HISTOPATHOLOGICAL ASSESSMENT OF DOLPHINS NECROPSIED ONBOARD VESSELS IN THE EASTERN TROPICAL PACIFIC TUNA FISHERY

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ABSTRACT

During chase and capture operations of the eastern tropical Pacific tuna purse-seine fishery, dolphins encounter multiple stressors that may cause injury. We examined tissue samples from 56 dolphins (26 *Stenella longirostris*, 28 *S. attenuata*, two *Delphinus* spp.) collected during shipboard necropsies on tuna vessels with the objectives of defining a background of naturally occurring disease, defining, if possible, the cause of death, and identifying any residua of disease or injury that might be attributed to "stress" related to the current or to prior chase and encirclement operations. Three categories of disease were defined for animals dying in the fishery operations: (1) naturally occurring diseases, unrelated to the fishery, (2) acute reactions, related to the immediate circumstances of encirclement, and (3) evidence of past injury, consistent with healing of acute injury related to the fishery or other major stressors. Parasitism was the major natural disease factor in these apparently healthy populations. Essentially all of the animals died of cardiac injury with implied impairment of cardiac conduction, arrhythmia, and widespread evidence of smooth muscle spasm, which we interpret as resulting from massive shock and stress. Acute necrosis of the renal tubules consistent with ischemia, was observed in almost all specimens. It is plausible that that in some cases, these types of injuries will cause delayed mortality, although no such mortality was demonstrated. We caution that lesions found are non-specific to the fishery, and it is possible that residua in these animals could have been produced by non-fisheries related stress in the normal environment.

INTRODUCTION

The process of chasing, encircling and releasing dolphins in the eastern tropical Pacific (ETP) tuna purse-seine fishery may result in the death of some individuals, either by recognized accident, such as net entanglement, or spontaneously, which has been attributed to "stress." Improvements in fishing technology have greatly reduced the number of deaths by recognized accident. What is not known is whether a dolphin that has been encircled and released, apparently unharmed, has suffered an injury that may result in unobserved death after release. Such hypothetical unobserved delayed deaths, not attributable to recognized trauma, might result from fishery induced stress.

Concerns regarding the effects of the tuna fishery on dolphin stocks in the ETP led to the passage of the International Dolphin Conservation Program Act (IDCPA; U.S. Public Law 105-42), a 1997 amendment to the Marine Mammal Protection Act. The law mandated that research be conducted by the Southwest Fisheries Science Center (SWFSC) to investigate the potential effects of fishery-induced stress on dolphins in the eastern tropical Pacific (ETP). The law also specifically required that the SWFSC conduct a necropsy sampling program over a three year period to examine tissues from dolphins killed during fishing operations, and "an experiment involving the repeated chasing and capturing of dolphins by means of intentional encirclement."

In accord with this mandate, the SWFSC developed a necropsy program in conjunction with the Government of Mexico and the Inter-American Tropical Tuna Commission (IATTC). The program placed trained necropsy technicians on board commercial tuna vessels to collect tissue samples from dolphins killed in the ETP fishery (mainly Pantropical spotted, *Stenella attenuata*, spinner, *S. longirostris*, and common dolphins, *Delphinus spp.*). The main objective

of the program was to evaluate the pathophysiological and immunological condition of dolphins killed in the fishery, with an additional goal of assessing overall health and disease status of the animals.

Also, a Chase Encirclement Stress Studies (CHESS) was conducted to evaluate the potential effects of fishery-induced stress on dolphins in the ETP. The studies were designed to repeatedly chase and capture a small group of dolphins and sample blood for evidence of tissue injury or changes in stress hormones and measurable immune parameters. In this way the studies could model fisheries operations in which dolphins might be set upon repeatedly during the course of a fishing season, but with identification of repeatedly encircled individuals.

This report describes the histopathology findings from examination of specimens collected in both the necropsy and CHESS programs. Results are based on gross and histopathologic examination of tissues. The three main objectives of the histopathology study were: (1) define a background of naturally occurring disease in the subject populations, (2) define, if possible, the cause of death in the subject animals, and (3) identify any residua (scars or other lesions) of disease or injury that might be attributed to "stress" related to prior chase and encirclement operations.

Background

During chase and capture operations of the ETP tuna purse-seine fishery, dolphins encounter multiple stressors. Curry (1999)2 identified specific stressors (e.g.; social separation, isolation and restraint, novelty) that could plausibly elicit an immediate physiological stress response of the hypothalamic-pituitary-adrenal (HPA) axis for dolphins involved in the fishery. In addition to eliciting an adrenocortical response, fisheries operations could cause muscle damage and hyperthermia. Finally, chronic stress or repeated acute stress can have maladaptive effects on immune responses, reproductive function and growth among others (Moberg 1987a , 1991; Rivier and Rivest, 1991; Chrousos, 1992; Chrousos and Gold, 1992; Chrousos, 1995; McEwen et al., 1997).

Available data on physiological effects of capture in cetaceans indicate that the process does elicit a stress response (activation of the HPA axis; Thomson and Geraci, 1986; St. Aubin *et al.,* 1996). Capture stress is also known to cause muscle damage in cetaceans (Geraci and Medway, 1973; St. Aubin and Geraci, 1989). Several effects related to immune function (changes in blood leukocytes and decreases in blood iron levels) have also been observed in captured cetaceans.

An Operational Definition of Stress

A single definition of "stress" has not been generally accepted by workers in the field of stress research (Levine, 1985; Moberg, 1987b; Chrousos *et al.,* 1988; Levine and Ursin, 1991; Fowler 1995). Here, we define the term stress as "demand for adaptation" with the recognition that organisms are in a constant condition of adjustment of physiological systems to maintain homeostasis. Stress is then specified in terms of the demand (stressor) applied (e.g. heat stress,

2 Curry, B. E., 1999. Stress in Mammals: The potential influence of fishery-induced stress on dolphins in the eastern tropical Pacific Ocean. NOAA Technical Memorandum. NOAA-TM-NMFS-SWFSC-260.

cold stress, crowding stress, etc). Thus, in cetaceans physiologic stress occurs in the adaptations needed to adjust to diving, for flight from predators or from intra-specific aggression, or to catch prey. It may also result from social interaction (Fowler, 1995). In addition, stress may occur whenever a wild animal is restrained or enclosed, however loose the restraint or enclosure might seem to the captor. Also of note, apprehension may be a mild psychological stressor that may intensify to become anxiety, fright or even terror (Fowler, 1995). In this context, it is clear that the issue is not whether dolphins are 'under stress' in a given condition, they are always under demand for adaptation, but whether the degree of stress experienced is physiologically damaging.

The effects of stress are expected to vary with the adaptive mechanisms of the subject, and will therefore vary with the species and its environment. Since stress may have a psychological component, which could be influenced by experience, it varies among individuals of the same species (see Hinkle, 1974; Lyons *et al.,* 1988a,b). These differences in the effects of stress on individuals occur in part because stress is not imposed, but is an expression of a response to a stressor. Unfamiliar or extreme stressors may evoke unusual or extreme responses. That is, damage may result from inappropriate or extreme adaptive responses, and, for example, in extreme situations some animals might undergo extreme, fatal cholinergic bradycardia (Fowler 1995).

Researchers have established some basic knowledge of the physiological responses to stress in cetaceans that can be applied to understanding the potential effects of fishery induced stress on dolphins in the ETP. This information was reviewed by Curry (1999) and is briefly outlined below.

Stress in Dolphins

Cetaceans exhibit the general mammalian response to stress, and there has been considerable research investigating the effects of stress on cetaceans. The physiological responses to stress that have been well documented in cetaceans include adrenocortical responses and effects on thyroid hormone balance. Elevated cortisol levels have been observed in cetaceans subjected to stressors such as capture, handling, and restraint, although the elevations appear to be modest in comparison to those known for other mammals experiencing similar stressors (Thomson and Geraci, 1986; St. Aubin and Geraci, 1990). St. Aubin and Geraci (1990) noted that, despite this apparently modest response, the systemic effects of cortisol are still evident in decreased circulating levels of eosinophils and reduced plasma iron, as well as in increased levels of glucose.

Also of note regarding the cetacean response to stress, aldosterone, not typically characterizing the adrenal response to stress in terrestrial mammals, is greatly increased in cetaceans (and pinnipeds) subjected to adrenocortical stimulation (Thomson and Geraci, 1986; St. Aubin and Geraci 1990). Aldosterone functions to enhance water and sodium reabsorbtion, so the response observed for cetacean and pinniped species studied thus far is thought to reflect the necessity of regulating these processes during stress (St. Aubin and Geraci, 1986; St. Aubin *et al.,* 1996).

Thyroid hormone balance in cetaceans appears to be sensitive to stress (Ridgway and Patton, 1971; Orlov *et al.,* 1988), and specifically to capture stress. St. Aubin and Geraci (1988, 1992) found that thyroid levels were suppressed during capture stress in free-ranging beluga whales, *Delphinapterus leucas*. Although chronic thyroid hormone imbalance can have

deleterious effects on growth and metabolism, the changes seen in these studies of captive cetaceans have been interpreted as adaptive responses to glucocorticoids, acting to realign thyroid hormone balance (St. Aubin and Geraci, 1988, 1992; and see St. Aubin *et al.,* 1996).

Dolphins killed in the ETP tuna purse-seine fishery were examined by Cowan and Walker (1979).3 The purpose of the study was to conduct necropsies on all available dolphin casualties occurring incidental to population stock assessment research and gear technology research on two dedicated cruises (April 17-June 5, 1978, and September 12-October 31, 1978). A total of 68 animals, 49 *S. attenuata* and 19 *S. longirostris* were available for examination. Detailed accounts of the manner and time of death and any observable behaviors were recorded for specimens examined. Cowan and Walker (1979) collected representative tissue samples from both diseased and apparently healthy organ systems, and provided descriptions of grossly evident lesions. The study also included descriptive accounts of parasitism and apparent related tissue changes.

Cowan and Walker (1979) defined three categories of disease: (1) naturally occurring disease, (2) tissue changes resulting from acute responses to terminal events, and (3) subacute pathological conditions that could hypothetically lead to death. Parasitism was found to be the most prevalent cause of naturally occurring disease. Acute tissue changes were described for the lungs, heart, adrenal glands, and spleen.

The authors concluded that there was no substantial evidence of "delayed mortality" related to the fishery (Cowan and Walker, 1979). However, they noted that several of the dolphins apparently died of massive cardiac reaction to stress and were documented to have cardiac lesions consistent with those produced in laboratory animals injected with catecholamine and humans thought to have died of stress cardiomyopathy (see Cebelin and Hirsch, 1980).
Here we report findings observed in dolphins necropsied onboard fishing vessels in the ETP. Our sampling protocol benefited from these previous works investigating the effects of stress on cetaceans, and the categorization of our results is broadly based on the study conducted by Cowan and Walker (1979), although myocardial sampling in the present study is much more extensive and systematic than in that early work.

An independent scientific peer review of this work was administered by the Center for Independent Experts located at the University of Miami. Responses to reviewer's comments can be found in Appendix 2.

MATERIALS AND METHODS

During the course of the necropsy study two training courses were conducted to prepare technicians for the shipboard collection of tissue samples. The first course included 10 technicians from the Mexican National Observer Program and the IATTC, and was conducted at the SWFSC, La Jolla, California, in January, 1999 (see Curry *et al.,* 1999). The second course was conducted at the SWFSC in June, 2001. The 15 technicians were from the Mexican, Venezuelan and Ecuadorian National Observer Programs and from the IATTC. The technicians

3 Cowan, D. F., and Walker, W. A. 1979. Disease factors in *Stenella attenuata* and *Stenella longirostris* taken in the eastern tropical Pacific yellowfin tuna purse seine fishery. Southwest Fisheries Science Center, National Marine Fisheries Service, NOAA, Administrative Report No. LJ-79-32C. 21 pp.

were made familiar with the objectives of the research, the protocols and sampling methods and had the opportunity to perform supervised complete necropsies on several dolphin carcasses.

The overall necropsy sampling procedure (Appendix 1) was conducted in two parts, levels one and two. This contingency strategy was developed to accommodate three general factors. The first factor was the relative importance of each tissue sample to investigating the potential effects of fishery-induced stress on the dolphins. Second, the expediency of collecting particular samples to avoid post-mortem changes that may have compromised the quality of organs or tissues collected. Third, shipboard constraints such as time, space and weather conditions had to be considered. In general then, the most important samples were collected first so that if the necropsy had to be abandoned at least some potentially valuable samples might have been collected.

Samples were collected onboard fishing vessels from November 1999 to October 2001. A total of 56 dolphins from fisheries operations was examined (Table 1). These included 26 animals identified as *S. longirostris* (10 male and 16 female), 28 *S. attenuata* (11 male and 17 female and two *Delphinus* spp. (1 male and 1 female). Two females, 1 *S. longirostris* and 1 *S. attenuata*, that died during CHESS, were sampled according to the necropsy protocol.
All tissues processed in the laboratory were observed to have been properly labeled and fixed in 10% neutral buffered formalin. All animals were represented by level 1 samples; many included level 2 samples (see Table 1). Tissues were trimmed to the appropriate size and processed through graded alcohols to dehydration, passed through xylene to infiltrate with paraffin, according to long-established standard procedure. Tissue blocks thus prepared were sectioned at 5 microns, placed on glass slides, stained with hematoxylin, phloxine and safranin (HPS) trichrome stain, and coverslipped. HPS is a trichrome stain used to help differentiate between collagen fibers and smooth muscle. Slides thus prepared were examined using ordinary light microscopy.

As requested, field technicians sliced the hearts in the transverse plane, held the sliced heart together with a looped cord, and returned the whole heart. In the laboratory, the entire heart was inspected, and in the absence of any specific grossly recognizable abnormality, a standard slice selected for sampling. The slice selected was the one that represented the level of the tip of the left anterior papillary muscle, an easily recognized standard landmark. Heart sections included anterior left ventricle, lateral left ventricle, posterior left ventricle, anterior right ventricle, lateral right ventricle, posterior right ventricle, and septum from the standard slice, and a sample of left and right atria. In addition to the sampling of every heart just described, any grossly observed lesion was also sampled for histologic study.

RESULTS

In the assessment of the incidence and extent of pathological findings obtained, three categories of disease were defined for animals dying in the fisheries operation.

(1) naturally occurring diseases, unrelated to the fishery.

(2) acute reactions, related to the immediate circumstances of encirclement.

(3) evidence of past injury, consistent with healing of acute injury related to the fishery or other major stressors.

The results relative to each of these three categories are described below.

1. Naturally occurring diseases, unrelated to the fishery

Parasitism was evidenced by occasional minor foci of inflammation in the lungs, associated with infestation with nematode lung worms, species not identified. Occasional small lung abscesses associated with parasitism were also observed. Small ulcerations of the gastric mucosa, probably associated with nematode parasitism, were evident, as were small foci of granulomatous inflammation of the gastric mucosa (second and third chambers) related to sites if invasion or attachment by small nematodes. Parasitic infestation of lymph nodes was observed in 5 of 52 animals examined.

In addition, there were several other pathologies considered to result from naturally occurring diseases. There were frequent (majority of animals) minor foci of intra-alveolar and interstitial pneumonitis. The cause was not determined. These foci were compatible with parasitic infestation, but parasites were not identified. There were two instances of microabscesses in lymph nodes, with cause not determined. Also, there were two instances of unilateral diffuse cortical hyperplasia of an adrenal gland. And, there were many (24 of 52 animals) instances of focal, occasionally multi-focal chronic inflammatory infiltration into the cortex of the kidney (focal interstitial nephritis); small renal cortical abscesses; some kidneys with patchy fibrosis or cortical scar.

2. Acute reactions, related to the immediate circumstances of encirclement

The major findings in this category were observed in the heart; more specifically, the myocardium. Among the specimens examined there was frequent blotchy mottling of the heart, with areas of pallor alternating with areas of congestion, suggesting circulatory disturbance.

Microscopic changes including hyalinized fibers and wavy fibers, perinuclear vacuolation, and contraction band necrosis (CBN; see Discussion) were nearly universal. These changes are widespread throughout the organ, but occur with greatest intensity in the subepicardial and subendocardial zones of the ventricles, especially the right ventricle, and both atria, and most severely in the right atrium. Similar findings were frequently observed in the fibers of the conduction system.

In addition to the myocardial changes, contraction banding was identified in the smooth muscle of intramural coronary arteries, and in a subset of animals, eccentric plaque formation in the intima of very small arteries, as well as disorder of arterial smooth muscle were identified. Indications of prolonged coronary artery spasm (intramural coronary arteries) were also found, including hyalinization of myocytes and architectural disturbance.

Also identified was contraction banding of smooth muscle of the viscera (intestine, urinary bladder) and in the media of vessels of many organs. Bronchospasm and pulmonary air-trapping was very common, almost universal. Acute necrosis of segments of the renal tubules (acute tubular necrosis or ATN), commonly with infraglomerular reflux, was a very common finding (46 of 52 animals).

3. Evidence of injury in the past, consistent with healing of acute injury related to the fishery or other major stressors

There was one instance of grossly recognizable superficial indentation (scar) of the anterior left ventricle, associated with patchy fibrosis of the atria.

Small patchy fibrous scars in the myocardium, of a size and in locations consistent with myocardial necrosis, as described in Category 2 above, were common, found in 20 of 56 animals (36%). Most of these scars were minimal. Patchy fibrosis in the atria was present in about a third of animals.

There was also fibrous plaquing of the intima of small intramural coronary arteries, consistent with the sequella of platelet thrombosis of the arteries.

Residue of smooth muscle injury in the viscera was not identified.

Within this sample set, there was apparently no difference in findings among species or geographic location of origin. The two animals that died from net entanglement in the CHESS study showed findings indistinguishable either in kind or severity from those collected during fishery operations by our trained technicians.

DISCUSSION

The results of the current study strongly indicate that the cause of death of the dolphins dying in the purse-seines was acute endogenously generated myocardial injury, leading to arrhythmia and sudden death. However, we must caution that lesions found are non-specific to the fishery in that they can be found in dolphins in a variety of locations, in a variety of circumstances. It is therefore possible that residua (scars and vessel abnormalities) in fisheries animals could have been produced by non-fishery related stress in the normal environment. The discussion to follow addresses two global issues. First we suggest that delphinid physiology, particularly cardiovascular adaptations to diving, may, under stress, predispose them to the types of lesions observed in this study. Second, we describe the nature of the lesions in detail, including their fate and the mechanisms by which they are known to occur.

Over several decades of examining beach stranded and net-caught dolphins at necropsy we have observed some recurring patterns of changes in organs and tissues that are similar to those described here for dolphins killed in the ETP fishery. In some cases, the changes we observed in dolphins studied elsewhere (e.g. Cowan *et al.,* 1986), like those described here, are consistent with injury caused by massive release of endogenous catecholamines (*alarm reaction*) or by vasospasm, including spasm of small cardiac arteries, with ischemia and reperfusion. This recurring pattern of pathology includes CBN of cardiac (Turnbull and Cowan, 1998) and smooth muscle; ischemic injury to the intestinal mucosa, especially the mucosa of the small intestine; and acute necrosis (ATN) of the proximal tubules of the nephron. The pattern appears to result from a stereotypic stress response, independent of the nature of the provoking stimulus.

We suggest that this recurring pattern of pathology in response to stress may be related to the physiological adaptations required for diving. A dolphin in free dive (voluntary dive) undergoes certain physiologic adjustments reflective of exercise, including a reflexive apnea, with voluntary over-ride, minimal cardiovascular adjustments, and a general maintenance of aerobic metabolism (Butler and Jones, 1997). Blood flow is reduced to the gut and kidneys, but maintained in the heart, brain and to a degree, exercising muscles. The animal is able to surface and dive repeatedly (foraging dive pattern), as there is little lactic acid build-up. This has been termed the "dive response." A dolphin in an *involuntary* dive situation undergoes a somewhat different set of adjustments, which have been termed the "dive reflex," but which may better be termed an alarm reaction. These adjustments include not only reflexive apnea, but also a change in heart rate and cardiac output, vasoconstriction with markedly decreased perfusion of gut, liver,

kidneys, and skeletal muscle, with substantial increase in production of lactic acid in these tissues, which is reflected in marked rise in blood levels on surfacing (Butler, 1982; Ridgway, 1986). The clear implication of the distinctive reactions to voluntary and involuntary diving is that the dolphin is responding to the environment *as it is perceived*; the triggering of the alarm reaction is a reaction to a situation interpreted by the dolphin as a dire threat, and is responded to by a marked autonomic reaction. Since the major threats to an aquatic, air breathing mammal are drowning and predation, the alarm reaction is an accentuation of the physiologic dive and escape responses.

In responding to a perceived threat, a dolphin will mobilize for an alarm reaction or "fight or flight," according to the established concept of stress (see Cannon and De La Paz, 1911). In terrestrial mammals, flight means acceleration of muscular activity, elevation of blood pressure, tachycardia and hyperventilation. For a diving animal, however, mobilization for flight means breath-holding, and re-directing the flow of blood away from non-vital to vital oxygen-dependent organs; i.e., the brain and the heart. This is physiologic, and non-injurious, provided the changes are coherent, and not extreme or overly protracted. In the instance of a novel threat perceived as extreme, the smooth coordination of the cardiovascular adjustments may break down and a massive release of adrenergic hormone from the adrenal medulla occurs (Eliot *et al.,* 1977). That is, a "sympathetic storm" (characterized by the pronounced release of catecholamines), occurs with spasm of small intramural coronary arteries preceding myocardial ischemia, as is true, for example, in human drowning (Lunt and Rose, 1987). This ischemia may be associated with arrhythmia, causing death. Alternatively an individual may survive, the ischemic injury having caused patchy necrosis of myocytes and scaring, or having left no evidence of residual injury.

The histopathogical evidence of myocardial ischemia and smooth muscle injury to visceral organs examined in the current study is definitive. The lesions of the myocardium observed in these dolphins are all well recognized in the clinical and experimental literature. All are attributable to catecholamine injury (Reichenbach and Benditt, 1970; Cebelin and Hirsch, 1980; Turnbull and Cowan 1998), or to ischemia (Mukherjee *et al.,* 1982; Muntz *et al.,* 1984). In fact, the final effector in ischemia may be the local release of catecholamines from the nerves in the heart. (Mukherjee *et al.,* 1982). Sudden cardiac death, that is death occurring either instantaneously, without preceding symptoms, or within minutes or hours after onset of symptoms could have occurred in some if not all of the specimens examined in the current study. Most cases of sudden cardiac death are attributable to a cardiac arrhythmia, especially ventricular fibrillation (Cobb *et al.,* 1974, Hinkle and Thaler, 1982), typically associated with ischemia.

Of particular note, the lesions of the vessels in these dolphins suggest spasm, which can produce ischemic injury with or without necrosis directly (Maseri and Chierchia, 1982), or by reperfusion, that is, interruption of blood flow followed after an interval by re-establishment of flow. The blotchy discoloration of the hearts observable on gross examination strongly suggests alternating areas of congestion and ischemia, which may be explained by irregular perfusion. Such cases of ischemia and reperfusion are known to produce several kinds of effects on myocardium. Profound functional changes precede appearance of microscopic evidence of injury.

A common feature of all the myocardial lesions described in the current study was vasospasm leading to ischemic injury, followed by reperfusion and reperfusion injury. We also found evidence of endogenous catecholamine injury to the myocardium, well known in human

medicine in experimental animals as an extreme stress response. A secondary effect of intestinal ischemia is to permit seeding of the blood with bacteria normally resident in the intestine, producing an often mixed bacteremia and sepsis. The types of cardiac injuries observed in the specimens we examined, and the functional changes associated with these injuries are described in detail below.

Catecholamine effects / catecholamine cardiomyopathy: Massive release of catecholamine from the adrenal medulla can cause cardiac injury and sudden death (Reichenbach and Benditt, 1970, Cibelin and Hirsch, 1980; Turnbull and Cowan, 1997). In myocardial ischemia, endogenous catecholamine (norepinephrine) is also released from adrenergic nerve terminals within the first hour after the onset of occlusion. Simultaneously, the number of exposed beta receptors detected in membrane fractions of homogenized cardiac myocytes increase (Mukherjee *et al.,* 1982). The cause of norepinephrine release is uncertain; however, re-uptake of norepinephrine may be decreased because it is an ATP-dependent process. (Muntz, *et al.,* 1984).

Coronary artery spasm: Coronary artery spasm, if prolonged, may cause myocardial necrosis (Maseri and Chierchia, 1982). Coronary artery spasm alone, or in conjunction with platelet aggregation has become a recognized cause of acute myocardial ischemia (Folts *et al.,* 1982; Maseri *et al.,* 1978; Oliva and Breckinridge, 1977). Reperfusion (see below) may follow relaxation of occlusive vasospasm.

Evidence for spasm of the intramural coronary arteries in dolphins: Spasm of small arteries can result in disruption of the vascular endothelium, with local thrombosis, and may also be associated with disturbance of the micro-architecture of the vessel, especially if individual smooth muscle cells undergo CBN. Architectural disturbance of the muscular media of small arteries is a common finding in dolphins in this study. Micro-thrombi are removed by a process of organization, with in-growth of fibrocytes, resulting in a fibrous plaque.

Ischemia of the myocardium: Within the first few minutes after the onset of ischemia, ultrastructural changes develop which include cellular and mitochondrial swelling, progressive loss of sarcoplasmic glycogen particles, and mild margination of nuclear chromatin (Jennings, Hawkin *et al.,* 1978; Schaper *et al.,* 1979; Jennings *et al.,* 1985). These early changes have been shown to be reversible in experimental studies, in that restoration of coronary blood flow after coronary occlusion up to 15 minutes in duration results in rapid recovery of myocyte ultrastructure even in the most severely ischemic zone. (Jennings, *et al.,* 1985).

With a longer duration of ischemia, however, these changes become progressively more pronounced. In addition, two ultrastructural features of injury develop that have been associated with the transition to irreversible injury. These are the development of amorphous densities within the matrix of mitochondria and the development of breaks within the trilaminar unit membrane of the sarcoplasm (Jennings and Reimer, 1981; Reimer *et al.,* 1983).

Hyalinized and wavy myocardial fibers: Hyalinized fibers are those fibers that bind more stain than surrounding fibers, and show homogenization of cytoplasm and nuclear condensation, all signs of cell injury. Wavy fiber refers to a change in the appearance of a myocardial fiber in which it becomes thin and attenuated, dense or hyalinized, and assumes an angulated or "wavy" accordion pleated appearance, as if it is longer than the space in which it is contained, and is

longitudinally compressed until it buckles. Wavy fibers, especially when associated with focal edema, are a characteristic sign of acute myocardial ischemia (Bouchardy and Majno 1971/72, 1974; Eichbaum, 1975). Beside their regular occurrence in early myocardial infarcts, wavy myocardial fibers are also frequently encountered in cases of acute adrenergic heart injury and after injection of high doses of catecholamines in experimental animals (Eichbaum, 1975). Ischemic wavy fibers are not necessarily necrotic, although they may well become so. This implies that with a period of recovery associated with restoration of perfusion, they may return to a normal appearance and function. If they do not recover, they may be replaced by scar.

Perinuclear vacuoles: This is the formation of intracytoplasmic spaces ("bubbles") at the nuclear poles. Perinuclear vacuoles are reported to result from ischemia, and are found in individuals suffering sudden cardiogenic death (Adegboyega *et al.,* 1996). Such vacuoles are found in many animals in this study.

Reperfusion injury: Ischemia followed by reperfusion of the ischemic area is held to produce both reversible and irreversible changes distinguishable from those occurring in a permanently ischemic focus. Four basic types of injury can result from reperfusion (Hansen, 1995). First, irreversible injury of myocardial cells can occur during the period of ischemia. On reperfusion they undergo explosive cell swelling, contraction banding, and calcium accumulation. Contraction banding is a manifestation of disruptive spastic contraction of contractile elements in a myocyte and in conduction fibers, producing a characteristic coarse cross-banding of the affected fiber. This is seen as acceleration of the process of necrosis. No recovery is possible. Second, reversibly injured cells on reperfusion may be transiently impaired. This is the so-called "stunned myocardium" from which full recovery is expected. Third, reperfusion arrhythmia, which may resolve or progress to ventricular fibrillation. The fourth type of injury is known as lethal reperfusion injury, which is defined as myocardial cell death due to reperfusion.

In addition to these cardiac injuries, many of the dolphins in this study showed expansion or over- expansion of alveolar spaces, when the normal elasticity of the lung should have caused collapse on opening the chest cavities. This is attributed to a readily observable spasm of the sphincter muscles of the distal airways. These smooth muscle structures also showed over-contraction, very similar to the over-contraction of the small intramural coronary arteries.
We are inclined to attribute this highly stereotyped pattern of smooth muscle spasm of arterial media, bronchial sphincters and visceral smooth muscle to a single cause - massive protracted autonomic discharge, or the sympathetic storm, which is accompanied by high levels of circulating catecholamines. This discharge occurs as the major manifestation of an extreme stress response in the dolphins.

Almost all dolphins in this study had lesions of acute necrosis of the renal tubule. This lesion is most commonly caused by prolonged renal ischemia (Meyers, *et al.,* 1986, Kaufman, *et al.,* 1991, Kashgarian, *et al.,* 1998). Infraglomerular tubular reflux is the movement or intrusion of detached proximal tubular epithelium into Bowman's space, around the renal glomerulus. It is generally taken as a very sensitive indicator of tubular epithelial damage (Waugh, *et al.,* 1964; Kashgarian *et al.,* 1998).

Curry (1999) reviewed the condition and its potential effects on dolphins involved in the ETP fishery. This finding of ATN in dolphins killed in the ETP is consistent with the histological observations of tissues from mammals suffering from a condition known as capture myopathy. Capture myopathy, a condition resulting from muscle exertion associated with

capture and restraint of wildlife, is characterized by a variable and lengthy list of clinical signs including ataxia, paralysis, myoglobinuria, and acute muscle degeneration (Hulland, 1985; Harthoon and Young, 1974; Bartsch *et al.*, 1977; Chalmers and Barrett, 1977; Basson and Hofmeyr, 1978). The condition can be induced by a combination of many stressors (e.g. terror, chase, capture, restraint), and that it is associated with exhaustion of the normal physiological reserves that provide energy for escape. Acute tubular necrosis and severe glomerular damage have been identified in numerous studies of mammals that died of capture myopathy and are considered to be the result of renal hypoxia caused by catecholamine activity related to shock or the sympathetic storm as described above (Haenichen and Barth, 1980; Spraker, 1993; Wallace *et al.,* 1987; Williams and Thorne, 1996) Myoglobinuric casts that could result from myopathy were not found in the renal tubules of dolphins in the present study.

It is worth emphasizing that myocardial injury related to "stress" is not limited to dolphins but can occur in many species, under both stringent artificial laboratory conditions, and under natural conditions. For a thorough review, see van Vleet and Ferrans (1986).

SUMMARY

The histopathologic study reported here had three main objectives: (1) define a background of naturally occurring disease in the subject populations, (2) define, if possible, the cause of death in the subject animals, and (3) identify any residua (scars or other lesions) of disease or injury that might be attributed to "stress" related to prior chase and encirclement operations.

(1) **Define a background of naturally occurring disease in the subject populations:**
As was documented by Cowan and Walker (1979), the major natural disease factor in the dolphin populations sampled is parasitism. Especially prevalent was parasitism by an unidentified nematode lungworm. The other common finding was of small, often multiple foci of chronic interstitial inflammation in the kidneys. These conditions were not considered to be a significant mortality factor. This background of disease is almost trivial when compared with parasitic disease found in some in-shore populations, and we consider that the population as sampled is generally healthy, with adequately functioning immune systems, evidenced by this low level of infectious and parasitic disease. However, it must be emphasized that the over-all sample size of 56 animals of 3 species is too small to reveal conditions of low frequency, in the less than 1-2% frequency range. We do not consider this objective to have been fulfulled. Further study using a much larger sample size is required to discover low frequency conditions.

(2) **Define, if possible, the cause of death in the subject animals:**

Essentially all animals sampled died with highly stereotyped findings; cardiac injury, with implied impairment of cardiac conduction, arrhythmia and muscle contractility and widespread evidence of smooth muscle spasm, including smooth muscle of small muscular arteries. All of the lesions are documented in the clinical and experimental literature, and mechanisms of production are reasonably well understood. These are interpreted, in the setting of the study, as resulting from massive shock and stress reactions.

Capture myopathy-related injury to the heart, suffered in acute shock, can cause death. Turnbull and Cowan (1998) hypothesized that dolphins are particularly susceptible to stress

cardiomyopathy. They found lesions typical of those attributed in other species to direct myocardial injury from catecholamines, or from coronary artery branch spasm induced by catecholamines or autonomic discharge (CBN), in stranded dolphins (Turnbull and Cowan, 1998). Given the conclusions of Cowan and Walker (1979), that stress cardiomyopathy occurred in dolphins killed in tuna purse-seine nets, and our own findings, it is seems that CBN and related changes could lead to the death of animals captured in the ETP fishery, although they cannot be thought of as specific to the fishery.

Our histopathologic findings from observation of tissues collected from dolphins killed in the ETP tuna fishery suggest that the individuals underwent a reflexive response to a perceived threat, or an alarm reaction, activating all the physiologic adaptations to diving or escape to an extreme or pathological level, resulting in widespread ischemic injury to tissues. This conclusion is supported by catecholamine concentrations measured in one of the two CHESS mortalities. Levels of epinephrine, nor-epinephrine and dopamine were all roughly an order of magnitude greater than the maximum values obtained for any of the living, surviving dolphins (Romano and St. Aubin, 2002). It should be noted that the sample was collected from the heart and hour or more after death, and the specific values may not actually represent circulating levels at the time of death. Those substances are labile, and actual levels may have been higher.
Given the near-universality of myocardial lesions, we are confident that the cause of death of the dolphins in this study is identified, and that the objective has been achieved.

(3) **identify any residua (scars or other lesions) of disease or injury that might be attributed to "stress" related to prior chase and encirclement operations:**

A significant number of animals examined had lesions of the heart and its small vessels that were quite consistent in size, location and type with being the outcome of the acute lesions observed. These include small patchy myocardial scars and abnormalities of small vessels consistent with mural injury due to spasm, and also microthrombi in the process of organization, or the presence of small plaques that are thought to result from endothelial injury and local thrombosis. The implication of this is that there are animals whose cardiac injury is of such a degree that they die in the nets, and other animals who suffer similar injury, but to lesser degree, who survive either to resolve the lesions without scar, or to replace damaged tissue with scar. The two animals who were known to have been set on and caught more than once (CHESS specimens) had similar lesions to those found in animals dying in fisheries operations. This indicates that fisheries animals too could have been set on more than once, which is consistent with observations.

An important related question is whether some animals can suffer similar injuries but survive to die later, after release. What is the fate of the myocardial injury; i.e.; is healing possible, or will survived injury inevitably be followed by scarring? It appears that some will heal, and some will scar. It seems obvious that an animal with a scar has survived the injury that caused the scar. A similar question applies to the renal lesions, which if severe and extensive, may be associated with renal failure. It seems plausible that in some cases these types of injuries will cause delayed mortality. This is speculative at this time, as the experimental studies that follow the course of these lesions in dolphins to provide definitive answers have not been done. The earlier study by Cowan and Walker (1979) found no evidence of lesions that might relate to delayed mortality . However, that study included only a limited sampling of myocardium, and, those authors did not have the advantage of insights gained from the considerable research done

on reperfusion and other forms of myocardial injury done over the intervening 23 years. Any delayed mortality, given the nature of the lesion seen in animals that have died would most likely be an infrequent event.

Again, however, it must be emphasized that the over-all sample size is too small to reveal conditions of low frequency, as defined above. We do not consider this objective to have been completely fulfulled. Further study using a much larger sample size is required to discover low frequency conditions.

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Table 1. List of specimens necropsied onboard tuna purse-seine vessels in the ETP and examined for histopathology. Abbreviations - Sa=*Stenella attenuata*, Sl=*S. longirostris*, D=*Delphinus* sp.

Specimen Number	Species	Sex	Total Length (cm)	Collection Date	Location	Level 1	Level 2
CEE001	Sa	F	175	10-Nov-99	08E46'N 123E28'W	complete	complete
CEE002	Sa	F	124	10-Nov-99	08E46'N 123E28'W	complete	complete
CEE003	Sl	F	140	12-Nov-99	13E38'N 116E32'W	complete	complete
CEE004	Sl	F	167	23-Nov-99	13E44'N 109E42'W	complete	complete
MJB001	Sl	F	154	13-Nov-99	09E20'N 127E05"W	complete	complete
JCJ001	Sa	F	179	02-Mar-00	15E12'N 105E22'W	complete	incomplete
JCJ002	Sl	M	153	02-Mar-00	15E12'N 105E22'W	complete	incomplete
JCJ003	Sl	M	142	02-Mar-00	15E12'N 105E22'W	complete	incomplete
LBZ001	Sl	F	142	09-May-00	22E09'N 106E36'W	complete	incomplete
MAG001	Sl	F	165	22-Apr-00	13E59'N 114E35'W	complete	complete
MAG002	Sa	M	135	06-May-00	13E32'N 114E53'W	complete	complete
JCJ004	Sl	F	163.5	01-Sep-00	11E46'N 099E27'W	complete	incomplete
JCJ005	Sa	F	80	01-Sep-00	11E46'N 099E27'W	complete	incomplete
LBZ002	Sa	F	190	06-Sep-00	12E30'N 100E22'W	complete	incomplete
LBZ003	Sa	M	191	01-Sep-00	13E34'N 113E57'W	complete	incomplete
CTC001	Sl	M	176	26-Sep-00	13E26'N 114E17"W	complete	incomplete
MAG003	Dspp	F	171	23-Aug-00	24E30'N 113E05"W	complete	complete
SBZ001	Sa	M	200	19-Sep-00	01E19'N 099E05"W	complete	complete
SBZ002	Sa	M	163	10-Oct-00	06E49'N 100E23"W	complete	complete
JOG001	Sa	F	188	28-Nov-00	13E06'N 106E08'W	complete	complete
JOG002	Sa	M	196	27-Nov-00	09E04'N 108E21'W	complete	complete
JCJ006	Sl	M	180	18-Nov-00	07E38'N 123E07'W	complete	incomplete
JCJ007	Sl	F	173	18-Nov-00	07E38'N 123E07'W	complete	incomplete
MAG004	Sa	F	189	06-Dec-00	06E97'N 111E01'W	complete	complete
LBZ004	Dspp	M	175	26-Feb-01	23E21'N 111E56'W	complete	complete
MAG005	Sa	M	163	09-Mar-01	07E05'N 098E03'W	incomplete	incomplete
MAG006	Sa	M	137	09-Mar-01	07E05'N 098E03'W	complete	incomplete
JCJ008	Sa	F	180	10-Mar-01	06E25'N 092E29'W	complete	complete
CTC004	Sa	M	171	08-Apr-01	05E52'N 090E04'W	complete	complete
JOG003	Sl	M	193	07-May-01	13E46'N 115E20'W	complete	complete
MJB002	Sa	F	175	19-May-01	08E18'N 121E43'W	complete	incomplete
MJB003	Sa	F	119	25-May-01	08E09'N 128E36'W	complete	complete
SBZ003	Sl	M	161	01-May-01	22E59'N 106E46'W	complete	complete
SBZ004	Sl	F	160	23-May-01	07E56'N 126E34'W	complete	complete
SBZ005	Sl	F	177	23-May-01	07E56'N 126E34'W	complete	complete
LBZ005	Sl	M	177	01-Jun-01	06E42'N 126E32'W	complete	complete
MAG007	Sa	F	198	13-Jun-01	10E22'N 127E90'W	complete	complete
CTC005	Sl	M	164	21-Jul-01	20E09'N 109E15'W	complete	complete
JOG004	Sa	F	197	30-Jul-01	14E17'N 098E58'W	complete	complete
JOG005	Sa	M	189	30-Jul-01	14E17'N 098E58'W	complete	complete
JOG006	Sl	F	162.6	31-Jul-01	14E28'N 097E59'W	complete	complete
RVC001	Sl	F	181.5	30-Jul-01	14E32'N 097E22'W	complete	Complete
JOG007	Sa	M	158.6	07-Aug-01	09E38'N 102E07'W	complete	Complete

JOG008	Sa	M	167.6	07-Aug-01	09E38'N 102E07'W	complete	Complete
MJB004	Sl	F	155	31-Jul-01	14E22'N 098E45'W	complete	Complete
GGT001	Sl	F	190	15-Jul-01	13E22'N 121E42'W	complete	complete
GGT002	Sl	F	150	01-Aug-01	15E32'N 098E16'W	complete	complete
RGV001	Sl	F	171	17-Jul-01	19E23'N 107E09'W	complete	incomplete
RGV002	Sa	F	178	08-Aug-01	08E59'N 103E46'W	complete	complete
RGC001	Sa	F	200.5	23-Jul-01	23E36'N 108E12'W	complete	incomplete
RGC002	Sa	F	172	06-Aug-01	11E49'N 120E16'W	complete	incomplete
RGC003	Sa	F	169	06-Aug-01	11E49'N 120E16'W	complete	incomplete
FGO001	Sl	M	147.3	27-Jul-01	12E03'N 097E24'W	complete	incomplete
FGO002	Sl	F	150	29-Jul-01	12E03'N 097E24'W	complete	incomplete
FGO003	Sl	M	163	06-Aug-01	09E39'N 098E30'W	complete	complete
ACB001	Sa	M	205	14-Sep-01	16E25'N 104E10'W	complete	incomplete

Appendix 1: Necropsy Data Forms

NECROPSY DATA FORM Page 1
General Information and External Examination

Name of Observer:_____ Set Number:_____
Cruise Number:_____ Position:_____
Date:_____ Time Initiated Backdown:_____
Time Necropsy Started:_____

Specimen Number:_____ Species:_____
Sex:_____
Lactating_____yes____no

Length (tip upper jaw to fluke notch):_____
Girth at axilla:_____
Girth at anterior insertion of dorsal fin:_____

COLOR PATTERN AND EXTERNAL EXAMINATION

* Sketch general color pattern features, dorsal fin profile and all external lesions, scars, and marks.

Teeth collected ? (lower left jaw):_____
Skin samples collected ? (collect two skin samples approximately $2cm^2$, place one sample in DMSO vial and wrap one in foil to place in liquid nitrogen):_____

NECROPSY DATA Page 2

INTERNAL EXAMINATION AND SAMPLING OF PRIORITY (LEVEL 1) ORGANS

TISSUES FOR HISTOPATHOLOGY AND IMMUNOLOGY

After collecting skin samples, open the animal in the following way: Cut through the blubber to the muscle at the head (just behind the eye). From the mid-dorsal line to the mid-ventral line. Make a similar cut just behind the anal opening. Connect the two cuts along the dorsal line and along the ventral line. Cut the blubber off, leaving the muscle behind.

Next, carefully open the abdomen and notice the condition of the intestine. Lift up the body wall and cut it away. Cut the diaphragm away from the ribs. Keep the cut close to ribs. Then cut the ribs off, using the big cutter for large animals, and the angled scissors for calf-size animals. This should expose all the organs.

After the carcass has been opened observe and sample these organs in the following order. Observations on visible abnormalities, where applicable, should be described in the space provided on this data sheet. When describing abnormalities, use ordinary words; that is, if something is white, say white, and don't try to use technical terms. Abnormalities should be measured and recorded in millimeters.

Record a comment for every organ. If it looks normal, say so. If necessary use the back of the data forms to diagram obviously diseased organs. If for some reason you were unable to examine or sample some of these organs please make a notation in the appropriate location on the data sheet as not examined or not sampled.

It is sometimes necessary to wash organs or slices of tissue to remove blood. However, while washing is acceptable, do not allow tissues or organs to soak in water, as this is damaging.

*****If there are 5 or more dolphins on deck sample priority level 1 tissues only on all animals. If there is 2-4 animals on deck at one time first conduct a priority level 1 tissue sampling on all animals. Once this is completed then sample priority level 2 tissues from all animals in the same sequence. **Note: Under these conditions there is a potential for mixing of tissue samples from different animals. When using this method of sampling you must take special precautions to insure proper labeling of samples and placing the tissues in the correct formalin containers.**

All tissue samples should be sliced thin (3-5 mm) before being put in formalin. Formalin fixation progresses from the surface inward, and the insides of large pieces of tissue do not fix well.

1) ADRENAL GLANDS: Remove both adrenal glands. Cut one in half and preserve both portions in formalin. Preserve the other remaining gland intact in formalin.

Since both adrenal glands are collected no observations on visible lesions are required.

2) KIDNEYS: Remove both kidneys and inspect for visible lesions. If a lesion is present describe below and cut a representative section from the abnormal tissue and preserve in formalin. Also,

take one section from the central area of the right kidney and preserve in formalin. Wrap the left kidney in aluminum foil, place in a labeled plastic bag and put in cooler for toxicology sampling.
Visible lesions present? (describe, if necessary draw a diagram of the organ and lesions on the back of this form)

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3) SPLEEN: Remove the spleen intact and inspect for visible lesions. This organ will be sampled for both histology and immunology as follows:
Immunology - - Cut the spleen in half then cut two thin tissue samples from the inner face and place in the small immunology formalin jar. Place the remainder of this half on a piece of foil (labeled on outside of foil and inside with a cardboard label) and place in liquid Nitrogen.
Histopathology - - From the other half of this organ remove a tissue section from the entire face and place in the 2 liter histology jar.
Visible lesions present? (describe, if necessary draw a diagram of the organ and lesions on the back of this form)

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4) Thymus: (attempt to sample Juvenile and adolescent animals only) - This organ has very loose texture and is located in the neck area just below the laryngeal cartilage. Expose this organ using the technique demonstrated during the training session. No visible observations on pathology are required. This organ will be sampled for both immunology and pathology in the following manner:
Histopathology Remove two thin sections and place in the 2 liter histopathology jar.
Immunology Remove 3-4 thin sections and place in the immunology formalin jar.
Place the rest of the thymus in aluminum foil (labeled outside of foil and inside with a cardboard label), and place in liquid nitrogen.
5) HEART: Remove the heart intact, make a series of slices as was demonstrated in the training session, then gently rinse the heart in clean water. Preserve the entire serially sliced heart in a bucket of formalin.
Since the heart will be returned intact to the laboratory observations on lesions are not required in this case.
6) LUNGS AND LYMPH NODE: Examine the surfaces of both lungs for visible lesions. Feel the surface of the lung with your hands. Parasitic lung worm, if present, will appear as raised, discolored, nodular areas on the surface which are firmer in texture than the surrounding normal tissue. Collect lung tissue as follows:

Abnormal tissue (if present) - - 3 sections from 3 different lesion sites.
Normal tissue - - 2 sections one from a representative area of each lung.
Lymph node on margin of lung --Cut node in half, and place both halves in the 2 liter histology container.
Visible lesions present? (describe, if necessary draw a diagram of the organ and lesions on the back of this form)

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7) MESENTERIC LYMPH NODE: Remove the entire node intact and cut it in half. Put one half on a piece of foil (labeled on outside of foil and inside with a cardboard label) and place in liquid nitrogen. Take the remaining half and remove two thin sections for the 2 liter histology jar. Remove a series of 3-4 similar sections and place in the immunology formalin container.
8) LIVER: Remove the liver intact. Examine the surface for presence of lesions. First, collect the posterior lobe of the liver, wrap in aluminum foil, place in a labeled plastic bag and place in cooler for toxicology. Then make 3 - 4 deep cuts almost through the organ and examine the interior for abnormalities. If present, lesions will usually appear as focal areas, which are paler in color than the surrounding normal tissue. Collect 2 sections of apparently normal tissue and preserve in formalin. If present, collect sections from up to 4 separate lesions and preserve in formalin.
Visible lesions present? (describe, if necessary draw a diagram of the organ
and lesions on the back of this form)

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9) REPRODUCTIVE TISSUES AND DATA: In order to assess the reproductive condition, male and female animals will be sampled as follows:
Males greater than 140 cm in length- - Collect a cross section of tissue from the right testis and preserve in formalin.
Females greater than 140 cm in length - - Place a tag on the uterine horn in front of the left ovary, cut the uterus at the cervix and remove the entire uterus intact. Open the uterus along its entire length with scissors and preserve in formalin. In cases where the animals are obviously pregnant do not attempt to save the uterus. In these instances tag the uterine tissue next to the left ovary then detach both ovaries from the uterus and preserve in formalin. Measure and record below the length of all fetuses. Do not save fetuses greater than 10 centimeters in length.

Fetus present? Y/N _____

Fetal sex? _____

Fetal length: _____

Fetal skin sample collected? _____

10) Tissues for toxicology: Sample an approximately 15cm^2 piece of blubber, place in aluminum foil, place in labeled plastic bag, and put in cooler. Transfer all toxicology samples that are in the cooler to the shipboard food freezer.

NECROPSY DATA Page 6

INTERNAL EXAMINATION AND SAMPLING OF PRIORITY LEVEL 2 ORGANS

ADDITIONAL TISSUES FOR HISTOPATHOLOGY

If work conditions and time permit these additional samples will be collected in the following order of importance.

1) Bone: Using the ribcage shears, cut out an approximately 5 centimeter long section of rib bone and preserve in formalin.

2) Stomach: Remove the stomach, open the forestomach and gastric compartment then dump the contents overboard. Gently rinse the stomach lining with clean water and examine for evidence of lesions. If present these will probably take the form of small ulcerations due to parasitism. Tissue samples of stomach lining will be collected and preserved in formalin as follows: Do not collect stomach worms unless they are attached to ulcerated tissue sampled.

Forestomach - - 1 section of normal tissue

Gastric stomach - - 1 section of normal tissue

Duodenal compartment - - 1 section of normal tissue

Stomach lesions (if present) - - 1 section diseased tissue from each stomach region.

Visible lesions present? (describe, if necessary draw a diagram of the organ and lesions on the back of this form)

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3) Intestine: Two regions of intestine will be collected and preserved in formalin as follows: Lesions in intestines are very difficult to assess visually so no observations on the potential condition are necessary.

Lower intestine: - - 1 section approximately 1/2 meter anterior from anal aperture.

Upper intestine: - - 1 section approximately 1/2 meter posterior to stomach.

4) Thyroid: - - This organ is also found in the neck region and is very difficult to locate. Expose this organ using the technique demonstrated in the training session and collect 1 section preserved in formalin. No observations are required on this organ.

5) Air sinus and inner ear complex: Remove excess tissue and detach the lower jaws from the head. Expose the pterygoid sinuses using the technique demonstrated during the training session.

Examine the epithelial lining of the sinuses for presence of parasites and ulceration. Collect tissue sample of the lining and preserve in formalin. If parasites are present collect a representative sample and preserve in a plastic bag with 10% formalin.
Visible lesions and parasites present? Describe, if necessary draw a diagram of the organ and lesions on the back of this form

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6) Brain and pituitary gland: Remove excess tissue from the head, make the appropriate cuts in the skull with the stryker saw, and break the skull cap loose with a chisel using the technique demonstrated during the training session. Wash the brain to remove surface blood, and inspect the surfaces carefully.
Look for spots of a different color, such as brown, tan, yellow, or red. Sample these, and put the samples into formalin. If you do not see surface abnormalities, then using a long sharp knife, slice the brain across from front to back, making slices about 1 centimeter thick. Inspect them for abnormalities. Sample any abnormalities and put the samples into formalin. Brain is very soft, so be careful handling this tissue, and do not crowd it into bottles. It is better to fix it separately until firm.
Collect the pituitary gland as shown in the demonstration, and save it in formalin without cutting it.

Appendix 2: Responses to CIE Reviewers

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May 01, 2002

Dear Dr. Forney;

This responds to the comments made by the CIE reviewers about our preliminary report (CIE-S11). These comments have been evaluated carefully, and where appropriate, incorporated into the final report, which has been sent to you. In general, the reviewers were very positive, and made comments that could be answered by clarification or elaboration of a point or two. This was mainly with regard to the specificity of lesions identified. That is, whether chronic lesions were compatible with old fishery-related injury, but might be caused by something else, or whether they were thought to be specific to the fishery. That issue had been addressed in the report, but strengthened to resolve any ambiguity. Several reviewers commented on the lack of 'controls' and of baseline studies in the entire project (not specific to the necropsy component). We certainly agree with that, but are not able to do anything about it. Clearly, any further studies must address those important matters.

Our necropsy study set out three objectives. To the final version of the report was added our evaluation of the degree to which those objectives were met. We think we have identified cause of death for animals dying in the net without a significant doubt, since findings were consistent and essentially universal. The other two objectives could only be partially met, because of the small sample size. We saw what we saw, but uncommon events (diseases, lesions) could easily be unrepresented in a sample of 53 animals of three species.

Sincerely,

Daniel F. Cowan, MD, CM
Professor of Pathology

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