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Stress-related changes in adrenal glands of stranded Harbour Porpoises (*Phocoena phocoena*) on the Dutch coast

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Summary

Stranded harbour porpoises (*Phocoena phocoena*) are submitted for post-mortem investigation to the Veterinary Pathological Diagnostic Centrum (VPDC) of the Department Pathobiology at the Utrecht University (UU). The main purpose of this investigation is to determine the cause of death and is commissioned by the Ministry of Economic Affairs, Agriculture and Innovation (EL&I). In this study the adrenal glands of these harbour porpoises were examined using routine histopathology and linked to the health problems they probably had before they died. Adrenal glands are releasing stress hormones in stressful situations. When animals suffer from chronic stress, adrenal glands are changing morphologically, histologically and in the way of releasing the stress hormones. In this study the adrenal glands are examined morphological and histological. The hypothesis was that the histological changes correlate with presence and duration of stress as judged by post-mortem investigation. Adrenalitis, congestion, haemorrhage, necrosis, apoptosis, hypertrophy, edema, cysts, extracellular eosinophilic material and vacuolar degeneration were found. All the adrenal glands had one or more of these abnormalities. Several examined examples are probably stress-related. The weights of the adrenal glands of the group with chronic stress were significant heavier than with acute stress. An adrenalitis in the transitional area of adrenal cortex and medulla has never been described in harbour porpoises and is recommended to be further investigated using IHC.

Content 1

Introduction

The Netherlands signed the "Agreement on the Conservation of Small Cetaceans of the Baltic, North East Atlantic, Irish and North Sea" (ASCOBANS) in 1994. Since this agreement the harbour porpoise (in these seas) is a protected animal. The stranded harbour porpoises on the Dutch coast are submitted for post-mortem investigation to the VPDC (Veterinary Pathologic Diagnostic Centrum) of the Department of Pathobiology at Utrecht University. This research is commissioned by the Ministry of Economic Affairs, Agriculture and Innovation (EL&I). The main purpose of these necropsies is to determine the cause of death. The causes of death are categorized in six groups: by-catch, emaciation, infectious disease, starvation, trauma and unknown. It is assumed that the porpoises had a certain amount of stress before they died and stranded or in cases of life-stranding followed by death. A common way to analyze stress is to study the components of the adrenal. It is supposed that frequent infection and pathological changes in organs are consequences that can be linked to chronic stress [1]. A better understanding about the reactions of the adrenal glands from the harbour porpoises in stress situations can be useful in determining causes of stranding on the coast, increase of numbers has (from 2009 to 2011) and responses of cetaceans to stress in general. Besides, further knowledge of the response of adrenal from harbour porpoises can be helpful in the development of protection plans and better triage protocols for use during treatment and rehabilitation efforts.

In this study the hypotheses is that histological changes in adrenal glands of stranded harbour porpoises correlate with presence and duration of stress as judged by post-mortem investigation.

1.1 Stress and the hypothalamic-pituitary-adrenal axis

Hans Selye made 'stress' a medical scientific idea. His definition of stress is "the nonspecific response of the body to any demand upon it" [2]. Selye's concept is about coping with a stressor, called "General Adaptation Syndrome" (GAS) [2]. GAS consists three phases: "alarm reaction" (flight or fight); "stage of resistance"; and "stage of exhaustion" [2, 3]. An organism enters the last phase if

the stress is too intense and takes too long (duration) the organism can not compensate anymore. A more recent view is that stress begins with the central nervous system perceiving a potential threat or disruption to homeostasis [44, 4, 5]. The stressors cause significant biological changes in the animal which are biological defense responses. This defense consists of some combination of the four general defense responses: behavioral response (avoiding the stressor), the autonomic response (cardiovascular system, gastrointestinal system, exocrine glands and adrenal medulla), the neuroendocrine response (hypothalamic-pituitary-adrenal axis), and the immune response (the immunosuppressive actions seem to be one of the major defense systems, and has direct regulation by the central nervous system) [4].

The response of the endocrine system to stress is by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic adrenomedullary system [6, 7]. In times of stress the hypothalamus is producing corticotrophin-releasing hormone (CRH) and this stimulates the pituitary to release adrenocorticotropic hormone (ACTH). ACTH stimulates the inner adrenal cortex to produce and release glucocorticoids, and the outer adrenal cortex to produce aldosterone. CRH also affects locus coeruleus which activates the sympathetic nervous system with secretion of norepinephrine in the periphery. The norepinephrine stimulates the adrenal medulla to produce catecholamines (norepinephrine and epinephrine).[7] Furthermore, there seems to be a concept of coordinated adrenocortical-adrenomedullary responses and coordinated adrenomedullary-symphatoneural responses in stress [5]. The glucocorticoid hormones stimulate the metabolism (to increase glucose) and have immune suppressive effects. The main physiological effect of this stress induced production of catecholamines is the increase of hart rate, blood pressure, and the blood flow towards the muscles. Together with the glucocorticoids, the body is ready for action, "fight or flight" response.[7] Aldosterone is not a typical characteristic of the adrenal response to stress, but it increases in cetaceans after adrenocortical stimulation [8-10]. This may be cetaceans' need to regulate body sodium and water during times of stress [8-10].

Chronic stress can occur if the stressors are frequent, intermittent, and repetitive, and can produce different responses: habituation, sensitization and desensitization [1]. There is a sustained activation of the hypothalamic-pituitary-adrenal axis, which results in repetitive, pulsative secretions of glucocorticoids [1]. Some researchers use the word distress, instead of chronic stress. Stress becomes distress when the stress response threatens the animal's well-being [4]. Distress by Goldstein (2002) is characterized by four characteristics. Distress is *aversive* to the organism, by learning to escape or avoid the stressor. The second characteristic is *consciousness*, for interpreting (and coping) the situation. The third is *communication*, and the fourth is *adrenal activation*. According to this, distress involves concurrent activation of the hypothalamic-pituitary-adrenal axis and adrenomedullary neuroendocrine systems. See figure 1 for an overview about the differences between acute and chronic stress, and stress versus distress.

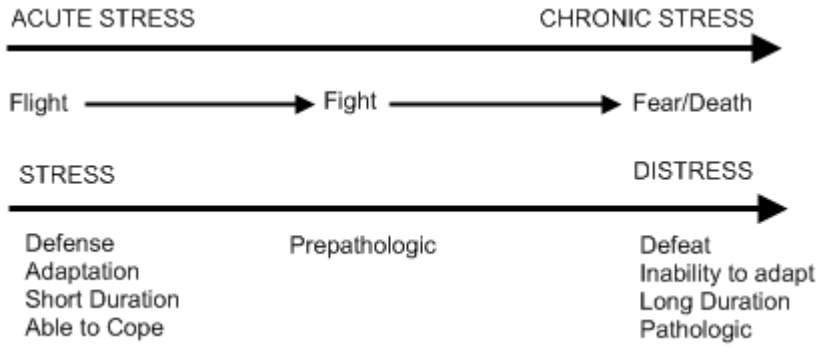


Figure 1. *Results of acute versus chronic stress [1].*

1.2 Morphology and histology of the adrenal gland from harbour porpoises

The adrenal glands in the porpoise lie very close to the dorsal abdominal midline [11]. The adrenal gland has a capsule, a cortex and a medulla [6]. The adrenal glands of porpoises are quit large and are extensively pseudolobulated [11, 12]. This lobulation is created by fibrous connective tissue septae extending from the capsule penetrating the cortex [11, 12]. Cetaceans' adrenal glands have large nerves, ganglia, and many blood vessels are associated with the hilus and the surface of the capsule [12]. The adrenal glands are probably growing because it is reported that the organs and glands of cetaceans continue to grow with their age [13]. However, the growth is slowing after the onset of sexual maturity [13].

The cortex consists of three zones. The inner zona reticularis and the zona fasciculata produce glucocorticoids, cortisol and corticosterone. The outer zona glomerulosa produces the mineralocorticoid aldesterone [6, 7]. There is not much known about the precise histological structure of the adrenal of harbour porpoises. More is known about the adrenal of the Atlantic bottlenose dolphin (*Tursiops truncatus*). The adrenal medulla of the Atlantic bottlenose dolphin has a distribution of chromaffin cells that is closest to that in hoofed mammals: the outer cells of the medulla are producing epinephrine and the cells centrally in the medulla norepinephrine [14-16]. There are also medullary protrusions observed, but the functional significance is unknown [14-16]. Between the adrenal cortex and the adrenal medulla there is a clearly visible layer of connective tissue found in the bottlenose dolphin [14-16]. The adrenal cortex of the Atlantic bottlenose dolphin is containing a zona arcuata instead of the zona glomerulosa [15]. The assumption that the zona arcuata is changing in zona glomerulosa during ageing is still not clear [15]. Vukovic et al. also found a thin layer of small cells below the zona arcuata, corresponding to the zona intermedia [15]. The difference between zona arcuata and intermedia is the cell arrangement. The cells in the zona arcuata are arranged in arches and the cells in zona intermedia has convoluted strands grouped in cords or clusters [15, 17]. The medulla and cortex interact with each other [6]. On the adrenal surface is a thick capsule of fibrous connective tissue and the connective tissue trabeculae is extending to a layer of connective tissue between the cortex and medulla and then spreads as very thin septa through the entire medulla [11, 14-16].

1.3 Stress induced pathological findings in other studies

1.3.1 Hypertrophy and Hyperplasia

Stress results in an increased ACTH stimulation, and the adrenal cortex reacts with both hypertrophy and hyperplasia as consequence [18]. Ulrich-Lai et al (2006) found substantial adrenal hypertrophy and hyperplasia in rats, which enhance sensitivity to ACTH [19]. Furthermore, animals with chronic stress are releasing more corticosterone responding to an increase in ACTH [6, 19]. The reported changes due chronic stress in the adrenal glands are hyperplasia in the outer zona fasciculata and hypertrophy in the inner zona fasciculata, decrease in size and cells of the zona glomerulosa and hypertrophy of the medulla [19, 20]. This may be a consequence from repeated activation by the sympathetic nervous system, or/ and by ACTH and glucocorticoids [19]. The different effects on the zones of the adrenal cortex could occur through multiple mechanisms [19]. Chronic stress may effect angiotensin II-stimulated aldosterone production [19]. Atrophy of the zona glomerulosa also occurs due hypoxia [20]. This is due to higher levels of PO2 (than in the deeper zones), which may cause profound effects if oxygen delivery changes are not sufficient [20]. Furthermore, the presence of a mitochondrial enzyme (18 hydroxylase) can cause such difference, or the ionic and osmotic sensitivity are different in the zona glomerulosa [20].

Kuiken et al. found adrenocortical hyperplasia and hypertrophy in harbour porpoises which died from a chronic cause of death and the adrenals were significantly heavier than harbour porpoises that died from an acute cause of death [21]. A similar increase in adrenal size and mass was reported in the Atlantic bottlenose dolphin [13] and in beluga whales (*Delphinapterus leucas*) cortical enlargement is also due to chronic stress [22].

1.3.2 Adrenal cysts

Lair et al. also found other changes in adrenal glands from beluga whales. Some belugas had cysts in adrenal glands with high cortisol levels, thus probably stress-related. However, it is possible that this is a normal aging process, because it seems to increase with age in the beluga whales [22]. Adrenocortical cysts were also found in harbour porpoises and Atlantic white-sided dolphins (*Lagenorhynchus acutus*) [23],[21].

1.3.3 Medullary and cortical nodular hyperplasia

Lair et al. (1997) also found nodular hyperplasia of the medulla and cortex [22]. The cortical nodules can represent a normal aging process in beluga whales or a regenerative reaction to degenerative changes such as vacuolar degeneration that also is observed [22]. The nodules in the medulla could be related to hypoxia caused by prolonged agony or by lung or cardiac diseases [22]. Furthermore, hypoxia and such disorders (are chronic diseases) can give chronic stress. In rats hypoxia also induced changes in the cortex of the adrenal, especially in the zona glomerulosa [20]. In contrast, these adrenals showed loss of weight because of a decrease of cells in de zona glomerulosa, and finally atrophy. However, the medulla showed hyperplasia. The ultrastructural changes found were a decrease in lipid droplet content, a marked increase in lysosome number, and presence of giant mitochondria [20]. The increase of lysosomes indicates stress [20, 24].

1.3.4 Vacuolar degeneration

A frequent finding of stranded animals was moderate to severe degeneration of the adrenal cortex. This was a bilateral lesion, and usually consisted of vacuolar degeneration of the zona glomerulosa and zona reticularis. This lesion is considered to be a nonspecific lesion associated with stress, disease states, or trauma [25]. The vacuolar degeneration found in the beluga whales are similar to bilateral adrenocortical vacuolar degeneration described in other stranded marine mammals [22 25]. However, some animals (particularly rats and cats) may develop vacuolar degeneration spontaneously in the adrenal cortex [26]. Furthermore, it can also be caused chemically [26]. The cortical degeneration in the adrenal glands found in the beluga whales, might also be the cause of presence of adrenocorticolytic metabolites [22]. Earlier, high levels of xenobiotics in the blubber of the beluga whales were demonstrated [27].

1.3.5 Lipid depletion and hyperemia

About 95% of 90 spinner dolphins (*Stenella longirostris longirostris*) and 172 spotted dolphins (*Stenella frontalis)* had macroscopically cortical darkness in association with continuous acute stress and/or vasogenic shock [28]. This darkened cortex is caused by lipid depletion and hyperemia (congestion) [2, 28]. In continuous acute stress the glucocorticoid secretion will increase by continuous stimulation of ACTH, which results in lipid depletion of cells and blood engorgement of cortical sinusoids [2, 28, 29]. The lipid depletion is because lipo-protein-derived cholesterol and endogenous cholesterol are utilized for adrenal steroidogenesis [30]. The color of the adrenal glands changes from light yellow-beige to dark reddish brown [2, 28]. Chronic hypoxia in rats causes ultra-structural changes in the zona glomerulosa: decrease in size and its cells, lipid depletion, increase of lysosomes, and presence of giant mitochondria [20].

1.3.6 Congestion and adrenal haemorrhage

Adrenal haemorrhage is associated with trauma, (neonatal) stress, anticoagulation, hematologic disorder, pregnancy, and adrenal masses [31-36]. Sepsis, burns, surgery, hypotension, pregnancy and exogenous steroids or ACTH administration are stress-related situations were adrenal haemorrhage can occur [31]. The increased level of ACTH during stress stimulates the release of catecholamines that increases the blood flow to the adrenal. The increasing blood flow causes an increasing secretion of glucocorticoids. [37] The increased blood flow and the vasoconstriction (catecholamine-induced) results in congestion with increased pressure in the capillaries [31, 36]. Haemorrhage is also more likely to occur due to hypoxia because of the damage to endothelial cells [36]. Furthermore, the adrenal is one of the favored organs in times of redistribution of the blood flow (for example with hypoxia) [36].

Adrenal haemorrhage is often associated with *Pseudomonas* infection, *Staphylococcus aureus, Klebsiella species, Escherichia coli, Proteus* bacterema, or with meningococcemia [31]. Hypotension or disseminated intravascular coagulation due septicemia increases the risk of haemorrhage [31].

Neonatal adrenal congestion and haemorrhage is associated with fetal hypoxia, asphyxia and neonatal stress, sepsis, difficult labor, renal vein thrombosis and extracorporeal membrane oxygenation [31, 35, 36]. The neonatal adrenal haemorrhage secondary to asphyxia is likely multifactorial [36].

1.3.7 Stress-related changes in the common seal (*Phoca vitulina vitulina*)

Adrenals of 112 common seals (juvenile and adult) were changed after distinct stress exposure. In the juvenile seals the cellular arcadiform arrangement of the zone arcuata is lost and thus changed to a zona glomerulosa which is typical for adult seals. The radial strands in the zona fasciculata were repeatedly disrupted by node-like assemblies of small cells. The zona reticularis was not present in the cortex. The rest of the adrenal cortex was increased. Adrenocortical cell islets whitin the medulla were more present. Finally the lipoid droplets in cortical cells were decreasing in numbers. [17]

In stressed adult seals, there was a progressive transformation. The capsule and trabeculae contained more regenerative cells (blastema cells) and contained degenerating cells. The adrenocortex was increased and there were degenerated cells in the zona glomerulosa. [17]

The increased incidence of immature cells in the cortex, the increased occurrence of transitional cellular stages and the enlargement of the cortex (zona fasciculata) was interpreted as a functional adaption in stress. [17]

1.4 Potential causes of stress

1.4.1 Effects of xenobiotics

In acute toxicity, cytoplasm vacuolation can occur [26]. These vacuoles coalesce to form larger vacuoles [26]. Usually the vacuolation starts in the inner cortex and progresses to the outer cortex [26]. Some chemicals like DDT derivative, can lead to severe, acute necrosis and haemorrhage of the adrenal cortex [26]. Chronic toxicity can lead to nodular degeneration, atrophy, fibrosis, or primary proliferation of cortical cells [26]. Hypertrophy of cells in the cortex can occur if the xenobiotic chemicals lead to increased circulating ACTH levels [26], like o,p'-DDT did in beagles [38]. Thus not only degeneration, but also hypertrophy in the cortex can be caused by stress or toxicity [18]. It is important to distinguish these two by typical changes in examination in endocrinology, clinical pathology and histopathology [18]. Typical changes related to stress are increased glucocorticoid secretion, and thymus atrophy/lymphocytolysis. Typical changes related to direct toxicity are degenerative lesions like necrosis and fibrosis. [18] Research into the effect of chlorinated hydrocarbons in adrenal glands from harbour porpoises stranded on the coast of Great Britain revealed that there is no correlation found between the adrenocortical hyperplasia and increased levels of chlorinated hydrocarbons. Hyperplasia was associated with chronic disease[21]. However, harbour porpoises from the Southern North Sea seem to have a poor health condition linked to an overload of zinc (Zn) [39, 40] or polychlorinated biphenyls (PCB's) [41] . Harbour porpoises from the Southern sea displayed higher zinc Zn, PCB's concentrations that those in Northern and Western European seas [40, 41]. In another study about porpoises stranded on the coast of England and Wales, which is the same sea as along the Dutch coast, health consequences due exposure to heavy metals were demonstrated [42]. Consequently, the pollution in the North Sea can be a factor that caused changes which may be found in the adrenal glands of the harbour porpoises stranded on the Dutch coast.

1.4.2 Noise pollution in the North Sea

The human activity on the North Sea is intensive and still growing (see figure 2) [4344]. Intentional sources are sonar sources from ships. Unintentional sources include shipping, pile driving for offshore construction, underwater explosions, dredging, operational oil and gas platforms, wind farms, pipe laying, cable laying, flow noise from pipelines, harbour noise and oil platform decommissioning. The harbour of Rotterdam is still growing, the numbers of drill platforms for oil or gas and windmill parks are increasing. [43, 44] Impact on harbour porpoises of construction of offshore wind farms is more severe than during their operation [45-47]. Most of the underwater noises produced by human are mainly at low frequencies (<1000 Hz) [48]. Namely air guns, shipping, pile driving and explosions are low-frequency sources, resulting in low absorption [44]. However, small motorboats and icebreakers are examples with significant noise above 1000 Hz [48]. Navigation echo sounders, fish-finding sonar's and military search sonar's are noises with high frequency (1-200 kHz) [44, 48]. The calls of most baleen whales are at 25-2000 Hz (low frequencies) [48]. The calls of most toothed whales are at 0, 5-20 kHz (moderate to high frequencies), and small odontocetes are mainly at very high frequencies 30-150 kHz[48].

The ears of fresh animals (maximal 12 hours dead) are sent to Laboratori d'Aplicacions Bioacústiques in Barcelona, Spain to investigate evidence of acoustic trauma. There electron microscopy is used to investigate the ears.

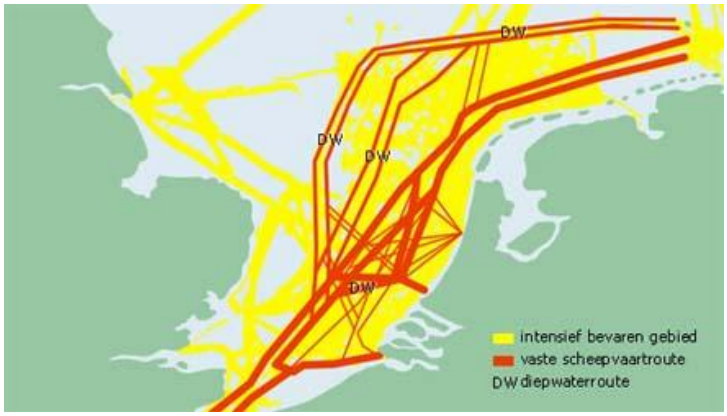


Figure 2. *This image shows the navigation of the ships. Yellow: intensive sailed area. Red: Fixed navigation routes. DW: Deepwaterroute [49].*

Effects on marine mammals

Underwater noise is likely to be an indicator for stress in marine mammals. Marine mammals are vulnerable to impacts from shipping noise because these sounds fall into their hearing ranges [50]. Marine mammals use sounds to communicate, orientate and for prey location.[51] Noise has behavioral impacts[48, 52], perceptual (masking) impacts [53-55] and physical (non auditory and auditory) impacts[53, 56-58]. There are also noise-related strandings reported [59].

Stress is a physiological effect of noise, therfore exposure to long term noise may cause stress in marine mammals. Intense sound exposure has been shown to increase norepineprine, epinephrine, dopamine and aldesterone in bottlenose dolphins and beluga's. [60] Recently researches found evidence that exposure to low-frequency ship noise may be associated with chronic stress. They measured lower metabolites of glucocorticoid hormones in the gathered feaces of the North Atlantic right whales (*Eubalaena glacialis*) in times of less ship traffic. [61]

The Harbour Porpoise and underwater noise

Harbour porpoises do have the most acute hearing and widest frequency range of all the odontocetes tested [62]. The best hearing frequency is around 100 kHz [62]. Most of the acoustic energy radiated from commercial shipping noise is below 1 kHz. Sounds of ships (also sonar) are in the hearing range of the harbour porpoises and may elicit stress and other undesired responses [50].

However, when ships are close to the animal, a potential for masking at higher frequencies exists [54]. The 'small' North Sea is a very busy sea, thus masking may be a happening. A study in captivity shows that the normal reaction of a harbour porpoise is to swim away from the sound and to slightly increase their respiration rate [50].

Content 2

Material and methods

2.1 Animals and sample collection

Harbour porpoises stranded on the Dutch coast are submitted for post-mortem investigation to the VPDC (Veterinary Pathologic Diagnostic Centrum) of the Department Pathobiology of Utrecht University. Harbour porpoises stranded between December 2009 and July 2012 were used for this study. Only a subset of the stranded harbour porpoises fulfilling specific selection criteria was included in this study. Selection criteria included the decomposition condition. All animals included were considered very fresh and fresh and were not frozen. To classify the carcasses the Department Pathobiology is using a standard classification to determine the states of decomposition of the harbour porpoises (Kuiken & Garcia Hartmann, 1993), with adjustment where necessary. This classification is the same as the condition code defined in the ECS proceedings. The Decomposition Condition Code (DCC) is based on external and internal decomposition signs of the dead harbour porpoises.

DCC 1: Very Fresh, less than 48 hours dead, may show signs of rigor mortis (<24h), blood still separates serum (24-48h), rigidity of eyes is diminished but not very flaccid, cornea is not cloudy.
DCC 2: Fresh, first signs of decomposition visible, eyes and surface quality of the skin reveal decomposition, otherwise good state, organs look intact, blood does not separate from serum, no smell of decomposition.
DCC 3: Putrefied, skin peeling, moderate but clear signs of decomposition (changes in color and consistency) of skin and organs, not suitable for bacteriology because of overgrowth, moderate smell of decomposition.
DCC 4: Very putrefied, advanced decomposition, skin and organs clearly altered, the loss of consistency changes the organ's shape, clear smell of decomposition, not suitable for any tissue analysis, even gross pathology is very unclear and can hardly be interpreted at all.
DCC 5: Remains, completely useless for pathological examination, organs are beyond clear recognition or absent, may be mummified, etc.

The study was carried out on 50 harbour porpoises, comprising 22 immature males, 8 mature males, 8 immature females, and 12 mature females. Age was determined by the development of the reproduction organs and the length of the animals (<90 cm, 91-130 cm, >130 cm). All animals died of natural causes or because of by-catch. The causes of death were categorized in six groups: by-catch, emaciation, infectious disease, starvation, trauma and unknown. As this research is about presence and duration of stress, the harbour porpoises were divided in different categories. The different causes of death were categorized according to the assumed duration of stress, namely acute stress, chronic stress and unknown. The harbour porpoises with assumed acute stress had died rapidly as a result of trauma, dystocia and other short term disorders (short term infectious). The harbour porpoises with assumed chronic stress died as a result of long-term disorders and long-term infections. It has to be noted that starvation is considered to be different from emaciation. Starved harbour porpoises are neonates which had not eaten recently, thus still have much blubber. Emaciated harbour porpoises lost weight presumably because they did not eat for a longer time. Thus these causes can be separated into belonging into the categories acute and chronic stress. The category by-catch consist of all animals which are thought to be possibly/probably/high probably and certainly by-caught, considered to be an acute situation which could cause acute stress.

2.1 Tissue sectioning and staining

During necropsy, tissue and organ samples are taken for diagnostics and other research. Firstly, the adrenals are evaluated in situ. The position can be changed or adhesion with other tissues may be present. Secondly, the left and the right adrenal glands are removed from each animal and

weighed on a platform scale (mg, only animals with DCC 1 and 2). The adrenal glands are also assessed out of the carcass, with an incision to look at the parenchyma. For histopathological evaluation, organs are collected from DCC1, 2, and 3 animals only, because of the state of autolysis. After fixation in formalin, the tissues are embedded in paraffin wax, sections are cut and stained with hematoxylin & eosin (H&E).

2.3 Histology

Slides were analyzed under an Olympus BX45 light microscope. The derogations per adrenal gland were written down. The slides of the adrenal glands of the 50 harbour porpoises were compared with each other. The capsule, cortex and medulla were visually screened, searching for any present change. The findings were compared to the necropsy reports made by pathologists, and completed with the histological changes found in this research. The found changes were used for a grading table (see table 1, 2 and 3) to do semi quantitative evaluation.

2.4 Statistical analyses

To compare the categories acute, chronic and unknown stress and to compare the animals per pathological finding between the five categories, semi-quantitative evaluation was used in this study. Grading was done as described below.

Table 1. *Semi quantitative grading system: for the pathological findings found in the adrenal glands which were used in this study. The adrenalitis is separated due to location into transition, medulla and cortex. 'Adrenalitis on transition of cortex and medulla' was only used if it was explicitly named. When entire adrenal gland was inflamed the two other adrenalitis columns were used.*

Grade	Adrenalitis on transition	Adrenalitis in medulla	Adrenalitis in cortex	Congestion	Haemorrhage
None (0)	Absent	Absent	Absent	Absent	Absent
Mild (1)	Mild diffuse/ multifocal of focal moderate	Mild diffuse/ multifocal of focal moderate	Mild diffuse/ multifocal of focal moderate	Mild diffuse or mild in area cortex/medulla/transition	Mild multifocal or focal moderate or mild in area cortex/medulla /transition
Mild to moderate (2)	Multifocal between mild to moderate	Multifocal between mild to moderate	Multifocal between mild to moderate	Multifocal between mild to moderate or in one area mild and another area moderate	Multifocal between mild to moderate or in one area mild and another area moderate
Moderate (3)	Moderate multifocal/diffuse or focal severe	Moderate multifocal/diffuse or focal severe	Moderate multifocal/diffuse or focal severe	Moderate diffuse or moderate in area cortex/medulla/transition	Moderate multifocal or mild diffuse or moderate in area cortex/medulla /transition or focal severe
Moderate to severe (4)	Multifocal between moderate and severe	Multifocal between moderate and severe	Multifocal between moderate and severe	Multifocal between moderate and severe or in one area moderate and another area severe	Multifocal between moderate and severe or in one area moderate and another area severe
Severe (5)	Severe multifocal/ diffuse	Severe multifocal/ diffuse	Severe multifocal/ diffuse	Severe diffuse or severe in area cortex/ medulla/ transition	Severe multifocal or moderate diffuse or severe in cortex/ medulla/ transition

Table 2. *Semi quantitative grading system: for the pathological findings found in the adrenal glands which were used in this study.*

Grade	Necrosis /apoptosis	Vacuolar degeneration	Eosinophylic material	Hypertrophy	cysts	Edema
None (0)	Absent	Absent	Absent	Absent	Absent	Absent
Mild (1)	Mild multifocal or focal moderate or mild in area cortex/medulla /transition or mild individual cells	Mild multifocal or focal moderate or mild in area cortex/medulla /transition or mild individual cells	or present	Mild multifocal or focal moderate or mild in area cortex/medulla /transition	Diameter: ≤ 5 mm, multifocal, bi- or unilateral	Mild
Mild to moderate (2)	Multifocal between mild to moderate or in one area mild and another area moderate	Multifocal between mild to moderate or in one area mild and another area moderate	X	Multifocal between mild to moderate or in one area mild and another area moderate	X	Mild to moderate
Moderate (3)	Moderate multifocal or mild diffuse or moderate diffuse or moderate in area cortex/medulla /transition or focal severe or moderate	Moderate multifocal or mild diffuse or moderate diffuse or moderate in area cortex/medulla /transition or focal severe or moderate	X	Moderate multifocal or mild diffuse or moderate in area cortex/medulla/transition or focal severe	Diameter: ≤ 10 mm, multifocal, bi- or unilateral	Moderate
Moderate to severe (4)	Multifocal between moderate and severe or in one area moderate and another area severe	Multifocal between moderate and severe or in one area moderate and another area severe	X	Multifocal between moderate and severe or in one area moderate and another area severe	X	Moderate to severe
Severe (5)	Severe multifocal or moderate diffuse or severe in cortex/ medulla/ transition or severe individual cells	Severe multifocal or moderate diffuse or severe in cortex/ medulla/ transition or severe individual cells	X	Severe multifocal or moderate diffuse or severe in cortex/ medulla/ transition	Diameter: > 11 mm, multifocal, bi- or unilateral	severe

Table 3. *Semi quantitative grading system: for the pathological finding adrenalitis.*

Grade	Adrenalitis
None (0)	Absent
Mild (1)	Mild multifocal, or 1 or 2 area's (medulla/ cortex/ transition)
Mild to moderate (2)	Two area's mild and one area moderate
Moderate (3)	Moderate multifocal or mild diffuse, one or two area's moderate or two area's moderate and one area is mild
Moderate to severe (4)	Two area's moderate and one is severe
Severe (5)	Severe multifocal or moderate diffuse. One or two area's severe or two area's severe and one area is moderate

The inclusion into each grade per pathology categories (adrenalitis, congestion, haemorrhage etc.) was based on the findings in the adrenal glands in this study. In table 1 the adrenalitis is separated in three categories according to the area affected: cortex/ medulla/ transition of cortex and medulla. This separation is because of a possible correlation between area and one of the 3 categories (acute stress, chronic stress, unknown). In table 3 the adrenalitis is not separated. Both ways were used to ensure that table 1 was not too detailed.

The grading system for the pathological finding 'cysts' followed the study from Lair 1997 [22]. They found that the adrenal cysts had various sizes with a diameter of ≤ 15 mm and mostly multifocal [22].

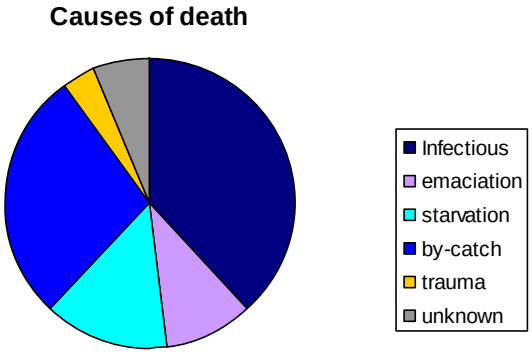
Results were compared with one-way ANOVA and Tukey (post-hoc test) by using SPSS 20. Two groups were compared with the independent two sample T-test. The one-way ANOVA was used to find any significant difference between groups. If there was a significant difference, the Tukey (post-hoc test) was used to determine which specific groups or categories differed from each other. In this study the hypothesis is that histological changes in adrenal glands have a significant correlation with presence and duration of stress (thus cause of death or disorder which they had before they died). Thus H0 was: There is no significant difference in adrenal gland abnormalities between the groups (acute stress/chronic stress/unknown).

Content 3

Results

3.1 Animals and collected information

Adrenal glands from 50 harbour porpoises stranded from 2009 to 2012 were examined. Of these 50 animals 38% (19 animals) died because of an infectious cause, 10% (5 animals) due to emaciation, 14% (7 animals) due to starvation, 4% (2 animals) due to trauma, 28% (14 animals) due to by-catch and 6% (3 animals) of the animals the cause was unknown (see graphic 1 and table 4).



Graphic 1. *In this pie graphic, the distribution of the causes of death is shown. Infectious is 38%, emaciation is 10%, starvation is 14%, trauma is 4%, by-catch is 28% and unknown is 6%.*

Table 4. *In this table the number of harbour porpoises used in this study are separated in 6 groups by cause of death, and separated in number of females and males in these 6 groups.*

	Infectious	Emaciation	Starvation	By-catch	Trauma	Unknown
Female	8 (42%)	4 (80%)	1 (14%)	5 (36%)	1 (50%)	1 (33%)
Male	11 (58%)	1 (20%)	6 (86%)	9 (64%)	1 (50%)	2 (67%)

Regarding sex distribution: infectious causes were identified in 42% (8) females and 58% (11) males, emaciation in 80% (4) females and 20% (1) males, starvation in 14% (1) females and 86% (6) males, trauma in 50% (1 female and 1 male) females and males, by-catch in 36% (5) females and 64% (9) males, and unknown in 33% (1) females and 67% (2) males.

For this study, animals were categorized differently, namely as animals with acute stress, chronic stress and unknown. The gender and age distribution is present in table 5.

Table 5. *In this table the percentage of acute stress, chronic stress and unknown animals are shown. In the left column there are 2 different factors: gender and age. Acute stress: animals that died due trauma, short-term infection, starvation or by-catch. Chronic stress: animals that died due long-term infection and emaciation*

	Acute stress (56%)	Chronic stress (38%)	Unknown (6%)
Female	25%	63%	33%
Male	75%	37%	67%
Neonate or juvenile	70%	50%	33%
Adult	30%	50%	67%

The pathological findings which was already done before starting this study, are shown in table 6 in appendix A. The information in this table is carcass number, date of stranding, age, gender, DCC, NCC, macroscopically findings, recent feeding, adrenal weights and probable cause of death.

3.2 Histological pathological findings and statistics

The histological pathological findings and the probable duration of stress (chronic or acute) are shown in table 7 in the appendix B. The pathological findings consist of adrenalitis, congestion, hemorrhagic, necrosis/apoptosis, hypertrophy, cysts and edema. The kind of stress per animal was based on the macroscopic and microscopic reports which were made by pathologists of Utrecht University.

For the age and gender distribution of the adrenal gland abnormalities, see table 8 and 9. Three types of adrenal gland abnormalities were highly prevalent in neonates and juveniles: hyperemia, adrenalitis on the transition of cortex and medulla, and haemorrhage. The three highly prevalent types of adrenal abnormalities in adults were hyperemia, adrenalitis in the cortex and necrosis/apoptosis. The three highly prevalent types of adrenal gland abnormalities in females were hyperemia, adrenalitis in the cortex and necrosis/apoptosis and adrenalitis on the transition of cortex and medulla. The three highly prevalent types in males were hyperemia, adrenalitis on the transition of cortex and medulla and necrosis/apoptosis. Thus, hyperemia, adrenalitis on the transistion of cortex and cortex, haemorrhage and necrosis were most commonly found.

Table 8. *Age distribution in adrenal glands abnormalities observed in the harbour porpoises.*

Age	Total examined	Adrenalitis			Hyperemia (%)	Hemorrhagic (%)	Necrosis /apoptosis (%)	Vac. deg. (%)	Eos. mat. (%)	Hypertrophy (%)	Cysts (%)	Edema (%)
		Transition (%)	Medulla (%)	Cortex (%)								
N/J	30	67	40	23	90	50	43	17	23	3	0	3
A	20	55	40	75	90	30	70	40	35	10	10	0

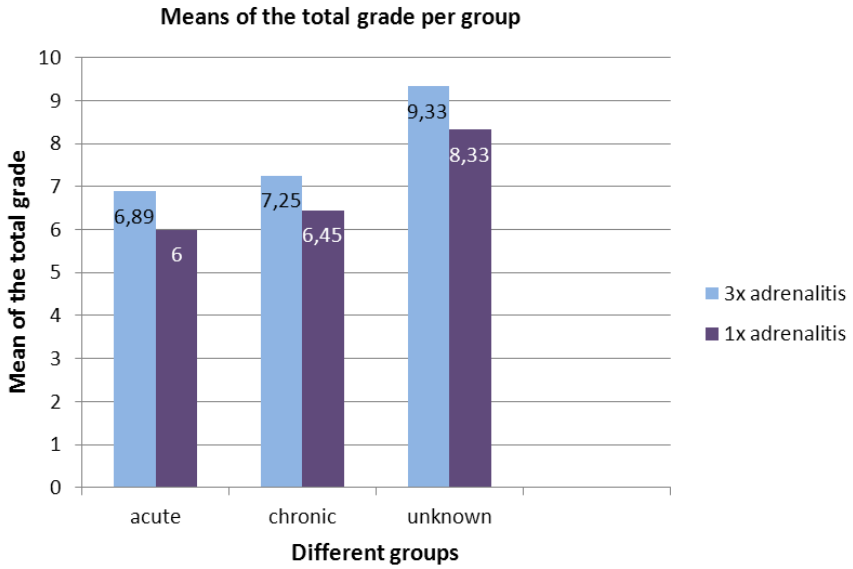
Table 9. *Gender distribution in adrenal glands abnormalities observed in the harbour porpoise.*

Gender	Total examined	Adrenalitis			Hyperemia (%)	Hemorrhagic (%)	Necrosis /apoptosis (%)	Vac. deg. (%)	Eos. mat. (%)	Hypertrophy (%)	Cysts (%)	Odema (%)
		Transition (%)	Medulla (%)	Cortex (%)								
Female	20	50	30	60	90	35	50	30	15	5	10	0
Male	30	70	43	30	90	43	53	20	37	7	0	3

The grades per harbour porpoise and per category (acute, chronic, by-catch, emaciation/starvation, unknown or acute, chronic, unknown) are shown in table 10 and 11 in appendix C. The statistics are calculated on 4 different ways:

- Acute, chronic and unknown: with 3 different adrenalitis categories and 1 adrenalitis category. Because the adrenalitis on the transition of cortex and medulla was striking during the evaluation of the slides, different groups were made for adrenalitis, to investigate whether there was a significant difference between the groups (acute stress, chronic stress, unknown) and area of adrenalitis.
- Acute and chronic: with 3 different adrenalitis categories and 1 adrenalitis category. The group 'unknown' is excluded because it consists only 3 harbour porpoises. To ensure that these 3 harbour porpoises did not interfered too much with the results of the 47 other harbour porpoises (because the results were compared with each other).

In graphic 2 the means of the total grades are shown.

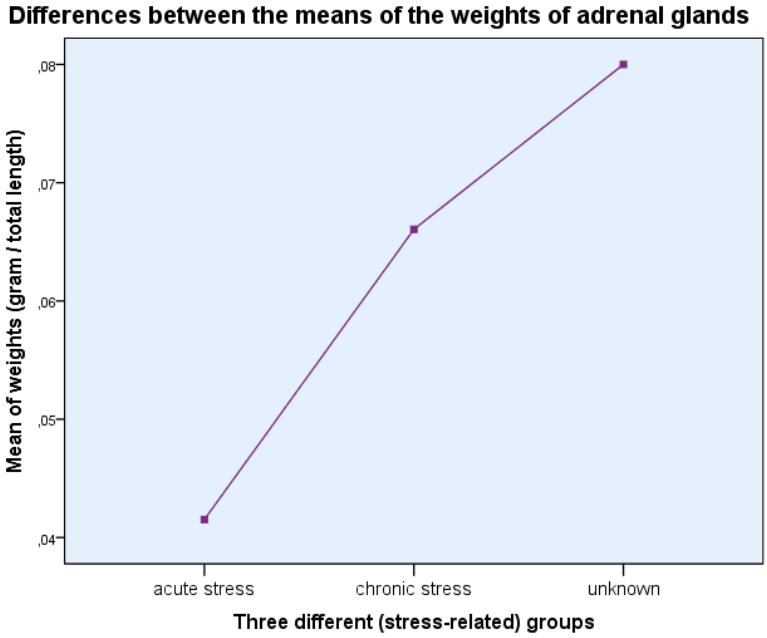


Graphic 2. *Means of the total grade per group: acute, chronic and unknown.*

Using the one-way ANOVA, there was no significant difference found between the groups (acute stress, chronic stress, unknown). Furthermore, there was no difference between the adrenal gland abnormalities (congestion/ haemorrhage/ cysts etc.) and the groups (acute stress, chronic stress and unknown).

3.3 Weights of the adrenal glands

The weights of both adrenal glands were reported for 44 harbour porpoises. This was compared in one-way ANOVA (and Tukey) and a significant difference was found between the acute stress and the chronic stress group (table 12 and 13 appendix D). The weights of the adrenal glands in group 'chronic stress' were significant heavier than the adrenal glands in group 'acute stress'. Juveniles or neonates do have smaller adrenal glands, thus the adrenal weights analyzed in relation to the total body length of the harbour porpoises. The total length was used because the age determination, used in the protocol, was done by measuring the total length. The body weight was not used because the presence of emaciation and starvation animals would induce bias. In graphic 3 the difference between the means of the weights of the adrenal glands are shown.



Graphic 3. *Means of the weights (gram / total length of harbour porpoise) of the adrenal glands per group: acute stress (0,0415), chronic stress (0,0661) and unknown (0,0800).*

Content 4

Discussion

The main goal is to discover which diseases or situations are causing stress in harbour porpoises which stranded on the Dutch coast. The effect of stress on the adrenal glands is documented in several marine mammals (see chapter 1). All histologically examined adrenal glands in this study had abnormalities. Vacuolar and granular degeneration, necrosis, and haemorrhage are common abnormalities due acute injury. Atrophy, fibrosis and nodular hyperplasia seem to be chronic reparative processes [26]. Vacuolar degeneration, necrosis, haemorrhage were also found in this study. These changes could be because of acute stress. However there was no significant difference found between acute stress and chronic stress harbour porpoises when considering these abnormalities.

Many harbour porpoises (42%) in this study had haemorrhage in the adrenal glands. Stress-related causes of adrenal haemorrhage are surgery, sepsis, burns, hypotension, hypoxia, pregnancy, exogenous adrenocorticotropic hormone, and exogenous steroids [31, 35, 36]. However, adrenal haemorrhage can be spontaneous due pregnancy, trauma or anticoagulant therapy [33]. Haemorrhage is also reported after acute injury [26]. All these situations seem to increase ACTH secretion to cause haemorrhage in the adrenal [37]. Only three harbour porpoises in presented study had sepsis and died probably with acute stress: these had hyperaemia. Thus in these animals acute stress could have caused this hyperaemia. Because the interpretation about the difference between haemorrhage, congestion and hyperaemia can vary between observers. Adrenal haemorrhage is found in adult and neonate/ juvenile harbour porpoises, and more in neonates and juveniles. This is probably because immature harbour porpoises are considered be less capable to cope with stress [63]. The development of the HPA axis and adrenal may also play a role that non-adult animals are less capable of coping with stress [64]. The hippocampal activation is furthermore rapid and robust in juvenile rats [65].

Congestion can be a result of stress because the increased ACTH results in increased catecholamines which cause increased blood flow [31, 36, 37]. Congestion and haemorrhage are often linked to each other. The adrenal gland is one of the favored organs in times of redistribution of the blood flow [35, 36]. Congestion was a very common found abnormality in this study. 90% of the adrenal glands had congestion and 38% of the adrenal glands with congestion also showed haemorrhage. In the acute group 89% had hyperaemia, the chronic group 90%, and the unknown group 100%. Because of this relative good distribution, there is no significant difference found. But because of the high prevalence in the different causes of death, it is possibly stress-related.

The extracellular eosinophilic material found in the medulla is probably leaked serum from the veins and arteries. Histochemical stainings using Congo red (amyloid), Van Giesson (collagen) and PAS (basement membranes) were negative. This leaked serum is probably due the present congestion.

Vacuolar degeneration is reported after acute injury [26]. However, vacuolization is also reported in fetuses (or rats) in acute and chronic stress due pregnancy [66]. Vacuolar degeneration found in the cortex seems to be more present in the outer cortex, thus zona glomerulas and/or zona fasciculata. This is different to another study in which vacuolar degeneration was identified more in the zona glomerulosa (outer zone) or zona reticularis (inner zone) [25]. This lesion could be associated with stress[25], chemical pollution or occur spontaneously [26]. The prevalence was higher in adults than immature animals. This could be because of the accumulation of the chemicals during their increased lifespan.

Necrosis is described as an abnormality due acute injury [26]. However there is no difference in this study between acute en chronic groups (both 76%). In the by-catch group it is even very low (5%). Together with haemorrhage and vacuolar degeneration these are changes due acute injury.

Three harbour porpoises had these three abnormalities together and one of these animals died acute. Necrosis was even more present (60%) in the chronic stress group than in the acute stress group (44%). Thus necrosis in these adrenal glands could be occurred by stress in general.

Many studies showed hypertrophy and hyperplasia of the cortex and/or medulla in relation to chronic diseases and/ or chronic stress (see chapter 1). They used different methods to determine hypertrophy of hyperplasia: the nucleo-cytoplasm ratio [20], cytoplasmic ratio [17], cell counting [19, 21], nuclei counting [19, 21], cortex-medulla ratio with point counting technique [13, 14, 16]. Unfortunately this was not possible on the histology slides in this study. It was not certain if the tissue samples were taken from the same place every time because different people (pathologists) took the samples. The methods used in the other studies are good methods because of the difficulty of determine the ratio of the adrenal glands which are lobulated. However Lair et al. analyzed the thickness of the adrenal cortex and medulla by measuring on the widest point of the gland [22]. The adrenal glands in this study were probably not handled the same and it is therefore not reliable to use only the widest point. Only one adrenal gland showed hypertrophy in the cortex. This was determined by visual examination assessing the space between the cells, color of the cells and the amount of cells.

The weights of the adrenal glands were also compared. The adrenal glands of the harbour porpoises in the group of chronic stress are significant heavier, than the adrenal glands of the harbour porpoises in the group of acute stress. This could be due hypertrophy or hyperplasia. Only one adrenal gland showed hypertrophy in this study. A more detailed method, like point counting technique, can be done in another research to determine the presence of hyperplasia or hypertrophy. Thus this relation can not be made. But the increased weight is possibly due chronic stress.

Adrenalitis is not described in association of stress, but sepsis is. Furthermore, chronic stress is immunosuppressive [4]. Adrenal glands can have varying degrees of inflammation and necrosis due bacterial and parasitic agents [67]. With bacterial sepsis, focal inflammatory may arise (usually suppurative) [67]. In dogs and cats granulomatous adrenalitis occurs due *Histoplasma capsulatum, Coccidioides immites,* or *Cryptococcus neoformans* [67]. *Toxoplasma gondii* causes necrosis with an infiltration of histiocytes in the adrenal cortex [67]. Large local concentrations of anti-inflammatory steroids in the adrenal cortex possibly permit progressive growth of cetain fungi, protozoa and bacteria due to suppression of the cell-mediated inmmunity [67]. But only three harbour porpoises had sepsis, thus sepsis is probably not the cause of this adrenalitis.

Sub-acute to chronic lymphoplasmacellular adrenalitis was the most common form of adrenalitis in this study. There was no difference between the acute or chronic group with regard to the type. However, stress hormones have negative impact on T lymphocytes activation and migration [68]. Overall, stress reduces the proliferation of lymphocytes [69]. This is in contrats to the findings in this study. Cells were identified only on their morphological characteristics based on H&E staining and the exact identity of these cells should be further investigated. Plasma cells are also found in this study. B cells will proliferate and differentiate in plasma cells after activation of T cells due presentation of fragments antigen on MHC molecules on B cells. Plasma cells will produce antigen specific antibodies, such as IgA, IgD, IgE, IgG, and IgM. Stress seems to affect the presence of these antibodies. Stress can cause an increase of IgA [70], or IgG, IgM and IgA [71], or decrease IgM (in rats during electric food shock stress) [72]. The recommendation is to do immunohistochemical (IHC) characterization of inflammatory cells in the adrenal glands, to determine the immune morphologic characterization.

The presence of adrenalitis at the transition of cortex and medulla was striking (see figure 3). 62% of the adrenal glands had adrenalitis at the transition. This can be related to the blood flow in the adrenal glands. The cortex and medulla have separated blood supplies [73]. The arteries for the medulla run straight trough the cortex and the arteries for the cortex are coming from the capsule[73]. But they have the same out flow: blood is going from the sinusoids of the cortex to the

medulla were the venous drainage is present [73]. In the zona reticularis irregular vascular channels are separating the rows and cords of cells [73]. It is possible that the blood flow from the cortex to the medulla is piled up on the transition because of the layer of connective tissue between the cortex and medulla [14-16]. However, besides in the transition the inflammatory cells should be present in the cortex because the blood flow goes from the outer cortex to the inner cortex. In this study most of the adrenal glands with adrenalitis on the transition did not have adrenalitis in the rest of the cortex or those much inflammatory cells. The terminal phase of haemorrhagic shock is commonly associated with distinct haemorrhage and inflammatory infiltration in the zona fasciculata and reticularis [74]. However, this was found in final disease stages and therefore in a different situation compared to the study, because there was no significant difference between infectious and emaciation or chronic and acute for example. The adrenal glands in the group unknown all had adrenalitis on the transition of cortex and medulla. Another explanation is a higher vascular density on the transition of cortex and medulla which may be typical for harbour porpoises.

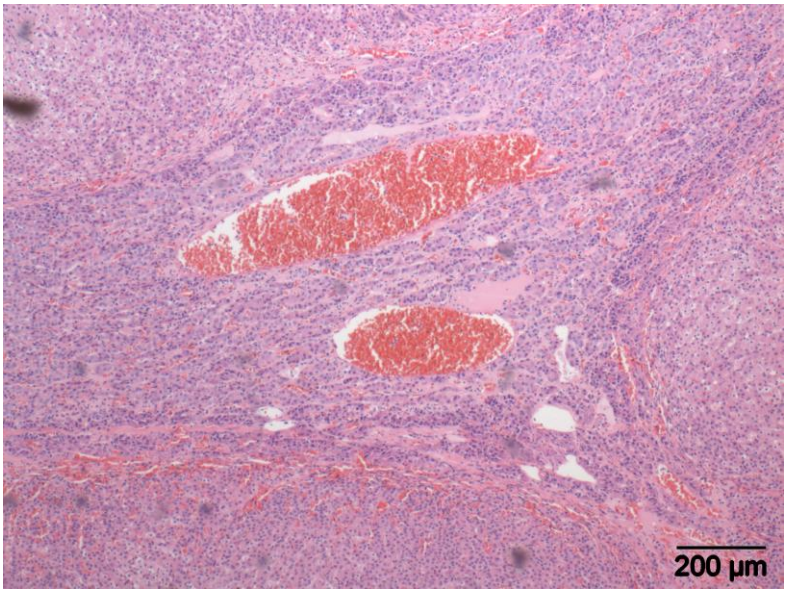


Figure 3. *Adrenal gland with congestion, haemorrhage and adrenalitis on the transition of cortex and medulla (see arrow). (4x1.25)*

There were several practical situations which are subject to discussion. First, the absence of slides presenting both adrenal glands. Most of the present microscopy slides of the adrenal glands were just from one adrenal gland, not from both adrenal glands. It is possible that the left adrenal gland showed different changes than the right adrenal gland. Some abnormalities were reported as bi- and unilateral, like vacuolar degeneration [22]. The recommendation for the coming necropsies is, if there is interest in more research in the adrenal glands of harbour porpoises, to sample both adrenal glands.

With the examination of the adrenal glands the terms mild, moderate and severe were used**.** This is a personal interpretation. However, many histological slides/ adrenal glands were used in this study, thus the terms mild, moderate and severe were probably used well because of the amount of material which was compared with each other. However, it is still a personal opinion and other staining methods can be used to make better differentiations and interpretations. Examples are ICH characterization or point counting technique (to determine the presence of hypertrophy or hyperplasia).

All the adrenal glands had abnormalities and some of them were described as stress-related in other studies (see chapter 1). A recommendation is to do a point counting technique to determine if the increased weights (in the group of chronic stress) of the adrenal glands are due hypertrophy or hyperplasia. The adrenalitis found on the transition of cortex and medulla is striking in this study

and is a good recommendation for further research using IHC for characterization of the inflammatory cells. Because all adrenal glands showed one or more abnormalities, investigation about situations which causes stress in harbour porpoises can be useful. Furthermore, it is possible that more general problems are responsible, like noise pollution.

Content 5

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Content 6

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Content 7

Appendix

7.1 Appendix A

Table 6. *Collected information about the harbour porpoises used in this study. A section of this information is from macroscopy - and microscopy reports, which were done by pathologists of the University of Utrecht.*

Carcass no.	Age	Sex	DCC	NCC	macroscopy	recent feeding	Adrenal weights (grams)		probable cause of death
							left	right	
2009									
HP 1	J	M	2	4		N	4	4	infectious
HP 2	N	M	2	3		N	2	3	starvation
HP 3	J	F	2	3.5		N	9	8	infectious
2010									
HP 4	J	F	2	4.5		Y	2	2	by-catch
HP 5	J	F	2	5		N	6	7	emaciation
HP 6	A	F	1	1.5	multifocal cysts: ca. 3-4 mm with multifocal red areas (irregular 3-4mm.)	N	14	13	unknown
HP 7	J	M	2	1		Y			infectious?/viral?
HP 8	A	M	1	1		N	8	7	live stranding/ infectious
HP 9	N	M	2	5		N	2	2	starvation
HP 10	A	M	1	2		N	8	9	by-catch
HP 11	A	M	1	2	diffuse pale cortex	N	7	7	by-catch
HP 12	J	M	1	3.5		N	4	5	starvation
HP 13	N	M	2	4.5		N	2	2	starvation
HP 14	A	M	1	4		N	20	17	sepsis
HP 15	A	F	1	4	unilateral the adrenal has 2 mm ø haemorrhage on cut surface.	Y	11	9	by-catch
HP 16	A	F	1	3		N	6	6	infectious
2011									
HP 17	A	F	2	4	The right adrenal contained three cysts 4 mm diameter, at the cranial tip stacked together, and 2 mm wide other cysts in the cortex on cut.	Y	19	17	infectious
HP 18	J	M	1	1		Y	2	2	infectious/ trauma
HP 19	A	M	1	2		Y	10	11	unknown
HP 20	J	M	1	2		N	4	4	sepsis
HP 21	J	M	1	1		Y	4	3	by-catch
HP 22	A	M	1	3		Y	12	8	by-catch
HP 23	J	M	1	4		Y	3	3	by-catch

HP 24	J	M	1	1		Y	4	4	by-catch
HP 25	A	F	1	5		N	11	12	infectious
HP 26	J	F	1	2		Y	5	6	by-catch
HP 27	J	M	1	4.5		N	4	4	infectious
HP 28	A	M	1	4.5	Marrow shrunken, surface is irregular, enlarged à 7 gram.	N			infectious
HP 29	J	M	1	5		Y	10	7	emaciation
HP 30	A	F	1	2		N	14	14	dystocia
HP 31	N	M	1	6		N	2	2	starvation
HP 32	N	M	1	5	Medulla dark red.	N	2	2	starvation
HP 33	N	F	1	2		Y	2	2	infectious
HP 34	J	M	1	4		N	6	6	infectious
HP 35	N/J	M	1	1		N			unknown
HP 36	A	F	1	5		N	9	8	emaciation
HP 37	J	M	1	6		N	5	5	trauma
HP 38	N	M	1	2		Y	1	1	by-catch
HP 39	J	F	1	6	Hemorrhage at transition cortex – medulla.	N	6	5	emaciation
HP 40	J	F	1	2		Y	4	5	by-catch
HP 41	A	M	2	6		N	6	8	by-catch
HP 42	A	F	1	4		Y	19	14	infectious
HP 43	A	F	1	5		Y	19	16	infectious
HP 44	J	M	2	5		Y			infectious
HP 45	A	F	2	4		Y			by-catch
2012									
HP 46	A	F	1	4		Y	14	14	infectious
HP 47	J	M	1	4	Diffusely pale. Decreased in consistency,	Y	5	11	infectious
HP 48	A	F	2	4	moderate	N			emaciation
HP 49	J	M	1	2	pale	Y	3	3	by-catch
HP 50	N	F	1	4		N	1	2	starvation

7.2 Appendix B

Table 7. *Histological findings in the adrenal glands used in this study, with the probable cause of death based by macroscopy and microscopy by pathologists of the University of Utrecht. The last column is about the probable kind of stress: acute or chronic, based on the macroscopy and microscopy reports.*

Carcass no.	histology	probable cause of death	acute/ chronic stress
2009			
HP 1	Mild hemorrhagic in medulla and cortex. Mild lymphoplasma infiltration and necrosis on transition of cortex and medulla and outer cortex. Vacuolar degeneration in cortex.	infectious	C
HP 2	Mild to moderate congestion and hemorrhagic in cortex and medulla. Mild lymphoplasma infiltration and necrosis on transition of cortex and medulla and in medulla.	starvation	A
HP 3	Severe congestion and mild multifocal hemorrhagic in cortex. Moderate lymfoplasma infiltration and necrosis on transition of cortex and medulla. Mild multifocal lymphoplasma infiltration in cortex.	infectious	C
2010			
HP 4	Moderate congestion and mild hemorrhagic. Mild diffuse lymphoplasma infiltration on transition of cortex and medulla.	by-catch	A
HP 5	Mild congestion and hemorrhagic. Mild lymphoplasma infiltration in cortex. Mild vacuolar degeneration in cortex.	emaciation	C
HP 6	Moderate to severe congestion and multifocal extensive hemorrhagic in medulla. Mild lymphoplasma infiltration on transition of cortex and medulla. Several individual necrotic cells in cortex. Mild focal eosinophilic cells in cortex.	unknown	
HP 7	Mild hemorrhagic in cortex. Mild lymphoplasma infiltration and hypertrophy in medulla.	infectious?/viral?	A
HP 8	Mild congestion and multifocal hemorrhagic in cortex. Mild lymphoplasma infiltration on transition of cortex and medulla and in cortex. Moderate vacuolar degeneration in outer cortex. More extracellular eosinophilic material in medulla: serum?	live stranding/ infectious	C
HP 9	Moderate lymphoplasma infiltration on transition of cortex and medulla and in medulla. More extracellular eosinophilic material in medulla: serum?	starvation	A
HP 10	Moderate congestion and multifocal hemorrhagic in cortex. Mild lymphocytic infiltration on transition of cortex and medulla and in medulla. Mild vacuolar degeneration in outer cortex.	by-catch	A
HP 11	Mild to moderate congestion. Moderate lymphoplasma infiltration on transition of cortex and medulla. More extracellular eosinophilic material in medulla: serum?	by-catch	A
HP 12	Moderate congestion. Mild to moderate lymphoplasma infiltration on the transition of the cortex and medulla. Mild extracellular eosinophilic material in medulla: serum? Mild focal edema in medulla (coupe 7).	starvation	A
HP 13	Moderate hyperemic (congestion) cortex and moderate hemorrhagic in medulla. Moderate lymphoplasma infiltration on transition of cortex and medulla. Mild multifocal necrosis in cortex.	starvation	A
HP 14	Mild to moderate congestion. Moderate to severe multifocal plasmacellulair infiltration and necrosis.	sepsis	A
HP 15	Moderate congestion in medulla. Mild multifocal plasmacellular infiltration in cortex and transition of cortex and medulla. Mild vacuolar degeneration and necrosis in outer cortex and medulla. Mild hypertrophy in medulla.	by-catch	A
HP 16	Moderate congestion. Moderate multifocal lymphoplasma infiltration and necrosis, Focal severe.	infectious	C
2011			
HP 17	Moderate congestion in medulla. Mild multifocal plasmacellular infiltration in cortex and medulla. Mild multifocal necrosis in cortex. Multilocular cysts.	infectious	C
HP 18	Mild congestion and hemorrhagic on transition of cortex and medulla. Mild plasmacellular infiltration and necrosis on transition of cortex and medulla.	infectious /trauma	A

HP 19	Mild to moderate congestion. Moderate plasmacellular infiltration in cortex and on transition of cortex and medulla. Moderate multifocal necrosis in cortex and on transition of cortex and medulla. Postmortem bacterial overgrowth.	unknown	
HP 20	Mild multifocal congestion, mild plasmacellular infiltration on transition of cortex and medulla. More extracellular eosinophilic material in medulla: serum?	sepsis	A
HP 21	Moderate congestion and mild hemorrhagic in cortex. Mild lymphoplasma infiltration (more lymphocytes) and mild necrotic cells in medulla, and moderate on the transition of cortex and medulla. Vacuolar degeneration in outer cortex (with congestion).	by-catch	A
HP 22	Mild congestion. Moderate lymfoplasma infiltration and focal vacuolar degeneration in cortex.	by-catch	A
HP 23	Mild to moderate congestion and moderate hemorrhagic. Multifocal necrosis in cortex. Moderate to severe lymfoplasma infiltration on transition of cortex and medulla.	by-catch	A
HP 24	Mild to moderate congestion and mild hemorrhagic in cortex. Mild lymphoplasma infiltration and necrosis on transition of cortex and medulla.	by-catch	A
HP 25	Severe hemorrhagic in medulla. Moderate lymphoplasma infiltration on transition of cortex and medulla and multifocal in cortex. Mild vacuolar degeneration and necrosis in cortex. More eosinophilic material in cortex: mineralization?	infectious	C
HP 26	Moderate congestion, mild to moderate subacute to chronic multifocal lymphoplasma infiltration (more plasma cells).	by-catch	A
HP 27	Mild congestion. Mild to moderate lymphoplasma infiltration and mild macrophage infiltration on transition of cortex and medulla. Mild vacuolar degeneration and necrosis in cortex. More extracellular eosinophilic material in medulla: serum?	infectious	C
HP 28	Moderate multifocal congestion. Moderate lymphoplasma infiltration on transition of cortex and medulla. Individual necrotic cells in cortex. Multifocal hypertrophic cortical cells. More extracellular eosinophilic material in medulla: serum?	infectious	C
HP 29	Moderate to severe congestion (more on transition of cortex and medulla). Mild lymphoplasma infiltration on transition of cortex and medulla. Mild apoptotic and necrotic cells in medulla. More extracellular eosinophilic material in medulla: serum?	emaciation	C
HP 30	Moderate congestion and mild hemorrhagic. Mild lymphoplasma infiltration on transition of cortex and medulla and necrosis. Moderate vacuolar degeneration in cortex.	dystocia	A
HP 31	Moderate congestion and mild hemorrhagic. Moderate multifocal lymphoplasma infiltration (more plasma cells) in medulla and on transition of cortex and medulla.	starvation	A
HP 32	Moderate to severe congestion and mild hemorrhagic. Mild focal lymphoplasma infiltration on transition of medulla and cortex and in medulla.	starvation	A
HP 33	Moderate congestion.	infectious	C
HP 34	Mild to moderate multifocal congestion. Mild multifocal lymphocyte infiltration in medulla and cortex.	infectious	C
HP 35	Moderate congestion. Moderate multifocal lymphoplasma infiltration (more plasma cells) on transition of cortex and medulla.	unknown	
HP 36	Moderate congestion. Mild plasmacellular and necrosis on transition of cortex and medulla. Moderate vacuolar degeneration in outer cortex.	emaciation	C
HP 37	Mild congestion. Mild necrosis round blood vessels in medulla. More extracellular eosinophilic material in medulla: serum?	trauma	A
HP 38	Moderate congestion and hemorrhagic. Moderate multifocal lymphoplasma infiltration (mainly transition of cortex and medulla).	by-catch	A
HP 39	Moderate congestion. Mild to moderate multifocal lymphoplasma infiltration on transition of cortex and medulla.	emaciation	C
HP 40	Moderate multifocal congestion. Mild vacuolar degeneration in outer cortex.	by-catch	A
HP 41	Moderate plasmacellular infiltration in medulla and cortex. Mild multifocal necrosis in cortex. More extracellular eosinophilic material in medulla: serum?	by-catch	A
HP 42	Mild congestion. Mild multifocal plasmacellular and histiocytic infiltration in cortex. Nice for picture.	infectious	C

HP 43	Mild multifocal congestion in cortex. Moderate multifocal plasmacellular adrenalitis in cortex and medulla. Moderate individual apoptotic cells in cortex.	infectious	C
HP 44	Moderate congestion (especially on transition of cortex and medulla). Mild multifocal hemorrhagic in cortex. Mild multifocal individual necrotic cells in cortex and mild focal lymphoplasma infiltration on transition of cortex and medulla. More eosinophilic extracellular material: serum?	infectious	C
HP 45	Mild congestion (especially on transition of cortex and medulla). Mild multifocal lymphoplasma infiltration. More extracellular eosinophilic material in medulla: serum?	by-catch	A
2012			
HP 46	Moderate congestion in cortex and medulla. Mild to moderate multifocal lymphoplasma infiltration and necrotic cells.	infectious	C
HP 47	Mild congestion and mild multifocal hemorrhagic. Mild lymphoplasma infiltration in medulla.	infectious	C
HP 48	Moderate congestion and hemorrhagic in cortex. Mild multifocal plasmacellular adrenalitis in cortex and on transition of cortex and medulla.	emaciation	C
HP 49	Mild congestion (especially on transition of cortex and medulla, and medulla). Moderate multifocal lymphoplasma infiltration in medulla and cortex. Mild individual necrotic cells.	by-catch	A
HP 50	Moderate congestion in cortex. Mild lymphoplasma infiltration on transition of cortex and medulla.	starvation	A

7.3 Appendix C

Table 10. *Semi quantitative grading system with three groups: acute, chronic and unknown.*

ACUTE

Harbour porpoise (HP)	Adrenalitis on transition	Adrenalitis in medulla	Adrenalitis in cortex	Hyperemia	Haemorrhagic	Necrosis	Vacuolar degeneration	Eosinophilic material	Hypertrophy	Cysts	Edema	Total
HP 7	0	1	0	0	1	0	0	0	1	0	0	**3**
HP 14	0	4	4	2	0	4	0	0	0	0	0	**14**
HP 18	1	0	0	1	1	1	0	0	0	0	0	**4**
HP 20	1	0	0	1	0	0	0	1	0	0	0	**3**
HP 30	1	0	0	3	1	1	3	0	0	0	0	**9**
HP 39	0	0	0	1	0	1	0	1	0	0	0	**3**
HP 4	1	1	0	3	1	0	0	0	0	0	0	**6**
HP 10	1	1	0	3	3	0	1	0	0	0	0	**9**
HP 11	0	0	0	2	0	0	0	1	0	0	0	**3**
HP 15	1	1	1	3	0	1	1	0	1	0	0	**9**
HP 21	3	3	0	3	0	2	1	0	0	0	0	**12**
HP 22	0	0	3	1	0	0	1	0	0	0	0	**5**
HP 23	4	4	0	2	3	1	0	0	0	0	0	**14**
HP 24	1	1	0	2	1	1	0	0	0	0	0	**6**
HP 26	0	0	2	3	0	0	0	0	0	0	0	**5**
HP 38	3	3	1	3	3	0	0	0	0	0	0	**13**
HP 40	0	0	0	3	0	0	1	0	0	0	0	**4**
HP 41	0	0	3	0	0	1	0	1	0	0	0	**5**
HP 45	0	0	1	1	0	0	0	1	0	0	0	**3**
HP 49	0	0	3	1	0	1	0	0	0	0	0	**5**
HP 2	1	1	0	2	2	1	0	0	0	0	0	**7**
HP 9	3	3	0	0	0	0	0	1	0	0	0	**7**
HP 12	2	0	0	3	0	0	0	1	0	0	1	**7**
HP 13	3	0	0	3	3	1	0	0	0	0	0	**10**
HP 31	3	3	0	3	1	0	0	0	0	0	0	**10**
HP 32	1	1	0	4	0	0	0	0	0	0	0	**6**
HP 50	1	0	0	3	0	0	0	0	0	0	0	**4**
Average	**1,148148**	**1**	**0,666667**	**2,0740741**	**0,74074074**	**0,59259**	**0,2962963**	**0,2592593**	**0,07407407**	**0**	**0,037**	

CHRONIC

Harbour porpoise (HP)	Adrenalitis on transition	Adrenalitis in medulla	Adrenalitis in cortex	Hyperemia	Haemorrhagic	Necrosis	Vacuolar degeneration	Eosinophilic material	Hypertrophy	Cysts	Edema	Total
HP 1	1	0	1	0	1	1	2	0	0	0	0	**6**
HP 3	3	0	1	5	1	3	0	0	0	0	0	**13**
HP 8	1	0	1	1	1	0	3	1	0	0	0	**8**
HP 16	0	3	3	3	0	3	0	0	0	0	0	**12**
HP 17	0	1	1	3	0	1	0	0	0	1	0	**7**
HP 25	3	0	3	0	5	1	1	1	0	0	0	**14**
HP 27	2	0	0	1	0	1	1	1	0	0	0	**6**
HP 28	3	0	0	3	0	1	0	1	1	0	0	**9**
HP 33	0	0	0	3	0	0	0	0	0	0	0	**3**
HP 34	0	1	1	2	0	0	0	0	0	0	0	**4**
HP 42	0	0	1	1	0	0	0	0	0	0	0	**2**
HP 43	0	3	3	1	0	3	0	0	0	0	0	**10**
HP 44	1	0	0	3	1	1	0	1	0	0	0	**7**
HP 46	0	2	2	3	0	2	0	0	0	0	0	**9**
HP 49	0	1	0	1	1	0	0	0	0	0	0	**3**
HP 5	0	0	1	1	1	0	1	0	0	0	0	**4**
HP 29	1	0	0	4	0	1	0	1	0	0	0	**7**
HP 36	1	0	0	3	0	1	3	0	0	0	0	**8**
HP 39	2	0	0	3	0	0	0	0	0	0	0	**5**
HP 48	1	0	1	3	3	0	0	0	0	0	0	**8**
Average	**0,95**	**0,55**	**0,95**	**2,2**	**0,7**	**0,95**	**0,55**	**0,3**	**0,05**	**0,05**	**0**	

UNKNOWN

Harbour porpoise (HP)	Adrenalitis on transition	Adrenalitis in medulla	Adrenalitis in cortex	Hyperemia	Haemorrhagic	Necrosis	Vacuolar degeneration	Eosinophilic material	Hypertrophy	Cysts	Edema	Total
HP 6	1	0	0	4	3	1	0	1	0	1	0	**11**
HP 19	3	0	3	2	0	3	0	0	0	0	0	**11**
HP 35	3	0	0	3	0	0	0	0	0	0	0	**6**
Average	**2,33333333**	**0**	**1**	**3**	**1**	**1,333333**	**0**	**0,333333333**	**0**	**0,333**	**0**	

Table 11 . *Semi quantitative grading system with three groups: acute, chronic and unknown, and one group about adrenalitis.*

ACUTE

Harbour porpoise (HP)	Adrenalitis	Hyperemia	Hemorrhagic	Necrosis	Vacuolar degeneration	Eosinophylic material	Hypertrophy	Cysts	Oedema	Total
HP 7	0	0	1	0	0	0	1	0	0	**2**
HP 14	0	2	0	4	0	0	0	0	0	**6**
HP 18	1	1	1	1	0	0	0	0	0	**4**
HP 20	1	1	0	0	0	1	0	0	0	**3**
HP 30	1	3	1	1	3	0	0	0	0	**9**
HP 37	0	1	0	1	0	1	0	0	0	**3**
HP 4	1	3	1	0	0	0	0	0	0	**5**
HP 10	1	3	3	0	1	0	0	0	0	**8**
HP 11	3	2	0	0	0	1	0	0	0	**6**
HP 15	1	3	0	1	1	0	1	0	0	**7**
HP 21	2	3	0	2	1	0	0	0	0	**8**
HP22	3	1	0	0	1	0	0	0	0	**5**
HP 23	4	2	3	1	0	0	0	0	0	**10**
HP 24	1	2	1	1	0	0	0	0	0	**5**
HP 26	2	3	0	0	0	0	0	0	0	**5**
HP 38	3	3	3	0	0	0	0	0	0	**9**
HP 40	0	3	0	0	1	0	0	0	0	**4**
HP 41	3	0	0	1	0	1	0	0	0	**5**
HP 45	1	1	0	0	0	1	0	0	0	**3**
HP 49	3	1	0	1	0	0	0	0	0	**5**
HP 2	1	2	2	1	0	0	0	0	0	**6**
HP 31	3	3	1	0	0	0	0	0	0	**7**
HP 32	1	4	0	0	0	0	0	0	0	**5**
HP 50	1	3	0	0	0	0	0	0	0	**4**
HP 9	3	0	0	0	0	1	0	0	0	**4**
HP 12	2	3	0	0	0	1	0	0	1	**7**
HP 13	3	3	3	1	0	0	0	0	0	**10**
Average	**1,666667**	**2,074074**	**0,740741**	**0,592593**	**0,296296**	**0,259259**	**0,074074**	**0**	**0,037037**	

CHRONIC

Harbour porpoise (HP)	Adrenalitis	Hyperemia	Hemorrhagic	Necrosis	Vacuolar degeneration	Eosinophylic material	Hypertrophy	Cysts	Oedema	Total
HP 1	1	0	1	1	2	0	0	0	0	**5**
HP 3	3	5	1	3	0	0	0	0	0	**12**
HP 8	1	1	1	0	3	1	0	0	0	**7**
HP 16	3	3	0	3	0	0	0	0	0	**9**
HP 17	1	3	0	1	0	0	0	1	0	**6**
HP 25	3	0	5	1	1	1	0	0	0	**11**
HP 27	2	1	0	1	1	1	0	0	0	**6**
HP 28	3	3	0	1	0	1	1	0	0	**9**
HP 33	0	3	0	0	0	0	0	0	0	**3**
HP 34	1	2	0	0	0	0	0	0	0	**3**
HP 42	1	1	0	0	0	0	0	0	0	**2**
HP 43	3	1	0	3	0	0	0	0	0	**7**
HP 44	1	3	1	1	0	1	0	0	0	**7**
HP 46	2	3	0	2	0	0	0	0	0	**7**
HP 47	1	1	1	0	0	0	0	0	0	**3**
HP 5	1	1	1	0	1	0	0	0	0	**4**
HP 29	1	4	0	1	0	1	0	0	0	**7**
HP 36	1	3	0	1	3	0	0	0	0	**8**
HP 39	2	3	0	0	0	0	0	0	0	**5**
HP 48	1	3	3	0	0	0	0	0	0	**7**
Average	**1,6**	**2,2**	**0,7**	**0,95**	**0,55**	**0,3**	**0,05**	**0,05**	**0**	

UNKNOWN

Harbour porpoise (HP)	Adrenalitis	Hyperemia	Hemorrhagic	Necrosis	Vacuolar degeneration	Eosinophylic material	Hypertrophy	Cysts	Oedema	Total
HP 6	1	4	3	1	0	1	0	1	0	**11**
HP 19	3	2	0	3	0	0	0	0	0	**8**
HP 35	3	3	0	0	0	0	0	0	0	**6**
Average	**2,333333**	**3**	**1**	**1,333333**	**0**	**0,333333**	**0**	**0,333333**	**0**	

7.4 Appendix D

Table 12. *Independent two sample T-test with 2 groups: acute, chronic.*

ANOVA

total length

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,008	2	,004	5,726	,006
Within Groups	,027	41	,001		
Total	,035	43			

Table 13. *One-way ANOVA with 3 groups: acute, chronic, unknown.*

Multiple Comparisons

Dependent Variable: adrenal gland / total length

Tukey HSD

(I) stress	(J) stress	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1,00	2,00	-,02454*	,00812	,012	-,0443	-,0048
	3,00	-,03848	,01899	,119	-,0847	,0077
2,00	1,00	,02454*	,00812	,012	,0048	,0443
	3,00	-,01394	,01932	,752	-,0609	,0330
3,00	1,00	,03848	,01899	,119	-,0077	,0847
	2,00	,01394	,01932	,752	-,0330	,0609

*. The mean difference is significant at the 0.05 level.