callosum and rostral brainstem), two pups (E07/08-42Ph and -53Ph) had diffuse axonal injury. One of these pups (E07/08-42Ph) had died due to head trauma, while the second (E07/08-53Ph) had meningitis.

The distribution of axonal injury within brains was compared for pups with meningitis and pups with subdural haemorrhage. Pups with diffuse vascular axonal injury were excluded from this section of the analysis, since this can obscure other patterns of injury (Dolinak and Reichard 2006). There was no significant difference in total axonal injury score for pups with meningitis versus those without meningitis, pups with subdural haemorrhage versus those without subdural haemorrhage, or for pups with meningitis versus those with subdural haemorrhage.

Pups with meningitis and pups with subdural haemorrhage were compared to a 'control'

Table 6.2. (*Overleaf.)* Details of βAPP immunoreactivity and cause of death for 2007/08 late season pups. Minimal immunoreactivity is defined as infrequent single positive axons (+) in one or more locations. Cause of death and concurrent syndrome classifications are as determined in Chapter 4. Note that two pups (E07/08-42Ph and -53Ph) had axonal injury in all four identified areas, consistent with a diffuse pattern of axonal injury. Pup E07/08-30Ph also had widespread axonal injury in these areas, but had a focal space-occupying lesion (cerebral abscess) which excludes it from the definition of diffuse axonal injury.

Pup ID	Axonal injury score	Pattern	Cause of death	Cerebellar Peduncles	Brainstem	Corpus callosum	Cerebral white matter
20Ph	26	vascular	infectious	n	n	y	y
21Ph	29	multifocal	starvation	y	y	n	y
22Ph	12	multifocal periventricular	trauma (head)	n	n	y	n
23Ph	16	multifocal	infectious	y	y	n	y
24Ph	6	minimal	infectious	n	n	n	n
25Ph	36	vascular	scavenged	n	n	y	y
26Ph	16	multifocal periventricular	hookworm	n	n	n	y
27Ph	45	vascular	scavenged	n	n	y	y
28Ph	5	minimal	infectious	n	n	n	n
29Ph	6	minimal	hookworm	n	n	n	n
30Ph	22	penumbral periventricular multifocal	infectious	y	y	y	y
31Ph	18	vascular multifocal	infectious	n	y	y	y
32Ph	13	multifocal	infectious	n	y	n	n
33Ph	23	vascular	trauma (head)	n	y	n	y
34Ph	7	minimal	trauma (abdo)	n	n	n	n
35Ph	2	minimal	infectious	n	n	y	y
36Ph	12	multifocal periventricular	infectious	n	y	n	n
37Ph	39	vascular	infectious	y	n	y	y
38Ph	7	minimal	infectious	y	y	n	n
39Ph	15	multifocal	infectious	n	y	n	n
40Ph	13	multifocal periventricular	infectious	y	y	n	y
41Ph	19	multifocal	infectious	y	y	n	y
42Ph	20	multifocal	trauma (head)	y	y	y	y
43Ph	17	multifocal	infectious	n	y	n	n
44Ph	3	minimal	autolysed	n	n	n	n
45Ph	2	minimal	infectious	n	n	n	n
48Ph	4	minimal	infectious	n	n	n	n
49Ph	3	minimal	infectious	n	n	n	n
50Ph	48	vascular	infectious	n	n	y	y
51Ph	2	minimal	infectious	n	n	n	n
52Ph	2	minimal	infectious	n	n	n	n
53Ph	23	multifocal periventricular	infectious	y	y	y	y
54Ph	7	minimal	infectious	n	n	n	n
55Ph	5	minimal	infectious	n	y	n	n
56Ph	25	multifocal	infectious	y	n	n	y
57Ph	2	minimal	hookworm	n	n	n	n

group of pups with no meningitis or subdural haemorrhage. Figures 6.6 and 6.7 show the details of these comparisons. Regions with statistically significant differences in immunoreactivity are described in the figure legends.

Figure 6.8 examines the effect of subdural haemorrhages in pups with meningitis by comparing pups that have meningitis without subdural haemorrhage to pups that have both meningitis and subdural haemorrhage.

Details of axonal injury distribution in pups with haemorrhage of the cerebellar vermis and those without this lesion are shown in Figure 6.9.

Details for individual pups are presented in Appendices 7 (Xara maps for individual pups) and 8 (βAPP scores for neuroanatomical areas).

6.3.2 Assessment of intracranial pressure

No pups had necrosis of either parahippocampal gyrus. Ten pups had haemorrhagic necrosis of the cerebellar vermis, indicative of cerebellar herniation.

In paediatric medicine, brain swelling is diagnosed when the brain weight is more than 10% above the normal range for the patient's age or body size (Reichard *et al.* 2003a), but normal ranges are not known for NZ sea lion pups. The brain weights for pups with vascular axonal injury were not significantly different from those without vascular axonal injury ($p = 0.49$). Assuming that head girth and possibly other parameters (body length, axillary girth, neck girth) might be correlated with age and with brain size, the ratio of brain weight to head girth was calculated for each pup. Similar ratios were calculated for body length, axillary girth, neck girth and skull weight. Comparisons of these ratios for pups with and without vascular axonal injury did not reveal any significant differences. Scatterplots and regression analyses of these

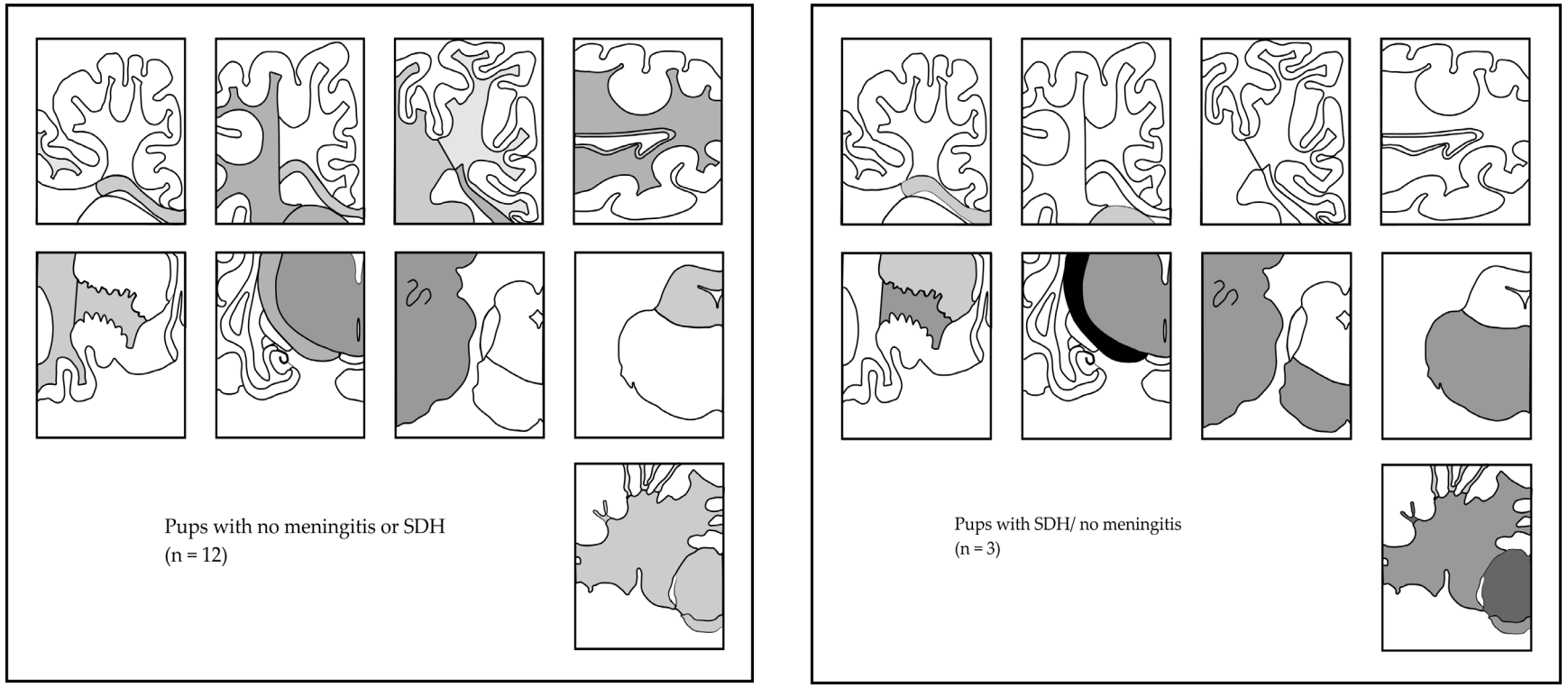


Figure 6.6. Relative distribution of axonal injury for pups with subdural haemorrhage compared with pups with neither meningitis nor subdural haemorrhage. Pups with subdural haemorrhage had higher scores in the perithalamic white matter/corona radiata region than those with no meningitis or subdural haemorrhage ($p = 0.05$).

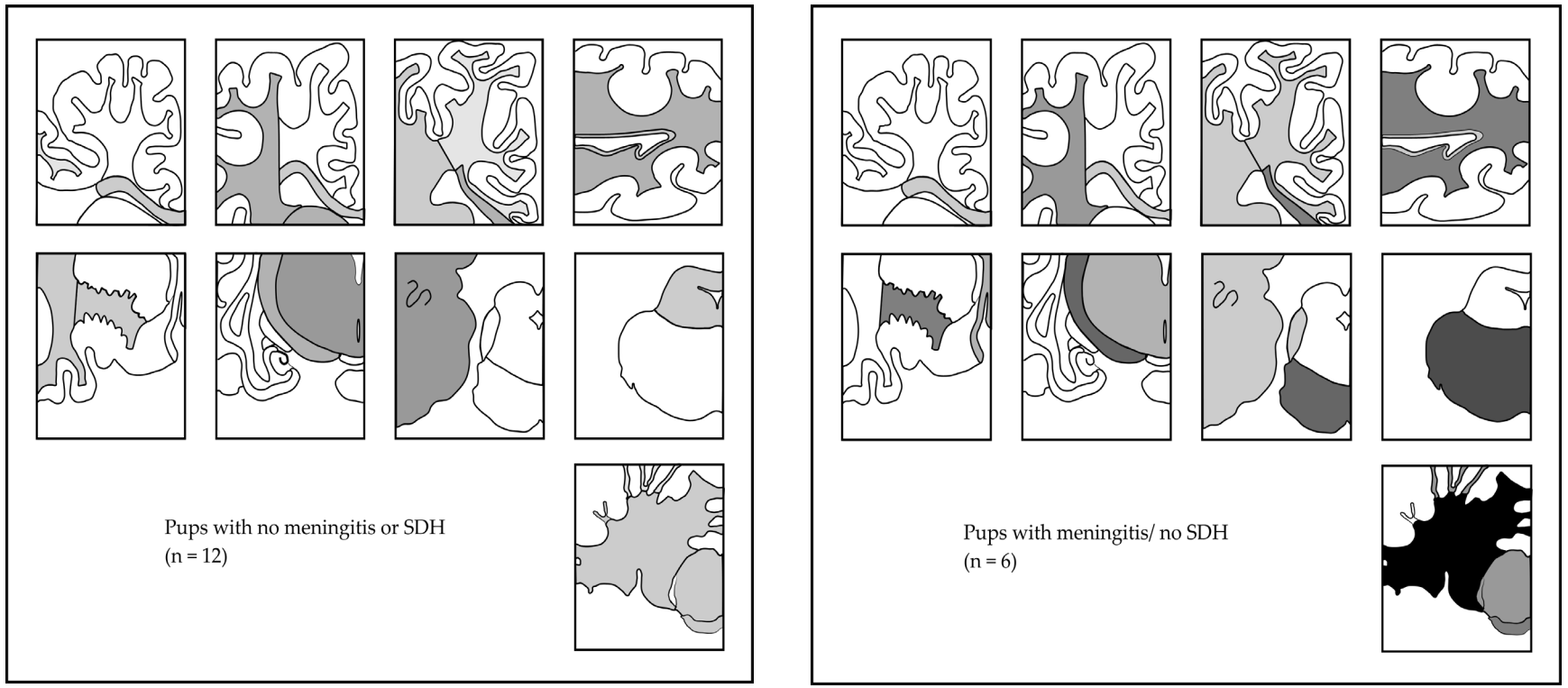


Figure 6.7. Relative distribution of axonal injury for pups with meningitis compared with pups with neither meningitis nor subdural haemorrhage. Pups with meningitis had significantly higher scores in the cerebellar peduncles ($p < 0.01$) and higher total axonal injury scores ($p = 0.04$) than in pups with neither meningitis nor subdural haemorrhage.

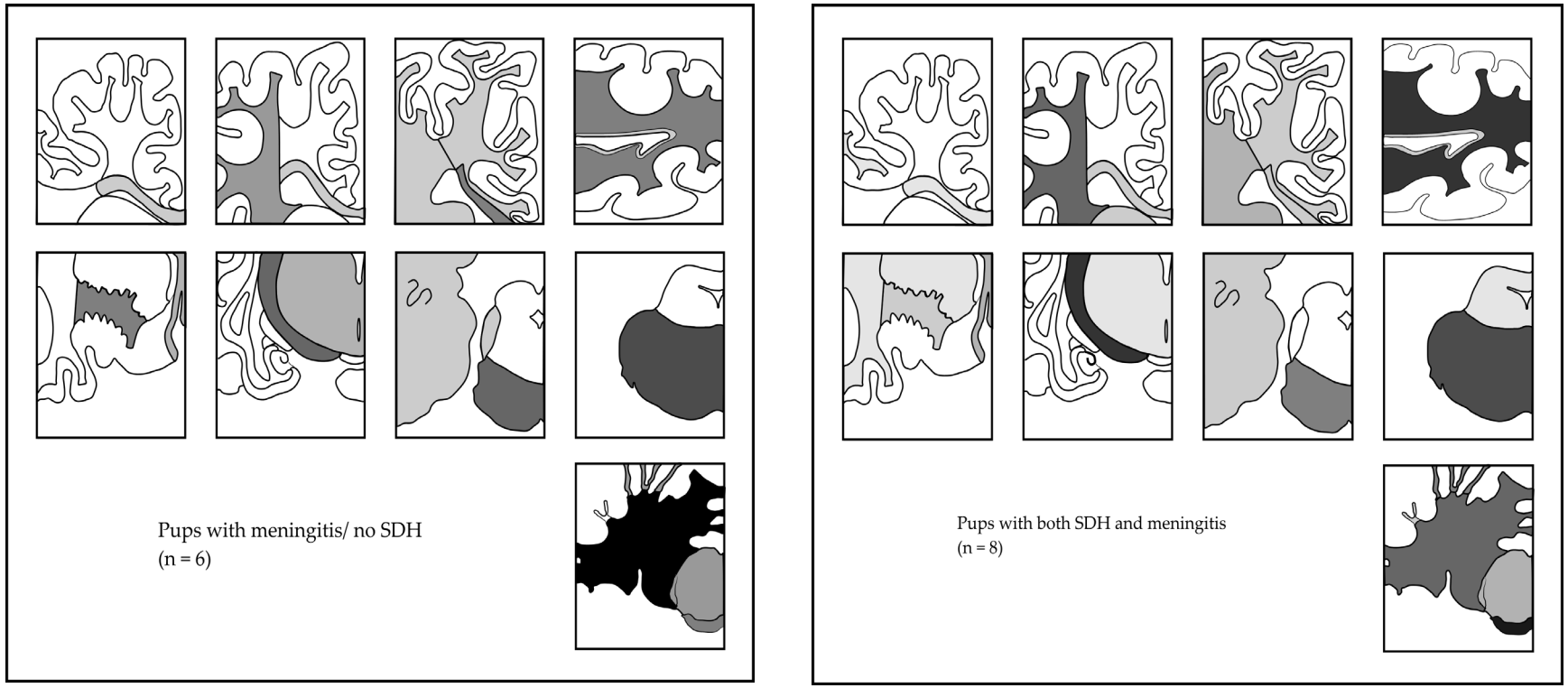


Figure 6.8. Relative distribution of axonal injury for pups with meningitis but no subdural haemorrhage, compared with pups that have both meningitis and subdural haemorrhage. There were no significant differences between axonal injury scores for any neuroanatomical regions, and no difference between total axonal injury scores for the two groups.

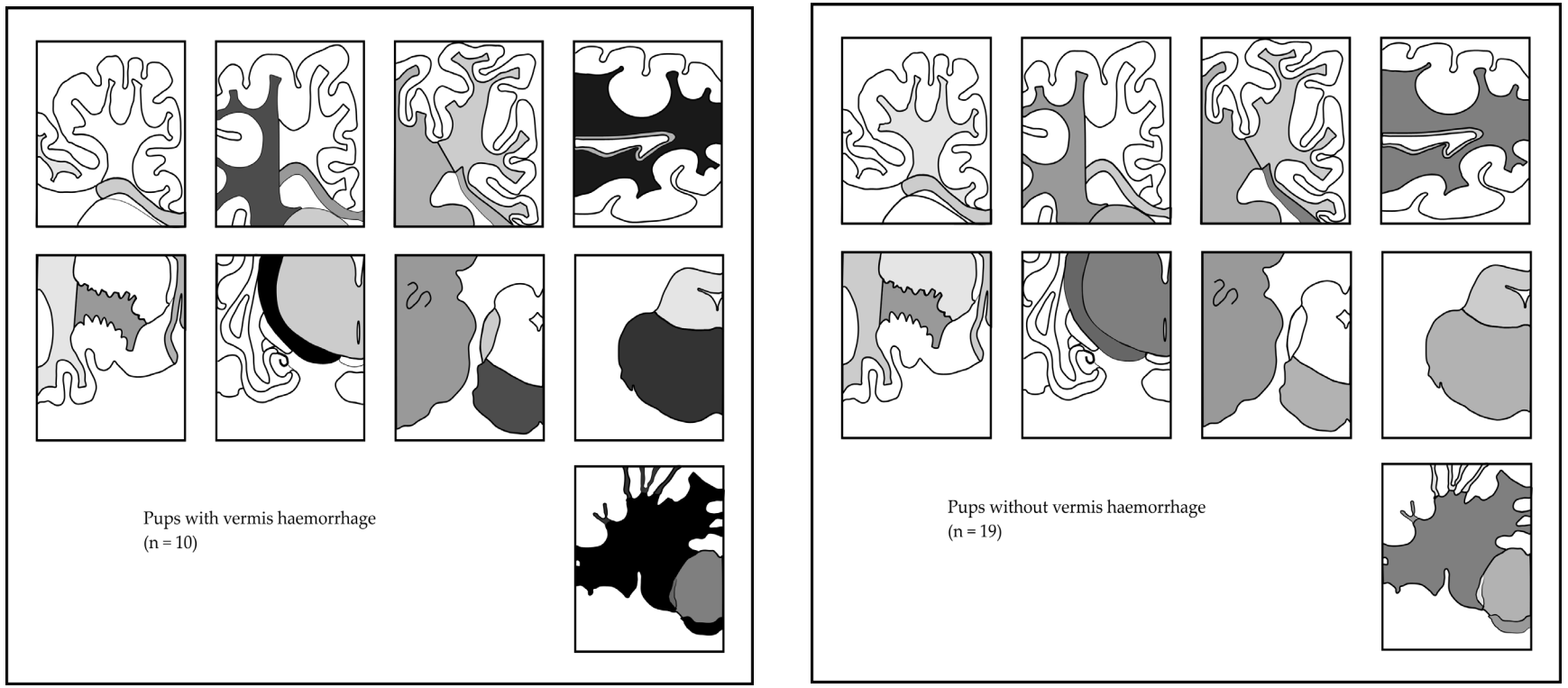


Figure 6.9. Relative distribution of axonal injury for pups with haemorrhage of the cerebellar vermis (left) and pups without haemorrhage of the vermis (right). The mean total axonal injury score was significantly higher for pups with vermis haemorrhage compared with those with no vermis haemorrhage ($p = 0.05$). Pups with vermis haemorrhage also had significantly more axonal injury in the ventral pons ($p < 0.01$), ventral medulla ($p = 0.03$), and central foliar white matter of the cerebellum ($p < 0.01$).

data also showed no consistent relationship.

6.3.3 Assessment of hypoglycaemia and hypoxia-ischaemia

Glucose levels in aqueous humor

Assays of aqueous humour glucose levels were conducted for 22 pups. Levels ranged from 0 to 1.68 mmol/l, with most pups (13/22) having no detectable glucose. There were no statistically significant associations between glucose level and cause of death or pattern or quantity of axonal injury.

Histological examination (H&E)

Acidophilic neurons (Figure 6.10) were present in the frontal and/or occipital cortex of 34/36 pups (94%). In one pup (3%) these were mainly in the superficial layer, in ten pups (28%) acidophilic neurons were found in all layers, and in 23 (64%) they were mainly in the mid and deep layers. Fifteen pups (42%) had no acidophilic neurons in the hippocampus, while in 12 others (33%) there were rare individual acidophilic neurons. A further seven pups (19%) had several acidophilic neurons in every 10x field, although the location varied. Only one of these pups had involvement of the dentate gyrus. 23/36 pups (64%) had acidophilic Purkinje cells; scores were not significantly different between groups. Overall, no pups had a distribution of acidophilic neurons that was typical of either hypoglycaemia or hypoxia-ischaemia as described for human patients.

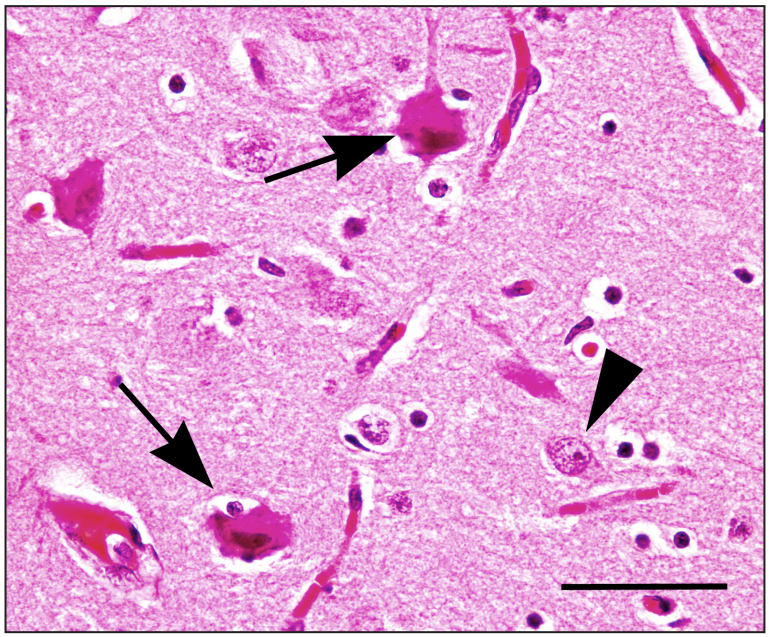


Figure 6.10. Microscopic appearance of acidophilic neurons, with shrunken angular borders of the neuronal soma, increased uptake of eosin, homogenisation of Nissl substance and shrunken densely stained nuclei with no apparent nucoleoli. The arrowhead indicates a normal neuron. Bar = 100 µm. (H&E)

6.3.4 Eyes

Eyes were available from 33/36 animals; 20 had two eyes available, and 13 had one (53 eyes in total). In the remaining three pups, both eyes had been scavenged. In all eyes, the retinal pigmented epithelial cells were strongly immunopositive for βAPP, as were many retinal ganglion cells and neurons in the outer nuclear layer. Anti-βAPP immunopositive axons were present in 33/53 available optic nerves, affecting 24 pups in total. Immunopositive axons were mostly in the form of swellings, bulbs and varicosities, although a few pups had granular to punctuate immunoreactivity (n = 6) or immunoreactive lengths (n = 2). Immunopositive axons were found at the lamina cribrosa (n=9), from 2 mm caudal to the lamina cribrosa with increasing intensity near the lamina cribrosa (n = 8), near the optic disc (n=7) or at the periphery of the nerve just caudal to the lamina cribrosa (n=1). Two pups had a vascular pattern of immunoreactivity

(E07/08-20Ph and -37Ph). Figure 6.11 shows examples of immunoreactivity in the optic nerves.

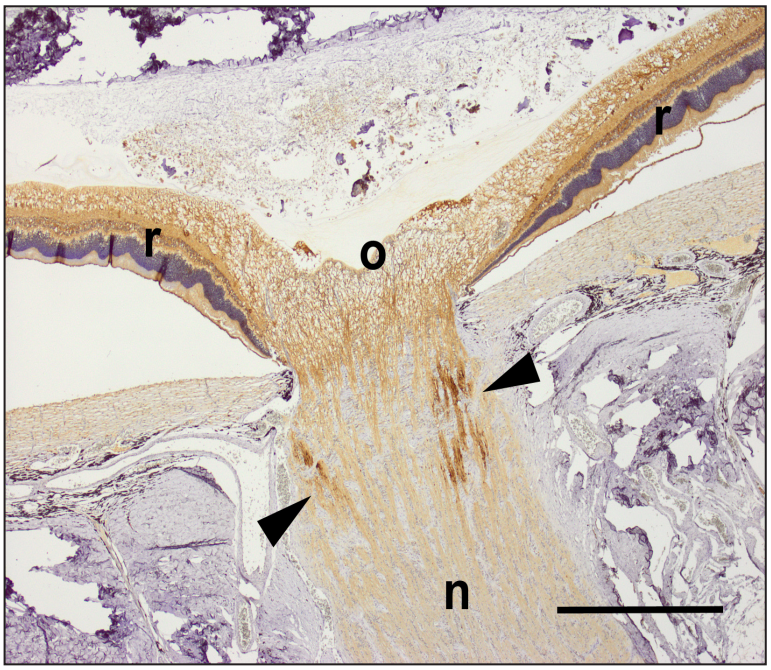


Figure 6.11. Groups of immunopositive axons (arrowheads) in the optic papilla of a sea lion pup (E07/08-20Ph). Immunopositive axons occur in distinct clusters characteristic of a vascular pattern of injury. (r = retina; n = optic nerve; o = optic papilla) Bar = 1 mm. (Anti-βAPP immunohistochemistry.)

Of the 20 pups with two available optic nerves, immunoreactivity was bilateral in eight, unilateral in six, and absent in six. In all pups with unilateral immunoreactivity, the stained nerve was scored as + and in the bilaterally stained nerves, only one pair differed by more than one score level. When comparing groups, the average axonal injury score for optic nerves was not significantly different for pups with meningitis versus those without meningitis, or for those with subdural haemorrhage versus those without subdural haemorrhage, but was significantly higher in pups with vascular axonal injury compared with those without vascular axonal injury ($p = 0.05$).

6.4 Discussion

The key aims of this chapter were to determine whether anti-βAPP immunohistochemistry was able to identify traumatic brain injury in pups, and whether the technique could detect injury caused by shaking. Anti-βAPP immunoreactivity highlighted some normal brain structures as well as demonstrating axonal injury in the form of variably distributed axonal swellings, bulbs, varicosities and immunoreactive lengths. In order to accurately attribute axonal injury to trauma, however, other causes of axonal injury must first be excluded. In adult human patients, the presence of macroscopic haemorrhages in the corpus callosum and pons is considered indicative of clinically significant traumatic brain injury, and is characteristic of grade III traumatic diffuse axonal injury. Although no pups had these focal haemorrhagic lesions, Dolinak and Reichard (2006) note that they rarely occur in infants and children. In addition, using a pig model of inertia injury, Smith *et al.* (2000) were able to induce coma and widespread axonal injury, but did not observe gross haemorrhage in the corpus callosum or pons. Accordingly, the absence of such haemorrhages in the pups does not exclude the possibility that clinically significant or even fatal traumatic brain injury was present.

In a number of well-controlled human studies, the use of anti-βAPP immunohistochemistry allowed reliable distinctions to be made between patients with traumatic brain injury and those without traumatic brain injury (Geddes *et al.* 2001b; Reichard *et al.* 2005; Dolinak and Reichard 2006). While the authors of these studies found little or no axonal anti-βAPP immunoreactivity in their control cases, all the sea lion pups presented here had at least some axonal immunoreactivity. Although it is theoretically possible that all of this axonal injury was traumatic, it is likely that non-traumatic factors were also involved. The pups in the study would have experienced agonal phases of varying durations, during which hypoglycaemia and hypoxia-ischaemia could have resulted in dysfunction of axonal transport, and therefore in

positive immunoreactivity for βAPP. Fifteen pups had a multifocal pattern of axonal injury, and two of these had a distribution and intensity of axonal injury consistent with traumatic diffuse axonal injury. One of these pups (E07/08-53Ph) had died due to *K. pneumoniae* septicaemia and meningitis, with no evidence of a traumatic injury to the brain. The presence of widespread axonal injury in locations similar to those seen in human diffuse traumatic axonal injury, therefore, is not exclusively associated with traumatic brain injury in sea lion pups.

In human medicine, differentiation between trauma, hypoxia-ischaemia, and hypoglycaemia as causes of axonal injury can be facilitated by assessment of H&E sections, analysis of gross lesions, and consideration of the clinical history (Dolinak *et al.* 2000b; Geddes *et al.* 2001b; Reichard *et al.* 2005; Dolinak and Reichard 2006). Although obviously the latter is not possible in this sea lion pup study, other ancillary investigations were used to aid interpretation of the βAPP findings, including measurement of glucose levels in aqueous humour to assess the role played by hypoglycaemia. Possible causes of hypoglycaemia relevant to sea lion pups include starvation and sepsis (Miller *et al.* 1980; Haymond and Pagliara 1983; Malouf and Brust 1985; Boyne and Benjamin 2003). Starvation is known to be a factor in NZ sea lion pup mortality (Castinel *et al.* 2007), and, as discussed in Chapter Five, sepsis was present in 25/36 of the pups in this study, suggesting that hypoglycaemia could be involved in axonal injury. Glucose levels in fasting pinnipeds appear to be quite variable, however, and are generally higher than those of other fasted species, although levels tend to decline with prolonged fasting (Castellini and Costa 1990; Rea *et al.* 2000; Arnould *et al.* 2001). In the current study, no statistical assocations were apparent between glucose levels and axonal injury, thus it was not possible to directly assess the role of hypoglycaemia.

In two human studies using anti-βAPP immunohistochemistry, cases of hypoglycaemia either mimicked grade I diffuse traumatic axonal injury (see Chapter Two) or, more commonly, resulted in a vascular axonal injury pattern as a result of raised intracranial

pressure. In those studies review of H&E stained slides enabled correct identification of hypoglycaemia as the cause of axonal pathology in all cases (Dolinak *et al.* 2000b; Reichard *et al.* 2005). While no sea lion pups in this study had acidophilic neuron distributions typical of hypoglycaemia, the predilection sites for hypoglycaemic damage have not been identified in this species, and may not be the same as for humans.

Hypoxic-ischaemic injury is another possible cause of axonal injury. Seven sea lion pups had a vascular pattern of axonal injury, which is believed to be caused by global hypoxia-ischaemia associated with raised intracranial pressure (Dolinak *et al.* 2000a; Blumbergs *et al.* 2008). Post mortem evidence of raised intracranial pressure in humans includes brain swelling, herniation, and necrosis of the parahippocampal gyrus. None of the seven pups with vascular axonal injury had necrosis of the parahippocampal gyrus, but the validity of this lesion as an indicator of intracranial pressure in animals has not been investigated. When pup brain weights were analysed, those with vascular axonal injury did not have significantly heavier brains than those without. While this could mean that these pup brains were not swollen, it is not possible to make accurate assessments, as normal age-specific weight ranges are not known for this species. In addition, Reichard *et al.* (2003b) note that detection of brain swelling in the young is inherently difficult.

Haemorrhagic necrosis of the cerebellar vermis, indicating herniation into the foramen magnum, was present in ten of the 36 pups in this study, but none of these pups had vascular axonal injury. Although this may seem counter-intuitive, Blumbergs *et al.* (2008) noted that herniation of the brain can occur during 'spatial decompensation', before there is an appreciable increase in intracranial pressure. Conversely, where intracranial pressure increases very rapidly, there may be little or no brain herniation (Blumbergs *et al.* 2008). The absence of herniation in the pups with vascular axonal injury, therefore, suggests either that raised intracranial pressure developed rapidly, or that axonal injury was a result of global hypoxia-ischaemia alone. Reichard *et al.*

(2005) suggested that anti-βAPP immunoreactivity may be an early marker of raised intracranial pressure, appearing before macroscopic evidence of herniation.

Causes of raised intracranial pressure include intracranial space-occupying lesions (including inflammatory infiltrate, and parenchymal and meningeal haemorrhages), cerebral oedema, increased intracranial blood volume, increased intra-abdominal and intra-thoracic pressure, and obstruction of CSF outflow (Bloomfield *et al.* 1997; Dolinak *et al.* 2000a). Of the seven pups with vascular axonal injury, three had syndromes associated with increased intracranial pressure: two had meningitis and one had been crushed. No cause of raised intracranial pressure was apparent in the remaining four pups.

The distribution of axonal injury in sea lion pups with herniation of the cerebellar vermis (see Figure 6.7) is consistent with interruption of axonal transport in areas that would be affected by compression of the herniating brain, i.e. the occipital lobe, cerebellar peduncles, ventral brainstem and corona radiata. This indicates that much of this axonal injury is likely to be due to hypoxia-ischaemia rather than directly to trauma.

Recently, meningitis has also been identified as a cause of axonal injury (Nau *et al.* 2004; Gerber *et al.* 2009), although much of this injury is probably mediated by ischaemia, either focal (e.g. thrombosis of parenchymal vessels) or diffuse (e.g. vascular compromise due to raised intracranial pressure). For the 16 sea lion pups with meningitis, total axonal injury scores ranged from 3 – 39, and were not significantly different from scores in pups without meningitis. The distribution of axonal injury in pups with meningitis was different from that in unaffected pups, however, with significantly more axonal injury in the cerebellar peduncles and ventral brainstem. Due to the low number of pups with traumatic brain injury in the absence of meningitis (n=3), it is unclear whether these differences could reliably be used to differentiate pups with meningitis from those with traumatic brain injury.

The second fundamental question posed in this chapter was whether anti-βAPP immunohistochemistry would be able to identify axonal injury due to shaking. Predicting the likely distribution of axonal injury in sea lion pups that have been subjected to injurious shaking is difficult, as it is not possible to conclusively determine whether their neural tissues will respond to insults in a similar way to human infants, children or adults. In humans, the gross and histological findings in inertial brain damage vary with patient age and mechanism of injury. In 'shaken' human infants (< 9 months old), the current consensus seems to be that most axonal injury found at post mortem is vascular in origin rather than traumatic (Geddes and Whitwell 2003a; Reichard *et al.* 2005; Dolinak and Reichard 2006; Finnie *et al.* 2010). Diffuse traumatic axonal injury as a result of inflicted head injury in this age group, and in toddler age children, is rare (Geddes *et al.* 2001a; Reichard *et al.* 2005; Dolinak and Reichard 2006), although lesser degrees of traumatic axonal injury are seen more frequently in toddlers (Geddes *et al.* 2001a). In both age groups, axonal injury within the cervical spinal cord and brainstem is a common finding, suggesting that the primary site of damage is the cervicospinal junction, and that encephalopathy is secondary to apnoea-induced hypoxic-ischaemic injury. Pure (manual) shaking injury has rarely been reported in adult humans (Pounder 1997; Carrigan et al. 2000), and the one paper that reported diffuse axonal injury in a shaken adult (Pounder 1997) was more likely to have been vascular axonal injury (Geddes and Whitwell 2003b).

Overall, these findings suggest that if a sea lion pup had sustained clinically significant shaking injury, then anti-βAPP immunoreactivity would likely indicate traumatic axonal injury in the brainstem and cervical spinal cord, possibly with concurrent diffuse vascular axonal injury. Fifteen pups in this series had axonal injury in the brainstem scored as either ++ or +++, and of these 15, only two had a diffuse pattern of vascular axonal injury (E07/08-31Ph and -33Ph). Neither of these pups had immunopositive axons in the spinal cord.

The possibility that meningitis could predispose pups to shaking-induced subdural haemorrhages was introduced in Chapter Five. If this were true, then pups with both subdural haemorrhage and meningitis would have increased amounts of axonal injury in the brainstem and an increased prevalence of vascular axonal injury compared to pups with meningitis only. This did not prove to be the case, hence there is no evidence that shaking is involved in creation of subdural haemorrhages in pups with meningitis.

In an ovine model of shaken baby syndrome, Finnie *et al.* (2010) found axonal injury in the cervical spinal cord, with positive immunoreactivity of neuronal soma in the cerebral cortex but no axonal injury in the brain. Control lambs had no neuronal immunoreactivity. This is consistent with an upregulation of βAPP production in neurons in response to trauma, as shown by Van den Heuvel *et al.* (2000) using an ovine head impact model. It follows, therefore, that sea lion pups that had clinically significant head injuries, even in cases with short survival times, might have an increased frequency of immunopositive neurons compared with those without brain injuries. Assessment of immunopositive neurons in the cerebral cortex, hippocampus and brainstem nuclei in sea lion pups, however, showed a high frequency of immunoreactivity in all pups (> 75% of neurons), suggesting that upregulation of βAPP synthesis is non-specific, and occurs in many conditions to include agonal hypoxia and inflammatory disease.

Reichard *et al.* (2004) investigated axonal injury in optic nerves of human infants with inflicted head injury, using anti-βAPP immunohistochemistry. They found immunoreactive optic nerve axons in 7/19 (37%) cases of blunt force head injury, and in 1/13 (8%) cases without head injury. All cases with axonal injury, irrespective of cause of death, also had both global hypoxic-ischaemic injury and brain swelling; no immunopositive axons were found in cases without both of these changes. Conversely, eight cases (seven traumatic and one suffocation) that had both global hypoxia-ischaemia and brain swelling did not have immunopositive axons in either optic nerve. Since

all eight had survival times greater than two hours, this indicates that axonal injury is not a universal consequence of global hypoxia-ischaemia with brain swelling. In individuals with head trauma, positive axons were scattered across the nerve between the lamina cribrosa and optic nerve head, while their near-drowning case had a well demarcated focus of immunoreactivity of almost all axons in one location at the optic nerve head. This latter case was interpreted as a vascular pattern. Gleckman *et al.* (2000) conducted a similar study, finding axonal injury in the optic nerves of 6/8 (75%) infants that had reportedly been shaken, with or without impact, compared with 0/3 control cases (non-traumatic deaths). They also included one case of head impact without shaking, where no optic nerve axonal injury was detected. Brain swelling and global hypoxia-ischaemia were not evaluated in these cases. Both sets of authors concluded that there was an association between optic nerve axonal injury and trauma.

Anti-βAPP immunoreactive axons were present in the optic nerves of 24 pups in this study and optic nerve axonal injury scores were significantly higher in pups with a vascular pattern of axonal injury. This suggests an association between hypoxic-ischaemic injury to the brain and the presence of axonal injury in the optic nerve, rather than a direct traumatic effect.

6.5 Summary

Although the use of anti-βAPP immunohistochemistry identified axonal injury in a considerable proportion of NZ sea lion pups, no single pattern of distribution of injury emerged as being distinctive of trauma. No pups had gross and immunohistochemical changes typical of grade III traumatic diffuse axonal injury in humans, and only one of the two pups that had axonal injury fitting the human histological criteria had convincing lesions of traumatic brain injury. Conversely, vascular axonal injury was comparatively frequent, indicating raised intracranial pressure during life. No pups had concurrent axonal injury in the spinal cord and brainstem that might suggest shaking as a cause

of the raised intracranial pressure. In addition, there was no evidence that shaking played a role in the development of subdural haemorrhages in pups with meningitis.

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Chapter Seven

MAP2 as a marker of ischaemia

7.1 Introduction

Microtubule-associated proteins (MAPs) are a group of cytoskeletal proteins that play a variety of roles in cytoskeletal integrity. MAP2 is a neuronal protein that is known to stabilise dendritic microtubules as well as having other, less well-characterised functions such as microtubule-associated transport (Dehmelt and Halpain 2005). Breakdown of MAP2 occurs in response to both hypoxic-ischaemic and traumatic brain injury, and decreased immunohistochemical expression of MAP2 has been used as a marker of these processes in several species (Kitagawa *et al.* 1989; Posmantur *et al.* 1996; Manavis *et al.* 1999; Kuhn *et al.* 2005; Lingwood *et al.* 2008). Anti-βAPP immunohistochemical

staining of pup brains from the 2007/08 season showed that 7/36 pups had a widespread vascular pattern of axonal injury, suggestive of hypoxic-ischaemic injury. The aim of this chapter is to determine whether anti-MAP2 immunohistochemistry can be used to investigate global hypoxia-ischaemia in NZ sea lion pups.

7.2 Immunohistochemical validation and optimisation of MAP2 in NZ sea lion brain

Marker validation and optimisation protocols were conducted using a monoclonal anti-MAP2 antibody produced in mice (clone HM-2; Sigma-Aldrich, Auckland, New Zealand). As this antibody is known to cross-react with rat MAP2, sections of rat hippocampus, cortex and cerebellum were used for optimisation. Paraffin embedded rat brain sections were cut at 5μm, mounted on positively charged glass slides, and de-waxed. Slides were then immersed in citrate buffer at pH 6.0, and subjected to 10 minutes at pressure in a pressure cooker, cooled for 10 minutes, then rinsed in TBS with 0.025% Triton-X (pH 7.6) for 5 minutes. Endogenous peroxidases were blocked by incubation in 0.3% hydrogen peroxide in TBS for 30 minutes, followed by incubation in 10% normal horse serum for 30 minutes, then incubation for one hour in primary antibody diluted with 1% bovine serum albumin in TBS at 1:500, 1:1,000 and 1:2,000. After two rinses in TBS, all slides were incubated with biotinylated universal secondary antibody (Vector Laboratories, Burlingame, California, USA) for 30 minutes, rinsed in TBS, incubated with ABC reagent (Vector Laboratories) for 20 minutes, and then rinsed again in TBS. Visualisation was conducted after incubation in DAB chromogen for 4 minutes, producing a brown reaction product. Sections of cortex and cerebellum from a dog and a NZ sea lion were run simultaneously. Negative isotype controls were run using an irrelevant antibody (anti-IBDV monoclonal antibody produced in mice, CSIRO/IBDV/17-82; TropBio Pty Ltd, Townsville, Queensland, Australia) to check specificity of primary antibody. Negative antibody controls were run by omitting primary antibody

to check specificity of secondary antibody binding. Evaluation of immunoreactivity showed that optimal positive reaction with minimal background reaction was achieved with a 1:1,000 dilution of primary antibody. Dendrites and neuronal soma were immunopositive on rat, dog and sea lion sections. No immunoreactivity was present on negative control slides.

7.3 Pilot Study: Effect of post mortem interval on MAP2 expression

While breakdown of cytoskeletal proteins can indicate ante mortem damage, autolysis can cause similar changes and in some species loss of MAP2 expression has been shown to occur with prolonged post mortem intervals (Irving *et al.* 1997; Kitamura *et al.* 2005). The anatomical location, time of onset and severity of decreased expression varies between studies, and could be affected by age, species, and post mortem conditions such as cooling rates (Schwab *et al.* 1994; Kitamura *et al.* 2005). The pilot trial discussed in this section was designed to evaluate MAP2 expression at several post mortem times in the brain of a dog.

7.3.1 Materials and Methods

A 12-year-old female neutered beagle was euthanised and the brain removed within 30 minutes of death. Sections of frontal cortex and hippocampus were taken from each hemisphere, and a single section of cerebellum was collected. All were placed into cassettes and then immersed in 10% neutral buffered formalin. The remainder of the brain was placed in a polystyrene container with a frozen ice-pack. At 12 hours, sections of frontal cortex, hippocampus, and cerebellum were taken immediately adjacent to the initial sample sites, placed in cassettes and stored in formalin as previously. These locations were chosen as being the areas known to be involved in hypoxic-ischaemic or traumatic brain injury in other species (Auer and Siesjo 1988; Kitagawa *et al.* 1989;

Dugan and Kim-Han 2006). The left side of the brain was used for the cortex and hippocampal sections. At 24 hours, tissue was collected from the right frontal cortex, the right hippocampus, and the cerebellum. These were again fixed in formalin. All cassettes were then left in formalin for a further 48 hours then processed into paraffin for sectioning.

Immunohistochemistry

Immunohistochemistry was carried out using a mouse monoclonal anti-MAP2 antibody from Sigma-Aldrich (M9942) as described above.

Microscopic evaluation and image analysis.

All sections were assessed using an Olympus BS51 light microscope. Digital images were captured on an Olympus BX51 with attached Olympus XC50 digital camera. Exposure levels were locked to ensure standardised light conditions for photomicrographs. Images were evaluated using ImageJ software (NIH). Each image was converted to 8 bit greyscale format, then the threshold was adjusted so that each converted image consisted only of labelled (black) and unlabelled (white) pixels. Relevant areas of interest (e.g. molecular layer of the dentate gyrus) were outlined using the polygon tool, and the percentage area showing immunoreactivity was measured using the 'analyse' function of Image J. For sections of frontal cortex, these measurements were made at the base of two sulci, and the areas averaged. Hippocampal sections were divided into four parts: the molecular layer of the dentate gyrus, the hilus/CA4 region, the CA3 region and the CA1 region. The results for each area were recorded as the area score. Area scores were added to attain a hippocampal score and a total MAP2 score.

Area scores were plotted against post mortem interval on scatterplots to detect trends over time.

Cerebellar sections were further examined semi-quantitatively, by counting 3 x 100 Purkinje cells in non-overlapping fields, and determining the average percentage of immunopositive Purkinje cells ('Purkinje cell count').

7.3.2 Results

All slides collected at 0 hrs had strong immunoreactivity of dendrites and neuronal soma in grey matter, with no detectable immunoreactivity in white matter. Negative controls showed no immunoreactivity.

Qualitative analysis

On subgross examination of sections of frontal cortex there was a distinct decrease in immunoreactivity over time. Microscopic examination of sections collected at 0 hours showed a faint laminar band of decreased immunoreactivity in the mid layers of the cortex, which became more pronounced with increasing post mortem interval. The severity varied between gyri, and was more prominent in the right cortex. This change was visible on subgross examination of the 24 hour sections as a continuous pale cortical ribbon, representing laminar loss of immunoreactivity ('pale cortical ribbon') (Figure 7.1A).

Consistent orientation of hippocampal sections was impossible due to friability of the brain at 12 and 24 hours, and the resulting variation in plane of section at each time interval made direct comparisons difficult. At 0 hours the majority of CA4, CA3 and CA2 neurons were faintly to moderately immunoreactive, whereas CA1 neurons were more densely immunoreactive. CA1 neurons on the right hippocampus showed fainter immunoreactivity than those on the left. At 24 hours there was slightly increased neuronal immunoreactivity throughout the hippocampus, with a concurrent mild decrease in dendritic immunoreactivity.

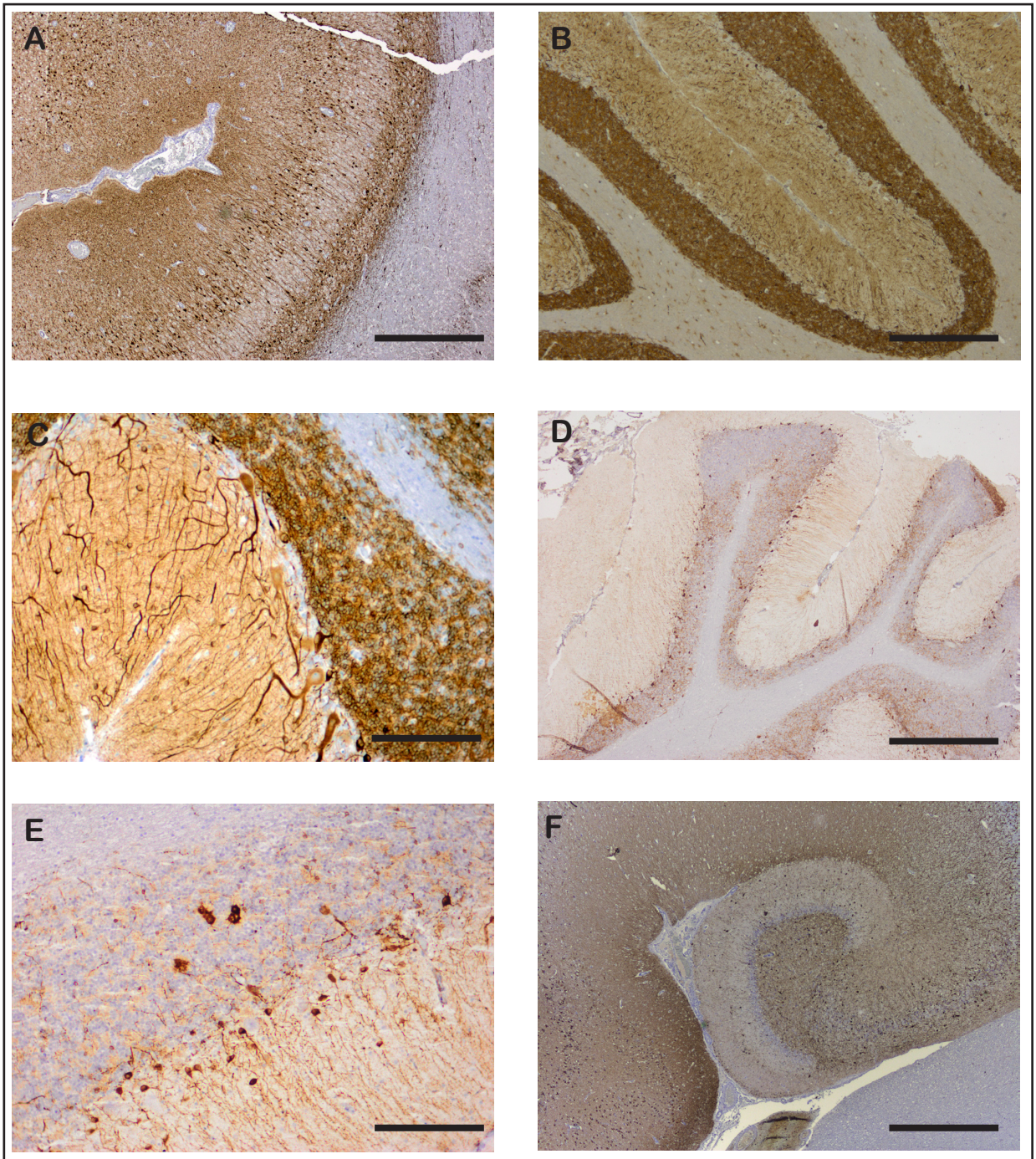

Figure 7.1. Anti-MAP2 immunostaining in sections of brain from a dog (A-E) and a subadult male Northern fur seal (F). **A.** Loss of dendritic staining in the mid layers of the cerebral cortex, creating a pale layer (pale laminar cortical ribbon). Bar = 100µm. **B.** Section of cerebellum from a dog at 0 hours post mortem interval. Note intense staining of the granular layer. Bar = 200µm. **C.** Higher magnification of cerebellum shown in 7.1B (0 hours post mortem interval). Bar = 100µm. **D.** Section of cerebellum from a dog at 24 hours post mortem interval. There is loss of immunopositive staining in the granular layer, and increased intensity of staining of some Purkinje cells. Bar = 200µm. **E.** Higher magnification of cerebellum shown in 7.1D. Note intensely immunostained neurons with loss of dendritic immunostaining in the granular layer and at the deep aspect of the molecular layer. Bar = 100µm. **F.** Hippocampus from a subadult male fur seal showing faint immunostaining of the dentate gyrus with more pronounced staining in the hilus and CA3 regions. Bar = 1mm.

The most marked change in the cerebellum over time was a loss of immunoreactivity in the granular layer. At 0 hours most Purkinje cell soma were faintly to moderately immunoreactive, and proximal dendrites were strongly immunoreactive, with even, dark immunoreactivity throughout the granular layer (Figures 7.1B and C). At 24 hours the granular layer was almost devoid of immunoreactivity, with only occasional faint lightly stained patches. The majority of Purkinje cells were unstained, with a few intensely immunopositive cells left, along with scattered dark-staining neurons in the molecular layer (Figures 7.1D and E).

Quantitative analysis

Analysis of scatterplots showed that all MAP2 scores decreased with increasing post mortem interval. The only area for which this trend was statistically significant was CA3, with a high proportion of the variability in anti-MAP2 immunoreactivity being explained by the prolonged post mortem interval ($p = 0.035$ and R-sq = 75.8%). Average Purkinje cell counts also decreased over time (Table 7.1).

PMI (hrs)	Average Purkinje cell count
0	93
12	43
24	49

Table 7.1. Counts of the percentage of Purkinje cells immunopositive for MAP2 antigen in dog brain tissue collected at differing post mortem intervals. PMI = post mortem interval; hrs = hours.

7.3.3 Analysis and conclusions

While this experiment demonstrated loss of MAP2 expression over a 24 hour post mortem interval, there were several limitations of this experimental approach. First, although it was useful to eliminate inter-animal variability by assessing change over time in one brain, serial sectioning of increasingly friable tissue as the post mortem interval increased made direct comparisons difficult, particularly for the hippocampus. Secondly, while the trial was directed toward identifying any trends rather than accurate

quantification of such trends, the use of only one animal is an obvious disadvantage. Thirdly, earlier studies indicate (Schwab *et al.* 1994; Lingwood *et al.* 2008) that some changes in MAP2 expression may be species dependent, so the changes found in this experiment may not be relevant to NZ sea lions. Finally, the post mortem conditions this brain was subjected to do not replicate those which NZ sea lion pups would be exposed to, particularly in terms of temperature.

In order to address some of these issues, a second experiment was designed, using Northern fur seals collected on St Paul, Pribilof Islands, Alaska, to more closely approximate the field conditions under which NZ sea lion pups exist, and to increase the sample size.

7.4 Effect of post mortem interval in northern fur seals

7.4.1 Materials and methods

Brains were sourced from subadult male Northern fur seals that were killed as part of the annual Aleut harvest. All tissues were collected under permit (National Marine Mammal Laboratories permit no. 13583). Fur seals were killed by acute blunt trauma to the head, and none had any discernable survival time. The heads were removed and kept outdoors at ambient temperature between death and brain removal. Environmental temperatures during the harvest range from 4 to 10^{o}C. This is similar to the temperature range on Enderby Island during the breeding season (5 – 12^{0}C).

Brains were removed at varying times after death (see Table 7.2), and then fixed in 10% formalin for eight weeks. Sections were cut from the frontal cortex, hippocampus and cerebellum and processed into paraffin. Tissue blocks were then sent to Massey University for MAP2 immunohistochemistry, which was conducted as described above. Image capture was undertaken using a Leica DM2500 light microscope and Leica DFC290HD camera, with standardised exposure conditions. Image analysis was

undertaken as described above. The number of measurements within each tissue block was maximised by measuring three sulci or folia where possible. For some animals two sections of hippocampus were included in the tissue block, thus two measurements were made for these cases. No hippocampus was available for SAM1 or SAM7.

Cerebellar sections were analysed by light microscopy to determine the percentage of anti-MAP2 immunopositive Purkinje cells as described above. Cortical sections were assessed for the presence of a pale cortical ribbon visible on subgross examination. These were scored as '0' (no pale cortical ribbon present), '1' (ribbon faint and discontinuous) or '2' (ribbon prominent and continuous). The location of loss of immunoreactivity (i.e. superficial, mid, or deep cortical neurons) was assessed microscopically, and the presence of intensely immunoreactive neurons noted.

Immunoreactivity of hippocampal neurons in the dentate gyrus, CA1, CA3 and CA4 was scored semi-quantitatively as '0' (none positive), '1' (a few positive neurons), '2' (approximately half neurons positive), or '3' (most neurons positive).

Statistical analysis

Trends in MAP2 expression over time were examined using scatterplots with linear regression fits. Mann-Whitney tests were used to identify statistically significant differences between time intervals. Indicators of prolonged post mortem interval were identified using linear regression (for ordinal data) and logistic regression (for binomial data). All analyses were conducted using Minitab software.

7.4.2 Results

Image analysis

Details are shown in Table 7.2. In all areas except the frontal cortex there was a statistically significant tendency for MAP2 scores to decrease with increasing post mortem interval. For most areas however, this relationship was not strong, with

R-squared values showing that post mortem interval explained only 10-30% of the variation. For each post mortem interval there was appreciable variation in anti-MAP2 immunoreactivity between individuals and between sites within an individual, particularly in the frontal cortex. While MAP2 scores were significantly lower at long post mortem intervals, the variability between and within animals, along with the small number of animals used at each time point, means that these differences should be interpreted with caution.

Manual, semi-quantitative and qualitative analyses

Results are shown in Table 7.3. The following variables were indicators of prolonged post mortem interval: a prominent continuous pale cortical ribbon ($p = 0.005$); intensely immunoreactive Purkinje cells ($p < 0.001$); and a pale cerebellar granular layer ($p = 0.010$). Average Purkinje cell count decreased significantly with post mortem interval ($p = 0.030$; R-sq (adjusted) = 51.6%). Immunoreactivity of neurons in the hippocampus was not associated with post mortem interval for any area examined.

7.4.3 Discussion

While several studies have suggested that post mortem delays of up to 24 hours do not affect MAP2 expression (Trojanowski *et al.* 1989; Kuhn *et al.* 2005; Oehmichen *et al.* 2009), the dog and fur seal experiments presented here show a loss of MAP2 expression over time. This conclusion is supported by other published studies, where the pattern and rate of loss has been suggested to be dependent upon temperature (Kitamura *et al.* 2005), species or age (Schwab *et al.* 1994). Despite this, valid interpretation of tissues collected from field cases could be made either if post mortem intervals were short, or if these changes were shown to be distinct from loss of MAP2 expression due to ante mortem pathological processes. For example, Hicks *et al.* (1995) proposed that widespread loss of MAP2 expression in all cortical layers was indicative of trauma.

PMI	Cerebellum (molecular)	Cerebellum (granular)	Dentate gyrus molecular layer	Hilus/CA4	CA3	CA1	Frontal cortex
2 hours (n=1)	13.9 (0.7)	80.0 (4.1)	n/a	n/a	n/a	n/a	49.2 (17.0)
3 hours (n=1)	39.6 (18.4)	96.7 (0.6)	70.2 (10.4)	56.5 (21.5)	71.6 (5.1)	59.3 (11.5)	30.6 (7.8)
4 hours (n=1)	13.0 (4.1)	79.3 (1.4)	2.0 (0.4)	26.0 (6.4)	21.0 (8.0)	24.5 (11.9)	11.0 (3.0)
7 hours (n=1)	25.3 (12.2)	58.9 (14.9)	89.9 (*)	44.6 (*)	84.1 (*)	58.9 (*)	35.6 (20.8)
10 hours (n=1)	13.2 (8.3)	81.5 (7.0)	0.2 (*)	0.1 (*)	0.7 (*)	1.4 (1.4)	14.9 (10.5)
20 hours (n=1)	2.2 (0.3)	21.9 (0.5)	0.9 (0.3)	11.9 (*)	17.4 (*)	12.9 (2.8)	13.9 (7.0)
25 hours (n=2)	4.2 (0.9)[1]	32.9 (7.0)[1,2]	1.7 (0.8)	8.8 (2.6)	1.3 (0.3)	8.7 (*)	27.2 (12.8)
30 hours (n=1)	9.2 (1.3)	78.6 (1.7)	25.3 (5.0)	26.1 (1.6)	16.3 (7.9)	27.6 (12.8)	60.3 (2.6)
45 hours (n=2)	1.9 (0.3)	25.4 (4.8)	0.4 (0.4)	1.4 (1.1)	4.7 (0.7)	1.7 (*)	35.8 (4.0)
70 hours (n=2)	4.5 (0.5)[1]	62.0 (4.6)[2]	0.8 (0.2)	6.5 (4.1)	4.9 (1.3)	11.5 (11.1)	15.1 (3.7)

Table 7.2. MAP2 scores in Northern fur seal brains following various post mortem intervals. PMI = post mortem interval. Results are presented as the mean percentage area immunopositive for MAP2 antibody. The standard error of the mean is presented in brackets. n/a = not available; * = not possible to calculate due to low number of observations.

[1] = statistically different to values at <2hours PMI (Mann-Whitney; $p < 0.05$)
[2] = statistically different to values at <3 hours PMI (Mann-Whitney; $p < 0.05$)

	Hippocampus neuronal				Cerebellum			Cerebral cortex	
Animal ID	Dentate gyrus	CA4	CA3	CA1	Pale granular layer	Intensely immuno-positive Purkinje cells	Average % immunopo-sitive Purkinje cells	Cortical ribbon score	Intensely im-munopositive neurons
SAM1	-	-	-	-	n	n	77	1	n
SAM2	3	0	3	3	n	n	97	0	n
SAM3	0	0	1	3	n	n	78	1	n
SAM4	3	2	3	3	y	n	42	0	n
SAM5	2	1	2	3	n	n	42	1	n
SAM6	0	3	2	3	y	n	35	1	n
SAM7	-	-	-	-	y	n	10	1	n
SAM8	0	3	0	3	y	y	19	2	y
SAM9	0	3	3	3	n	n	65	1	n
SAM10	3	2	3	3	y	y	27	1	n
SAM11	0	0	1	1	y	y	16	2	y
SAM12	0	0	2	0	y	y	6	2	y
SAM13	0	2	2	3	y	y	11	2	n

Table 7.3. Results of manual, semi-quantitative and qualitative analyses of MAP2 immunoreactivity in Northern fur seals. '-' = tissue not available.

The fur seal and dog experiments presented here, however, show that prolonged post mortem interval can also lead to loss of MAP2 expression in all cortical layers, hence caution should be used in attributing this change to trauma in cases where the post mortem interval is prolonged or unknown. Other changes detected in the fur seals, such as loss of immunoreactivity in the hippocampus, are similar to those described in hypoxic-ischaemic injury (Kitagawa *et al.* 1989; Tomioka *et al.* 1992; Miyazawa *et al.* 1993; Lingwood *et al.* 2008).

The presence of intensely immunoreactive neuronal soma has been noted in other studies. Schwab *et al.* (1994) believed this to be a post mortem change, and noted that the intensity of immunoreactivity increased with post mortem time. Buddle *et al.* (2003) attributed the same change to ischaemia. These authors proposed that redistribution of MAP2 could occur due either to active dendritic transport or to dissociation of microtubule-MAP2 complexes. Since dendritic transport requires energy (Sheetz *et al.* 1998), this is unlikely to be the mechanism of MAP2 redistribution in post mortem tissues.

Some authors propose that the confounding effects of post mortem loss of MAP2 can be minimised by using post mortem interval-matched control tissues (Schwab *et al.* 1994; Irving *et al.* 1997). However, while every effort was made during the Enderby Island field season to decrease autolysis by collecting pups twice daily, exact post mortem intervals for individual pups were rarely known, and five pups were in a state of decomposition when found, hence must have been missed on first examination of the colony. In addition, the necropsy examinations of five pups were delayed until the day after they were found due to other research commitments. A useful outcome of this fur seal trial, therefore, was the identification of changes that indicate prolonged post mortem interval. The studies presented above therefore act as a useful basis from which to interpret MAP2 expression in the NZ sea lion pups, which is described in the next section.

7.5 Evaluation of MAP2 expression in 2007/08 field cases

7.5.1 Materials and methods

Sections of frontal cortex, hippocampus and cerebellum were processed and analysed as described above. In addition, indicators of autolysis were assessed as determined by the post mortem interval experiments: a continuous pale cortical ribbon (score 2); loss of immunoreactivity in the cerebellar granular layer; and intensely immunoreactive Purkinje cells. Each pup was allocated a score of one for the presence of each of these indicators, resulting in a total autolysis score between 0 and 3.

Statistical comparisons were made using Student's t-test for normally-distributed data, and the Mann-Whitney test for data that were not normally distributed.

7.5.2 Results

Qualitative analyses

Many pups had diffuse (n = 20) or patchy (n = 2) loss of immunoreactivity in the granular layer of the cerebellum, accompanied by intensely immunoreactive neurons, often with thickened proximal dendrites. The average number of immunopositive Purkinje cells ranged from 0 to 94%.

The most common change in the frontal cortex was loss of neuronal immunoreactivity to form a pale ribbon localised to the mid (n = 9), superficial plus mid (n = 7) or all (n = 6) cortical layers. In addition, marked loss of immunoreactivity of dendrites was also present in the mid (n = 4), superficial and mid (n = 2) or all (n = 2) layers.

Comparison between groups

All pups with vascular axonal injury, as indicated by βAPP immunohistochemistry, had loss of immunoreactivity of the neurons in the superficial layers of the cortex. This change was significantly more common in pups with vascular axonal injury than

in those without (p = 0.016). Hippocampal CA1 MAP2 scores were also significantly lower (p = 0.040) in pups with vascular axonal injury. No significant differences were found for pups with contusions, pups dying due to traumatic brain injury, pups with intracranial subdural haemorrhage, or pups with haemorrhage of the cerebellar vermis. Pups with traumatic brain injury as the cause of death (n = 2) had *higher* dentate gyrus (p = 0.023), CA3 (p = 0.028), hippocampus (p = 0.035) and total MAP2 (p = 0.038) scores than pups with other causes of death.

Analysis of autolysed pups

Pups that were known to have a post mortem interval of more than 12 hours were analysed as a distinct group ('prolonged post mortem interval pups') and compared with unknown post mortem interval pups. Nine of ten prolonged post mortem interval pups had an autolysis score of 3, and the tenth had a score of 2. Prolonged post mortem interval pups had significantly higher autolysis scores than unknown post mortem interval pups ($p < 0.001$). There were no significant differences in total MAP2 scores between these two groups.

The effect of prolonged post mortem interval on MAP2 expression in each area was assessed by comparing values for pups with an autolysis score of 3 (n = 11) to values for pups with a score of 0 (n = 10). MAP2 scores were significantly lower in the autolysed group for the dentate gyrus molecular layer (p = 0.001), hilus/CA4 ($p < 0.001$), CA3 ($p < 0.001$) and cerebellar molecular layer (p = 0.002), while there was no significant difference between groups for CA1 or cerebral cortex.

A total of 10 pups had an autolysis score of 0. When qualitative analyses were repeated using only these pups there were no significant differences in MAP2 immunoreactivity in any area for pups with vascular axonal injury (n = 2) compared to pups without vascular axonal injury (n = 8).

7.5.3 Discussion

The results of the study described in this section show that NZ sea lion pups with vascular axonal injury have a significant loss of MAP2 expression in the hippocampal CA1 region and in the mid layers of the cortex, consistent with hypoxic-ischaemic injury. A similar pattern has been demonstrated in experimental ischaemic brain injury in rats, gerbils and piglets (Kitagawa *et al.* 1989; Tomioka *et al.* 1992; Miyazawa *et al.* 1993; Lingwood *et al.* 2008). In contrast, two recently-published human studies found that hypoxia-ischaemia resulted in significant loss of MAP2 expression in CA2, 3, and 4, but not CA1 (Kuhn *et al.* 2005; Oehmichen *et al.* 2009). These human studies used brain tissue obtained up to 48 hours after death, and although the authors stated that they found no correlation between MAP2 loss and post mortem interval, the data supporting this were not presented, and it is possible that the effects of autolysis could have confounded these comparisons.

This sea lion pup study also validated the indicators of autolysis identified in the previous experiment using Northern fur seal brains, by analysing a group of pups known to have prolonged post mortem intervals. In turn, comparing pups with high autolysis scores with those that had low autolysis scores demonstrated that there was no significant effect on MAP2 scores for CA1 or on MAP2 expression in the mid layers of the cerebral cortex, and showed that any differences observed in these areas were unlikely to be due to autolysis. Under the field conditions on Enderby Island, collection of tissues twice daily was sufficient to adequately preserve MAP2 protein. Further validation of this method of detecting brain injury in pinnipeds could be achieved by repeating the fur seal experiment using brains collected at 0, 12 and 24 hours, in order to determine whether statistically significant loss of MAP2 expression occurs in any area during this time.

In contrast to the identification of hypoxic-ischaemic injury, traumatic brain injury

was not detectable in NZ sea lion pups using anti-MAP2 immunohistochemistry. A number of published studies have examined the effects of experimental traumatic brain injury on MAP2 expression, finding decreased anti-MAP2 immunoreactivity at sites of impact or contusion as well as distant to affected parenchyma, particularly the dentate hilus and cortex (Hicks *et al.* 1995; Lewen *et al.* 1996; Posmantur *et al.* 1996; Manavis *et al.* 1999). MAP2 expression in contused parenchyma was not evaluated in this study, and statistically significant differences in MAP2 expression were not found for NZ sea lion pups with contusions.

A limitation inherent in detection of brain injury using the methods described here relates to survival time, which could affect the magnitude and location of loss of MAP2 immunoreactivity. Kitagawa *et al.* (1989) found very early loss of MAP2 expression in CA1 of gerbil brains in response to ischaemia, with later decreases in other areas of the hippocampus. The size of affected regions also increased over time, suggesting that MAP2 loss is progressive. Some studies have also shown recovery of MAP2 expression over time, interpreted as reversible injury (Ota *et al.* 1997; Huh *et al.* 2003). These factors mean that, as discussed in the last chapter with respect to βAPP immunoreactivity, pups that had brain injuries but very short survival times might not be detected by the methods used here.

7.6 Summary

Loss of MAP2 expression in the middle layers of the frontal cortex and the CA1 region of the hippocampus of NZ sea lion pups with vascular axonal injury are consistent with the hypothesis that these pups had hypoxic-ischaemic injury. In contrast, pups with intracranial subdural haemorrhage or haemorrhage of the cerebellar vermis did not have indications of ischaemic damage, and MAP2 expression changes shown to be consistent with blunt trauma in other species were not detected in NZ sea lion pups

that had lesions of traumatic brain injury. This may reflect a true absence of effect (for example, herniation of the cerebellar vermis may not result in hypoxic-ischaemic damage) or the results could have been obscured due to short survival times.

7.7 References

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Chapter eight

General discussion and conclusions

8.1 The pathogenesis of subdural haemorrhages in NZ sea lion pups

The central hypotheses of this thesis were that subadult male sea lions cause appreciable pup mortality, and that the subdural haemorrhages seen in some pups at necropsy were due to shaking. The existence of shaking and throwing attacks at the Sandy Bay colony was confirmed by observation, but no pups were seen to be killed during these attacks, and follow-up studies of shaken pups were not possible. The number of pups with intracranial subdural haemorrhages certainly increased in the second half of the season, which is the period during which most attacks by subadult

males are thought to occur, but further analysis showed that only two of these pups had died due to traumatic brain injury. Of the nine non-traumatic intracranial subdural haemorrhage cases, eight had meningitis and one had septicaemia.

The causal agent of these infections was a mucoid phenotype of *K. pneumoniae,* which is responsible for an increasing number of human meningitis cases. While 80% of NZ sea lion pups with *K. pneumoniae* meningitis had subdural haemorrhages, this has not been reported in other species, suggesting that factors other than the agent itself play a role in development of these haemorrhages. Furthermore, while subdural collections in meningitis are usually haemorrhagic effusions, this was not the case in the sea lion pups, where the majority of subdural haemorrhages were blood with no inflammatory infiltrate.

So what caused the subdural haemorrhages seen in these pups? The traditional explanation of traumatically disrupted bridging vessels might explain the haemorrhages present in the two pups with head trauma, but cannot be the mechanism behind the remaining haemorrhages. An alternative explanation is that subdural haemorrhage arises from intradural vessels, involving the parasagittal venous plexus and the unlined intradural vascular channels. These unlined channels were first described in 1875 (Christensen 1944) and are now believed by some to play a role in resorption of CSF (Mack *et al.* 2009; Squier *et al.* 2009). Numerous studies have shown that tracer injected into the subarachnoid space passes into the dura, possibly along nerve roots and exiting blood vessels, as well as via arachnoid granulations. Similarly, tracer injected into the dural interstitium moves into the dural venous sinuses, possibly via these unlined intrdural channels (Fox *et al.* 1996; Papaiconomou *et al.* 2004; Mack *et al.* 2009; Squier *et al.* 2009). This thesis has shown that unlined intradural channels are present in both the intracranial and cervical spinal dura mater of NZ sea lion pups.

Since CSF flow into the dural sinuses occurs passively along a pressure gradient (Papaiconomou *et al.* 2004), if the pressure within the venous plexus were to exceed

that in the unlined channels, blood could reflux into the channels. This theory is supported by the observation of blood-filled unlined channels in histological sections of intracranial dura mater from several pups. Some sections also showed coalescing intradural haemorrhages that dissected into the subdural compartment. A disruption of the normal pressure gradient between the venous plexus and the unlined channels could occur in several situations. Firstly, the presence of exudate in the subarachnoid space of pups with meningitis could result in obstruction of movement of CSF into the dural channels. Meningitis could also result in increased venous sinus pressure due to seizures or increased cerebral blood flow (hyperaemia). Coagulation disorders associated with sepsis might also predispose to haemorrhage in the face of altered pressure differentials.

Increased dural venous sinus pressure in the absence of meningitis could also occur with increased intra-thoracic pressure or abnormal cerebral blood flow regulation. Pressure autoregulation is well developed in neonatal animals, but can be disrupted by hypoglycaemia, hypoxia or seizures (Pryds 1991). Cerebral autoregulation can also be disrupted by traumatic brain injury (Udomphorn *et al.* 2008), providing another mechanism (in addition to direct damage to bridging veins) of subdural haemorrhage in pups with head injury. Increased intra-thoracic pressure could predispose to intra- and sub-dural haemorrhage in pups during parturition and in pups that are crushed by adult sea lions. The latter might explain the intracranial subdural haemorrhage in one early-season pup (E07/08-05Ph) that had a crushed thorax in the absence of injury to the head or neck.

One unexpected finding in this study was the lack of evidence of raised intracranial pressure in pups with subdural haemorrhage. Geddes *et al.* (2003) argued against a direct traumatic cause of subdural haemorrhages in human infants, instead proposing that a combination of raised intracranial pressure and hypoxia resulted in intradural haemorrhages which then coalesced in the subdural compartment. In Chapter Six of

this thesis, the pattern of βAPP immunostaining demonstrated vascular axonal injury in seven pups, indicating raised intracranial pressure, but none of these had subdural haemorrhages. One possible explanation for this is that intradural haemorrhages develop due to rapid, transient disturbances in dural venous pressure, and they may in fact be less likely to occur with more gradual, sustained increases in intracranial pressure, since CSF absorption through the dural route increases at high intracranial pressures (Papaiconomou *et al.* 2004). This would in turn elevate the pressure within the unlined channels, and thus decrease the likelihood of reflux of blood from the venous plexus.

The presence of haemorrhage within the spinal meninges also requires explanation. Rupture of bridging veins cannot be the cause, as there are no bridging veins in the spinal cord (Nicholas and Weller 1988). Some authors suggest that subdural haemorrhage in the spinal cord results from redistribution of intracranial subdural haemorrhage under the effect of gravity (Russell and Benoit 1983; Gilad *et al.* 2007; Case 2008; Yang *et al.* 2009), and continuity of intracranial and spinal haemorrhages has been observed (Lecouvet *et al.* 2003; Koumellis *et al.* 2009). This mechanism does not seem plausible in sea lion pups for several reasons. Firstly, six pups had spinal meningeal haemorrhage in the absence of intracranial subdural bleeding. Since no pups had any indication of chronic or resolved subdural haemorrhage, migration after resolution of intracranial haemorrhage, as proposed by Moscovici *et al.* (2011) and Bortolotti *et al.* (2004), is unlikely. Secondly, in the sea lion pups spinal sub-meningeal haemorrhages were present in both the subdural and the subarachnoid compartments, sometimes simultaneously. This distinction is of importance when considering the possible pathogenesis of spinal sub-meningeal haemorrhages in any species, and a notable finding of this thesis is that gross examination of the spinal cord and meninges is not sufficient to determine the anatomical location of haemorrhage.

If spinal sub-meningeal haemorrhages are not a consequence of migration, then they

must be originating within the vertebral canal itself. Payer *et al.* (2010) suggested two possible sources of sub-meningeal haemorrhage: veins in the subarachnoid space or the extra-arachnoid veins on the inner surface of the dura. The authors proposed that these veins could rupture in the face of trauma or increased intra-abdominal or intra-thoracic pressure, or could bleed spontaneously due to coagulation disorders. While these mechanisms might also be involved in the sea lion pups, the most likely interpretation of the histological sections in this thesis suggests an alternative *source* of bleeding: the intradural venous plexus. Many pups had intradural haemorrhages surrounding the spinal nerve roots, and there was a strong statistical correlation between large intradural haemorrhages and the presence of sub-meningeal haemorrhages. In several sections, blood could also be seen dissecting through the dura into either the subdural or subarachnoid compartments.

Arachnoid proliferations are found around spinal nerve roots, and are believed to be routes of CSF absorption in the spinal cord of a number of species (Gomez *et al.* 1974; Kido *et al.* 1976; Bozanovic-Sosic *et al.* 2001; Brodbelt and Stoodley 2007). Kido *et al.* (1976) found that some arachnoid structures were embedded within the dura, and were closely associated with venous sinuses. It is possible, therefore, that alterations in pressure gradients between the arachnoid granulations, unlined vascular channels and the venous sinuses could result in haemorrhages as seen histologically in the sea lion pups.

8.2 Importance of brain injury in pup mortality and morbidity

The studies described in this thesis were able to confirm that pups were picked up and shaken by subadult males, but the preceding discussion shows that these attacks were not responsible for the majority of the subdural haemorrhages that occurred. This thesis used gross examination and routine histochemistry as well as MAP2 and βAPP

immunohistochemistry to evaluate the role of traumatic brain injury in pup mortality, and found that this was the cause of death in 3/36 late season pups.

While fatal traumatic brain injury therefore seems to have been relatively uncommon in 2007/08, one intriguing finding was that *all* pups examined had at least some axonal injury, while many had widespread axonal injury throughout white matter. None of these pups had grade III diffuse axonal injury as described in human brain injury cases. The techniques used in this thesis were not able to definitively attribute axonal injury to a particular cause, but the evidence points to much of it being non-traumatic. Irrespective of origin, brain injury was present in all pups necropsied, and could have contributed to mortality in many. A vascular pattern of axonal injury, in particular, was a frequent finding and occurred in pups with varying causes of death, suggesting that white matter damage due to decreased blood supply was a common occurrence. These findings have implications for veterinary medicine in general: if axonal injury is this prevalent in sea lion pups, then it is also likely to occur in other non-human mammalian species, and therefore should be considered in the diagnosis and treatment of veterinary patients.

8.3 Does shaking alone lead to pup mortality?

The discussion above indicates that most subdural haematomas were not due to shaking, and no pups had other gross, histological, or immunohistochemical findings in the brain that could be unequivocally attributed to shaking. In human infants, ocular lesions such as retinal haemorrhage, optic nerve sheath haemorrhage and optic nerve axonal injury are considered to be strong indicators of child abuse (Tongue 1991; Duhaime *et al.* 1992; Leestma 2005). While some researchers believe that these injuries are a direct result of tearing of nerves and blood vessels due to shear or stretch forces (Caffey 1974; Greenwald *et al.* 1986; Gleckman *et al.* 2000; Levin 2009), others have pointed out that increased intracranial pressure or increased venous pressure should

also be considered as potential mechanisms (Duhaime *et al.* 1992; Ommaya *et al.* 2002; Geddes and Whitwell 2004).

Some authors believe that axonal injury in the optic nerve is strongly associated with inflicted trauma (Gleckman *et al.* 2000; Reichard *et al.* 2004). Interestingly, this lesion was seen in the majority of sea lion pups examined. Since axonal injury cannot be considered to be synonymous with trauma however, other potential causes must be evaluated before this change could be assumed to indicate shaking injury. One possibility is that optic nerve axonal injury in these pups was due to increased intraocular pressure. Several papers have previously described blockage of fast axonal transport in the optic nerve in response to experimentally elevated intraocular pressure (Hayreh 1972; Anderson and Hendrick 1974; Hayreh *et al.* 1979). Anderson and Hendrick (1974) found that even moderate increases in intraocular pressure caused interruption of fast axonal transport at the level of the lamina cribrosa. Intraocular pressure is known to increase due to elevated intracranial pressure (Lehman *et al.* 1972; Lashutka *et al.* 2004), and pups in this study that had a vascular pattern of axonal injury, indicating increased intracranial pressure, had significantly more axonal injury in their optic nerves than those without vascular axonal injury.

Alternatively, axonal injury in the region of the lamina cribrosa could be a direct ischaemic effect. McLeod *et al.* (1980) demonstrated axonal injury following ligation of the short posterior ciliary arteries, which supply the lamina cribrosa, at least in primates (Steele and Blunt 1956; Hayreh 1972). Since these arteries penetrate the dural sheath of the optic nerve, increased pressure within the sheath, for example due to the presence of inflammatory exudate or to distension of the sheath in response to increased intracranial pressure (Hansen and Helmke 1997), could decrease blood flow to the lamina cribrosa. Conversely, generalised hypoxia-ischaemia could cause more widespread axonal injury within the nerve. In all but two pups, βAPP immunostaining was within or immediately adjacent to the lamina cribrosa.

The conclusion here is therefore that axonal injury in the optic nerve does not necessarily indicate shear force trauma to the optic nerve. Axonal injury observed in cases of trauma may in fact be more likely to arise from interrupted axonal transport due to ischaemia and elevated intraocular pressure, particularly when the lamina cribrosa and adjacent regions are affected. When this is considered along with an analysis of the gross lesions and axonal injury patterns in the brain of sea lion pups, it seems there is very little evidence to suggest that shaking of pups by subadult males is important in pup mortality.

8.4 The role of attacks by subadult males in pup mortality

The next question becomes: are attacks by subadult males important in overall mortality? Only four of the 2007/08 late season pup mortalities were due to trauma: three to traumatic brain injury, and one to abdominal crushing. This is a comparatively low figure, particularly in light of the fact that trauma is believed to be a major cause of mortality in this population, with a peak of traumatic deaths caused by subadult males in the second part of the season (Castinel *et al.* 2007).

Although it could be considered that the 2007/08 season had an atypically low number of traumatic deaths, part of the reasoning behind the belief in a late-season peak of trauma is the fact that each year clusters of pups are found dead with gross lesions that appear to be due to traumatic brain injury. Indeed, the 2007/08 pups had an impressive array of lesions that have all been attributed to inflicted trauma in human infants: optic sheath haemorrhages, scleral and retinal haemorrhages, intracranial subdural haemorrhages, spinal sub-meningeal haemorrhages, and optic nerve axonal injury. However, as is apparent from the preceding studies, most of these pups actually had *K. pneumoniae* meningitis, thus the impression of appreciable late-season traumatic deaths in the past may well be wrong. The possibility that all of these lesions could be caused by alterations in vascular, intra-ocular, intracranial or subarachnoid pressure warrants

further investigation.

The involvement of *K. pneumoniae* infection in the majority of late season pup mortalities could also have important management implications. Although at present no direct intervention strategies are included in NZ sea lion population management, if pup mortality rates were ever deemed sufficient to warrant intervention, based on the findings of this thesis there would be little point in attempting to prevent or decrease attacks by subadult males. Instead, attention would be better directed at decreasing pup susceptibility to infection by *K.pneumoniae*, for example by development and application of an effective vaccination protocol. Currently the true extent of *K. pneumoniae* mortality is unknown, and the studies presented here indicate that deaths due to this bacterium are still occurring at the time the science team leave the island at the end of the season.

8.5 Study limitations

MAP2 and βAPP immunohistochemistry proved to have some limitations in assessing brain injury in this study. First, although quantitative or semi-quantitative scoring systems were applied, these do not necessarily correlate with clinical severity of damage. Secondly, in field situations where the circumstances and time of death are unknown, valid interpretations of marker expression cannot always be made. For example, as shown in Chapter Seven, MAP2 protein is not stable in post mortem tissues, and a prolonged post mortem interval can mimic hypoxic or traumatic brain injury. Similarly, the cause of widespread axonal injury, as indicated by βAPP immunohistochemistry, cannot always be surmised without clinical information, particularly when survival times are short or unknown. These problems are by no means novel to this study, and apply equally to human forensic investigations. While some of these issues have been well articulated by other researchers, the limitations of these techniques are not always fully recognised (Schwab *et al.* 1994; Irving *et al* 1997; Graham *et al.* 2004; Kitamura *et*

al. 2005; Kuhn *et al.* 2005; Reichard *et al.* 2005; Dolinak and Reichard 2006; Oehmichen *et al.* 2009).

One particularly notable although confounding factor in this study was the high frequency of inflammatory brain disease in the sea lion pups. Since meningitis affects cerebral blood flow and intracranial pressure (Pfister *et al.* 1994), it can cause axonal injury (Nau *et al.* 2004; Gerber *et al.* 2009), and, presumably, alter MAP2 expression through hypoxic-ischaemic effects. In turn, because most late-season pups had meningitis, it was very difficult to evaluate the effects of trauma alone. In order to determine whether the techniques used in this study are able to reliably distinguish between hypoxic-ischaemic brain injury and traumatic brain injury, a different study population is required.

8.6 Conclusions and future directions

Despite the difficulties inherent in conducting experiments using tissues from animals in which the circumstances and time of death are unknown, this thesis has shown that subadult male NZ sea lions do not cause appreciable pup mortality, and that subdural haemorrhages in pups are not caused by shaking. In addition it has been shown that brain injury, particularly non-traumatic axonal injury, is common in NZ sea lion pups. In the process of demonstrating these findings, several hypotheses have been generated, and warrant further investigation. First it is proposed that reducing the incidence of *K. pneumoniae* infection would result in markedly decreased pup mortality. Secondly, it is hypothesised that axonal injury occurs commonly in non-human mammals, and that much of this injury is due to hypoxic-ischaemic events including raised intracranial pressure. A third hypothesis is that subdural haemorrhages occur due to disrupted pressure regulation between vascular and CSF compartments.

Investigating the first of these hypotheses could include developing an effective and safe

vaccination protocol that could be used on young NZ sea lion pups at Enderby Island. The second hypothesis could be tested by applying similar methods to those used in this thesis to other non-human mammalian species. More specifically, the ability of MAP2 and βAPP immunohistochemistry to detect and distinguish between hypoxia-ischaemia and trauma could be more closely evaluated by testing these markers in a population in which there is little infectious disease. Additional biomarkers such as S100B protein could be used to identify raised intracranial pressure and to further characterise hypoxic-ischaemic and traumatic brain injury. The final hypothesis, that of the intradural origin of subdural haemorrhages, would likely require investigation in experimental models, but the importance of this in human forensic medicine means that the pathogenesis of subdural haemorrhages is a tantalising area for ongoing research.

8.7 References

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