The Veterinary Journal 194 (2012) 326–331

Contents lists available at SciVerse ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Freezing and thawing of pinniped carcasses results in artefacts that resemble traumatic lesions ☆

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ARTICLE INFO

Article history:
Accepted 29 March 2012

Keywords:
Pinniped
Marine mammal
Post-mortem examination
Freezing
Artefact
Trauma

ABSTRACT

The objective of this study was to assess whether the freezing and thawing of pinniped carcasses prior to post-mortem examination could create artefacts that resembled lesions caused by trauma. Necropsy findings in New Zealand fur seals (*Arctocephalus forsteri*), captured incidental to commercial fishing, and either chilled ($n = 5$) or frozen ($n = 5$), were compared. Changes in frozen, but not in chilled, carcasses included: pseudo-bruising of subcutis; the accumulation of thick dark red fluid (resembling haemorrhage) in the thoracic cavity, pericardial sac and abdominal cavity; apparent subcapsular renal haemorrhage; pseudo-contusions of the brain; apparent haemorrhage from the nares; and blood-staining of the anterior ocular chamber.

The processes of freezing and thawing were strongly associated with subcutaneous pseudo-bruises, the presence of thick, dark red abdominal fluid and renal subcapsular 'haemorrhage' ($P = 0.004$). These artefacts probably develop due to a combination of autolysis and 'freeze–thaw' effects including lysis of cell membranes, fluid shifts into the extracellular space, and disruption of blood vessel walls. The results of the study demonstrate that artefacts resembling traumatic lesions are created during freezing and thawing of pinniped bodies. Such changes must be taken into consideration at post-mortem examination of previously frozen carcasses.

Introduction

The accurate interpretation of grossly and microscopically visible lesions post-mortem relies, to a large extent, on good tissue preservation, and is hampered by the autolysis that follows a prolonged post-mortem interval. Bodies of animals, particularly wildlife, are often frozen to limit autolysis when a delay between death and necropsy is anticipated. In the case of wild marine mammals, there is often a long distance between the site of death and a diagnostic facility so post-mortem examinations are frequently undertaken on previously frozen carcasses (Dunn et al., 2002; Norman et al., 2004; Ketten, 2005; Duignan and Jones, 2007; Roe, 2009, 2010). Despite this, there is very little information in the veterinary literature relating to artefacts that can occur as a result of freezing.

Between 1998 and 2009 a series of necropsy projects were conducted on pinnipeds that had been accidentally captured and drowned in the New Zealand arrow squid fishery at the Auckland Islands in the sub-Antarctic. The carcasses were frozen on board the fishing vessels and transported back to the New Zealand mainland where they were thawed and examined. One objective of the project was to document traumatic lesions, and a number of gross lesions consistent with trauma were consistently found, including subcutaneous bruising and the accumulation of blood-tinged fluid in the abdominal cavity. However, the difficulty in interpreting such lesions in previously frozen bodies was noted (Roe, 2010).

The effects of freezing and thawing cells have been well studied (Mazur, 1970; Pegg and Diaper, 1988; Ishiguro and Rubinsky, 1994; Scott et al., 2005), and it is widely recognised that freezing results in cell damage, cell lysis, and in the accumulation of extracellular fluid (Baraibar and Schoning, 1985, 1986; Schafer and Kaufmann, 1999). The effects of freezing and thawing on whole bodies, however, have not been widely reported but are particularly relevant for larger bodies such as those of pinnipeds, where the time taken to freeze and thaw the carcass increases the likelihood that autolysis will be superimposed on the changes caused directly by the freeze–thaw process. The objective of the present study was to investigate and document artefacts that occur as a result of the freeze–thaw process in pinniped carcasses.

☆ The results of this work were presented at the 2010 Annual Conference of the New Zealand Society of Veterinary and Comparative Pathology, at Palmerston North, New Zealand.

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http://dx.doi.org/10.1016/j.tvjl.2012.03.028

Materials and methods

Animal selection and transport

Ten otherwise healthy male New Zealand fur seals (*Arctocephalus forsteri*) were sourced from animals incidentally captured during commercial fishing operations in Cook Strait, New Zealand, between June 2008 and September 2009. This is a cold-water fishery, operating at depths of up to 400 m, and at water temperatures of 10–12 °C. Seals were retrieved dead from nets, packed in two layers of polythene, placed on ice and transported to port. From there the carcasses were transferred to a refrigerated transport vehicle and shipped to Massey University, Palmerston North, New Zealand. On arrival, they were allocated alternately into one of two groups: 'frozen' or 'non-frozen'. Those in the frozen group were stored at −20 °C for 4–8 weeks, then removed, placed in dorsal recumbency, and thawed at room temperature (13–18 °C) prior to necropsy. Animals in the non-frozen group were subject to post-mortem examination within 12 h of receipt.

Post-mortem examination

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 expose the 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. The two brain halves were then placed into 10% neutral buffered formalin and fixed for at least 2 weeks prior to a second gross examination, at which time any lesions were photographed and recorded. Sections of formalin-fixed brain, kidney and subcutaneous tissue were processed into paraffin for examination by light microscopy. Paraffin-embedded 4 μm thick sections were stained with haematoxylin and eosin (HE) and with Martius Scarlet Blue trichrome (MSB).

Statistical analysis

Comparisons between group characteristics were performed using two sample *t* tests (for transit time and bodyweight), and using Fisher's exact test (for capture date-to-vehicle transfer). The differences in 'lesion' frequency between the groups were analysed using Fisher's exact test.

Results

Ten by-caught male fur seals were examined between July 2008 and September 2009. Death was attributed to drowning in all cases, and all animals were in good body condition with no evidence of concurrent disease. Table 1 gives details of the transport time and weight for each animal. There was no significant difference between transit times ($P = 0.406$, two sample *t* test), capture date-to-vehicle transfer ($P = 1.000$, Fisher's exact test) or bodyweight ($P = 0.970$, two sample *t* test) for frozen vs. non-frozen animals. The gross lesions found are summarised in Table 2. The non-frozen animals were classified as minimally autolysed, with no bloating, firm muscle tissue, normal colour and consistency of internal organs, and minimal discolouration of blubber.

Examination of subcutaneous tissues

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 referred to here as 'pseudo-bruising'), within the blubber or muscle along the ventral midline (Fig. 1A), 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, and one had several millilitres of non-clotted, dark-red fluid beneath the muscle fascia in the apparently bruised area. One fur seal from the frozen group had severe haemorrhage of the muscles and blubber of the right side of the neck, extending from the inner surface of the skin to the level of the cervical vertebrae. The superficial and deep musculature of this region was crushed and torn. There were no penetrating wounds to the skin.

Examination of body cavities

All five frozen fur seals (but none of the non-frozen animals) had thick, dark-red, non-clotted fluid in the abdominal cavity. The volume of intra-abdominal fluid varied from approximately 10–380 mL. In all cases intra-abdominal organs and vessels were intact. Smaller, non-quantified volumes of similar fluid were found in the pericardial sac and thoracic cavity of three frozen animals. The intra-abdominal fluid was red–brown and somewhat opaque with low cellularity (>10.4×10^9 nucleated cells/L). Cytological examination of centrifuged precipitate revealed 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 (Fig. 1B). 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, and two of these had several mL of dark-red fluid within the tunica vaginalis. Segments of intestine were discoloured dark-red in all frozen animals but not in the non-frozen bodies. Other lesions, found only in the frozen group, were blood-tinged fluid in the ante-

Table 1
Details of intervals from capture-to-necropsy and bodyweights of 10 by-caught New Zealand fur seals from Cook Strait, New Zealand.

Case number	Capture-to-transfer[a]	Transit time (days)[b]	Days frozen	Days thawed	Weight (kg)
1	D	5	0	0	105
3	D	2	0	0	80
5	N	3	0	0	54
7	N	3	0	0	75
9	D	2	0	0	55
2	D	3	25	3	80
4	N	5	36	4	52
6	N	3	24	4	55
8	D	4	52	4	120
10	D	3	44	4	65

D, transferred on day of capture; N, transferred next day.
[a] Time of transfer to refrigerated transport relative to capture.
[b] Transit time, time between loading onto refrigerated transport and delivery to necropsy facility.

Table 2
Comparison of gross lesions and pseudo-lesions of by-caught New Zealand fur seals either chilled ($n = 5$) or frozen/thawed ($n = 5$) prior to necropsy.

Case number	Frozen?	Abdominal fluid (mL)	Renal	Vas def.	Head/neck	Brain (focal)	Brain (mottled)	Nares	Ant. chamber	S/C
1	N	N	N	N	N	N	N	N	N	N
3	N	N	N	N	N	N	N	N	N	N
5	N	N	N	N	N	N	N	N	N	N
7	N	N	N	N	N	N	N	N	N	N
9	N	N	N	N	N	N	N	N	N	N
2	Y	100	Y	Y	N	Y	N	N	N	Y
4	Y	10	Y	Y	Y[a]	N	Y	Y	Y	Y[b,c,d]
6	Y	180	Y	Y	Y	Y	Y	Y	N	Y[c,d]
8	Y	260	Y	Y	N	N	N	N	Y	Y[b,d]
10	Y	380	Y	Y	Y	Y	Y	Y	Y	Y[b,c,d]

S/C, subcutaneous 'bruising'; abdo., abdominal; renal, apparent bruising of renal capsule; vas def., apparent haemorrhage around vas deferens; head/neck, apparent bruising of soft tissues of head and/or neck; brain (focal), pseudo-contusion; brain (mottled), mottled discolouration around sulci and meningeal vessels; nares, apparent haemorrhage from nares; ant. chamber, apparent haemorrhage into anterior ocular chamber.
[a] Extensive bruising of skin, blubber and muscle associated with tearing of muscle.
[b] Pseudo-bruising at ventral midline.
[c] Pseudo-bruising at axillae.
[d] Pseudo-bruising at shoulders.

rior ocular chamber ($n = 3$) and haemorrhage from the nares ($n = 3$).

Examination of brain

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. Mottled red–brown discolouration within sulci and along meningeal vessels was present in three frozen animals, and larger focal areas of red–brown- to black-staining resembling contusions were present in three (Fig. 1C). The only gross finding in non-frozen brains was congestion of the meningeal vessels ($n = 3$), with the congestion being unilateral in two of these.

Statistical comparisons

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

Histopathological examination

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, and expansion of the subarachnoid space by homogenous to finely stippled eosinophilic material (Fig. 2A). There was a general loss of cellular detail, and numerous 'fissures' (narrow clear spaces 20–40 μm wide and up to 5 mm long) were present throughout the parenchyma. Within the cortex and subarachnoid space, intact and lysed erythrocytes were present both within and outside blood vessels (Fig. 2B), and vessel walls were often discontinuous or indistinct. The subarachnoid space in some areas contained granular to globular eosinophilic deposits. The MSB stain demonstrated scattered, individual intact erythrocytes both within and surrounding blood vessels.

Sections of subcutaneous pseudo-bruises revealed brightly eosinophilic granular material (interpreted as fluid) dissecting along connective tissue layers within the blubber (Fig. 2C). Underlying muscle was unaffected. Sections of kidney exhibited distension of the space between the two capsular layers by variable amounts of eosinophilic fluid admixed with lysed and, less frequently, intact erythrocytes. Sections of tissue from non-frozen animals showed minimal autolytic changes, with the majority of erythrocytes intact, and no fragmentation of blood vessel walls. There were fine clear vacuoles present throughout the outermost 1–2 mm of the superficial cortical grey matter. No fluid was present in the subarachnoid space.

Discussion

Despite extensive research into the mechanisms of freeze–thaw damage at a cellular level, there are few publications that describe the gross changes that occur due to freezing and thawing of whole bodies. Changes interpreted as freeze artefact have been reported and include dark red fluid in the body cavities of a human infant (Tabata et al., 2000) and blood-tinged discharge from the nares of a rat (Micozzi, 1986). Both of these changes were also present in the frozen fur seals in this study, along with several other changes resembling traumatic lesions. While the small sample size means that for some of these changes an association with freezing did not reach statistical significance, our results do indicate that accumulations of intra-abdominal fluid resembling non-clotted blood, pseudo-bruising of subcutaneous tissues, apparent haemorrhage into the renal capsule and brain pseudo-contusions, are artefacts created during the freeze–thaw 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 the direct membrane damage caused 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 pinnipeds, therefore, is likely to be partly composed of accumulated extracellular fluid tinged with haemoglobin from lysed red blood cells.

Autolysis is likely to play an important role in the development of the artefacts seen in this study. While the aim of freezing a carcass before necropsy is to limit autolysis, with large bodies (such as pinnipeds) the time taken to freeze and thaw increases the likelihood of autolytic changes. In addition, several authors have noted that autolysis is accelerated in the external tissues of frozen bodies (Micozzi, 1986; Zugibe and Costello, 1993). Post-mortem autolysis has been extensively studied in human forensic medicine. Passive

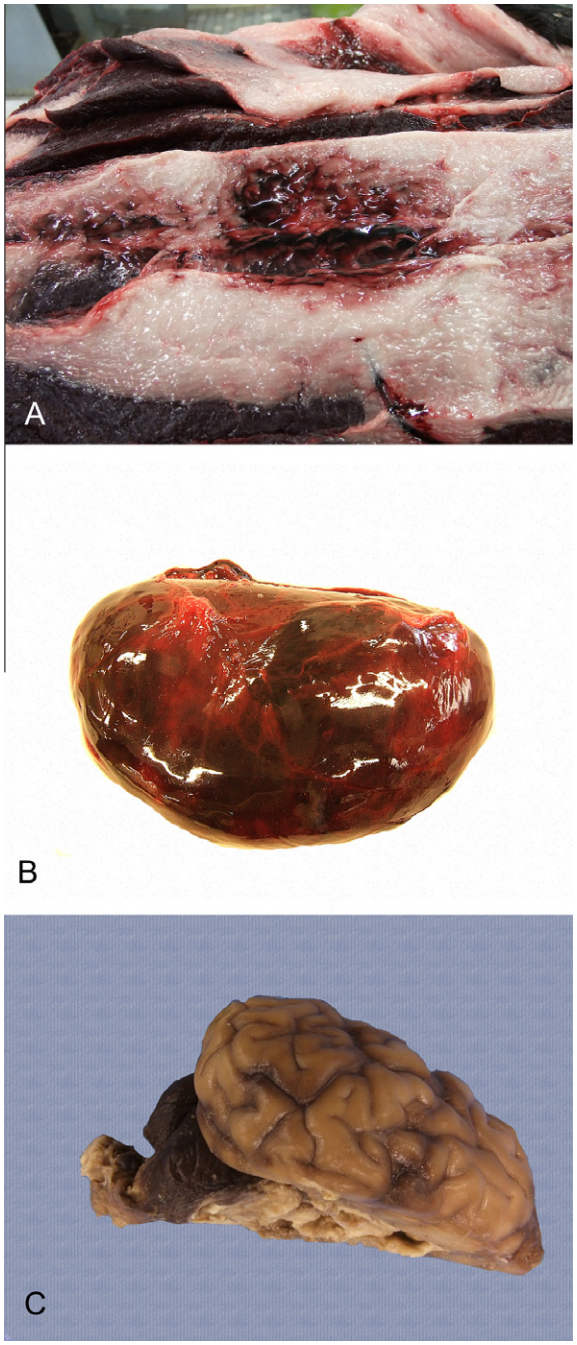

Fig. 1. (A) Dark-red, gelatinous discolouration (pseudo-bruise) in the ventral blubber of a frozen New Zealand fur seal (case No. 10). This resembles an ante-mortem bruise, but the superficial blubber and overlying skin are unaffected. (B) Renal subcapsular space from a frozen and thawed New Zealand fur seal body distended by dark-red gelatinous material resembling haemorrhage, most prominent on lumbar surface (case No. 6). (C) Dark-red discolouration of cerebellum and red mottling within sulci and along meningeal vessels of a frozen New Zealand fur seal (case No. 10).

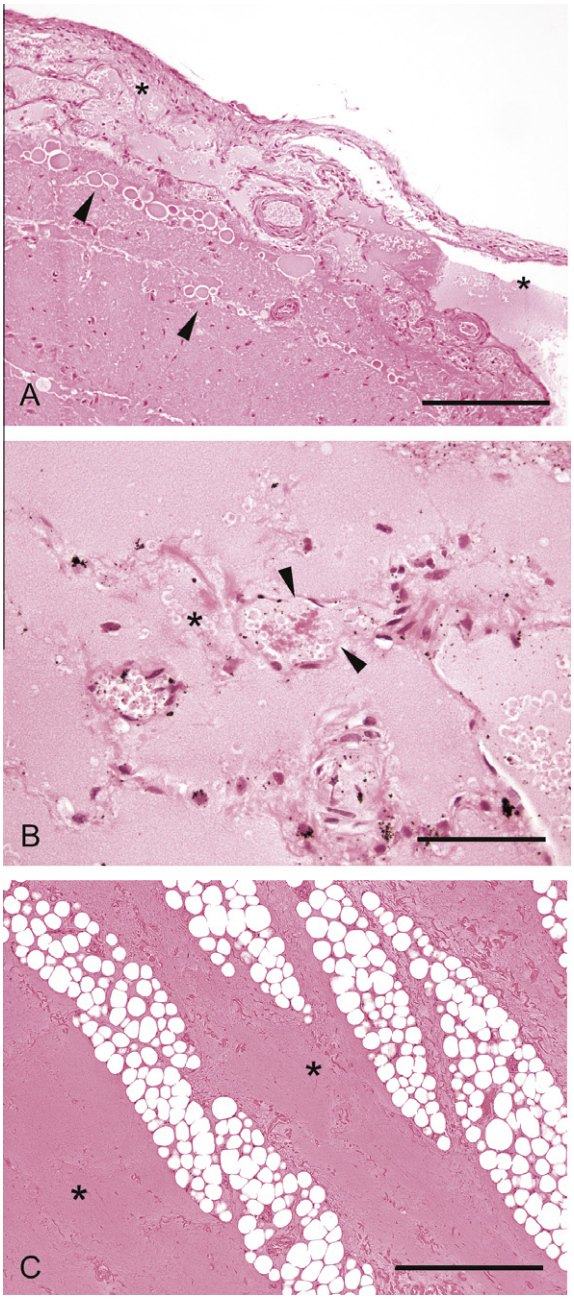

Fig. 2. (A) Cerebral cortex of a New Zealand fur seal (case No.4) illustrating a pseudo-contusion. Subarachnoid space contains homogenous to faintly stippled eosinophilic material (asterisk), with lysed and intact erythrocytes. Rows of brightly eosinophilic globules (arrowheads) present within superficial cortex. Bar = 200 μm, HE stain. (B) High power view of subarachnoid space of cerebral cortex of a New Zealand fur seal (case No. 4). Note the disrupted blood vessel wall (arrowheads), with extravascular accumulation of erythrocytes (asterisk), most of which are lysed. Bar = 200 μm, HE stain. (C) Connective tissue within blubber of previously frozen New Zealand fur seal (case No. 10), expanded by eosinophilic fluid (asterisks), grossly resembling a bruise. Bar = 400 μm, HE stain.

accumulation of blood due to 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 show erythrocytes outside blood vessels, whereas in hypostasis erythrocytes should remain 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 occurs after death (i.e. in pseudo-bruises), particularly within areas of hypostasis (Vanezis, 2001; Saukko and Knight, 2004; Bockholdt et al., 2005; Pollanen et al., 2009).

In a study of human cadavers, Pollanen et al. (2009) induced 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

autolytic, congested venous plexuses as hypostatic pressure progressively increases. Pollanen et al. (2009) proposed that these changes would be particularly likely to occur in tissues with an extensive system of thin-walled vascular channels surrounded by loose connective tissue. Blood leaking from autolytic vessels would contribute to fluid accumulations in body cavities of frozen-thawed fur seals, including the tunica vaginalis and the anterior ocular chamber, as reported in our study.

Hypostatic congestion and autolysis, along with haemoglobin staining, may also explain the staining we report in the renal capsule. 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. Renal subcapsular haemorrhage has previously been reported in other marine mammals, including beaked whales (Evans et al., 2001; Saliki et al., 2006), California sea lions and Northern fur seals (Smith et al., 1974; Gilmartin et al., 1976). While in some cases these haemorrhages have been attributed to pathological processes, the possibility that autolysis may have contributed to their occurrence remains.

Congestion, autolysis and haemoglobin staining could also have caused the changes resembling brain contusions in the seals examined in our study. Macroscopic examination of the superficial vasculature in the brains of two non-frozen animals revealed marked asymmetrical congestion of the meningeal vessels over the cerebellum and caudal occipital lobes, most likely reflecting hypostatic congestion that developed during transit. In an animal frozen and then thawed, autolytic damage to 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 hours after death.

Although this study was not designed to investigate the precise pathogenesis of the artefacts that occur following the freeze–thaw of pinniped bodies, the identification of autolysis and hypostasis as contributing factors has some implications for necropsy investigations. While it seems likely that similar changes to those seen here in pinnipeds could occur in other species, the magnitude and distribution of such changes is likely to depend on factors such as time taken to freeze and thaw, and body position during these processes. The methods used in the present study are typical of those used in field investigations, where economic and space constraints, for example on commercial fishing vessels, mean that body position during freezing cannot be standardised, and the time taken for a body to freeze cannot be controlled. In order to avoid misinterpretation of necropsy findings from previously frozen bodies, it is important that the examiner is aware of the artefacts that can develop.

The pattern of subcutaneous pseudo-bruising consistently present in the ventrum, axillae and shoulders of the seals in this study is 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 seal carcasses were thawed in dorsal recumbency making it improbable that there was a gradual, gravity-dependent increase in intravascular pressure in the ventral subcutaneous vessels during thawing. The exact cause of the pattern of distribution of pseudo-bruises is unknown, but could be related to the anatomy of the vascular supply to the blubber and skin. Further work would be required to clarify this.

Misinterpretation of artefacts is less likely to occur when necropsies are conducted by experienced veterinary pathologists. For example, the severe bruising and muscle damage present in the neck of one frozen seal was interpreted as a true ante-mortem lesion on the basis of significant damage to muscle in conjunction with haemorrhage in affected muscle, overlying blubber and skin. Similarly, pseudo-contusions of the brain are unlikely to be misinterpreted by experienced pathologists as traumatic lesions in the absence of soft tissue bruising to the head. However, given that pseudo-contusions could 'mask' true traumatic lesions, the assessment of possible head trauma should be performed on non-frozen animals whenever possible.

Histopathological examination of tissues can help to 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, however, and ageing using histological criteria is not possible in very recent bruising (Thornton and Jolly, 1986; Vanezis, 2001). Thus these criteria cannot differentiate between peri-mortem bruising and post-mortem 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 reveals extravasation of erythrocytes in a manner indistinguishable from true bruising (Prinsloo and Gordon, 1951; Pollanen et al., 2009), thus limiting the use of routine histochemistry in differentiating these changes from ante-mortem bruises. We found similar bruise-like changes in the brains, renal capsules and subcutaneous tissues of the seals examined in the current study.

Conclusions

Freezing and thawing of pinniped bodies results in artefacts that can resemble traumatic lesions. Autolysis and hypostasis play a role in the development of these artefacts, although the relative contribution of these processes remains to be elucidated. When the use of previously frozen carcasses cannot be avoided, necropsies should ideally be conducted by veterinary pathologists experienced in the interpretation of pseudo-lesions.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

Acknowledgements

All work was conducted under permit to the New Zealand Department of Conservation. This work was partly funded by the New Zealand Deepwater Group and the New Zealand Ministry of Fisheries. The authors would like to thank: Richard Wells and John Cleal for organisation of transport and other logistics; Laureline Meynier, Mike Hogan and Nicola Wallace for assistance in the post-mortem room; Evelyn Lupton and Eugene Ndeke for the processing of histology sections; Sandra Forsyth for clinical pathology expertise; and Professors I.G. Mayhew and R.D. Jolly for their useful comments.

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