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Decrease Stress; Train Your Animals: The Effect of Handling Methods on Cortisol Levels in Harbour Porpoises (*Phocoena phocoena*) Under Human Care

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Abstract

Circulating cortisol levels are accepted as a sensitive indicator of acute stress in marine mammals, particularly in relation with capture and handling. The present study provides the first long-term monitoring of cortisol levels in four harbour porpoises held in human care—an adult male and adult female and two juvenile females. It also compares levels in blood obtained after removing the animal from the water (OWR sampling) with levels in blood obtained at poolside under voluntary husbandry behaviours (VHB sampling). Cortisol levels differed significantly between the four porpoises, although they all exhibited quite high variations in cortisol levels, with averages of 64.9 and 70.5 µg/l in the adult male and female, respectively, and 90.7 and 51.4 µg/l in the juvenile females. OWR sampling induced significantly higher cortisol levels than VHB sampling, with a dramatic threefold decrease in circulating cortisol levels obtained under VHB sampling compared to levels obtained under OWR sampling (16.6 and 20.2 µg/l compared with 64.9 and 70.5 µg/l in the adult male and female respectively). Even if the porpoises showed some habituation to handling, regular and frequent handling over several years did not suppress a significant stress response in the porpoises when they were removed from the water, pointing to the advantage of using VHB for limiting stress in husbandry practices.

Key Words: Harbour porpoise, North Sea, *Phocoena phocoena*, cortisol levels, human care, handling methods, voluntary husbandry behaviours

Introduction

As a result of the increased concern for improving the well-being of marine mammals held in human care for research, display, and rehabilitation, more effort has been put into assessing stress. The ability to measure stress is indeed necessary for identifying the least stressful methods for capturing and handling marine mammals.

Elevated serum concentrations of adrenal corticosteroids and particularly cortisol are considered as an index of the stress response in terrestrial mammals (Dantzer & Mormede, 1983; Moberg, 1987; Morton et al., 1995). A similar increase has been observed in bottlenose dolphins (*Tursiops truncatus*), white whales (*Delphinapterus leucas*), and pilot whales (*Globicephala melas*) (Thomson & Geraci, 1983, 1986; Parker & Schroeder, 1987; Orlov et al., 1988, 1991; St. Aubin & Geraci, 1989; Copland & Needham, 1992; St. Aubin et al., 1996; Waples & Gales, 2002), as well as harp seals (*Phoca groenlandica*) (St. Aubin et al., 1979). Bearing in mind that an assessment of stress based on a number of biochemical and hematological variables is preferable to the use of a single measurement (e.g., Moberg, 1987; Hattingh, 1988; Dierauf, 1990; Kock et al., 1990; St Aubin & Dierauf, 2001), circulating cortisol levels have been accepted as a sensitive indicator of acute stress in marine mammals, particularly in relation with capture and handling (Thomson & Geraci, 1986; Orlov et al., 1991; Koopman et al., 1995; Morton et al., 1995; St. Aubin et al., 1996; Waples & Gales, 2002).

In harbour porpoises, cortisol levels have only been reported as point data for wild porpoises bycaught in herring weirs and tagged before release (Koopman et al., 1995; Teilmann et al., 2004). In 1997, the Fjord & Bælt initiated a monitoring of cortisol in harbour porpoises held in human care (Buholzer et al., 2004). The present study provides the first long-term monitoring of cortisol levels in harbour porpoises; it compares levels observed in blood obtained after removing the animal from the water and blood obtained at poolside under voluntary husbandry behaviours.

Materials and Methods

The Porpoises

The four porpoises, a maximum of three at any time, were kept together in an outdoor, semi-natural enclosure of about 9,600 m^3, spanned by nets on two sides. The habitat and the animals were exposed to natural environmental conditions for water quality, tidal currents, temperature, light, and variation in flora and fauna. The biological characteristics for the porpoises at the beginning and end of the study are indicated in Table 1.

Blood Sampling Procedures

During the study period, blood was sampled at least once a month for routine medical check and up to four times a month for shorter periods during specific research projects conducted under permission from the Danish Ministry of Justice (1998-561-110). In Eigil, Freja, and Sif, the cortisol was measured on samples taken when the animals were considered to be healthy based on clinical and blood examination, except in two cases for Freja where the white blood cells count (WBC) was greater than 5 10^3/mm^3, which indicated a bacterial infection. For Nuka, however, the WBC was greater than 5 10^3/mm^3 in the majority of the samples.

Blood was collected in S-Monovetten® KE (Sarstedt), was centrifuged immediately, and serum was separated and frozen in 1 ml aliquots. Within the next few days, serum cortisol was measured by radio-immunoassay following standard techniques in a commercial laboratory in Germany (Gemeinschaftspraxis Dr. Kramer und Kollegen, D-21494 Geesthacht) with X-Counter (Wallace, Lisle, Ireland). The minimum detectable level was 2 µg/l, the intra-assay coefficients of variation was 3 to 5%, and the inter-assay coefficients of variation was 4 to 6%. The nonspecific binding was not evaluated, but experience showed that in the standard deviation, identical results were found whether or not nonspecific binding was taken into consideration.

Blood Sampling Out of the Water on a Restrained Animal or OWR Sampling—The animals were gated into a holding pool (HP) to which they usually had free access. The porpoises were caught one at a time, and in varying order, by a trainer in the HP and placed into a stretcher. They were then lifted out of the water, examined on the pontoon, or transported to an investigation table close-by. The amount of time the animals were removed from the water ranged from 10 to 40 min, depending on the examination. The blood sample was taken from the dorsal side of the flukes within 5 to 15 min of the animal being removed from the water. The procedure was kept similar over the years, with the same core people involved, although a progressive changing in staff occurred.

Blood Sampling at Poolside Under Voluntary Husbandry Behaviour or VHB Sampling—The porpoises were trained, through operant conditioning and positive reinforcement, to present their tail flukes for blood sampling at poolside. The animal laid in the water in a horizontal position, its tail supported by one trainer kneeling on the pontoon while a second person took blood from the dorsal side of the flukes. The procedure lasted from 1 to 3 min. Blood was collected this way since July 1999 with Freja, September 2001 with Eigil, and October 2005 with Sif.

Table 1. Biological characteristics of the four harbour porpoises kept in human care at the Fjord & Bælt during the cortisol study

ID	Sex	Start of study	Length (cm)	Weight (kg)	End of study	Length (cm)	Weight (kg)
Freja	Female	7 April 1997	127	40	14 May 2004	150	56
Eigil	Male	7 April 1997	130	37	14 May 2004	140	43
Nuka	Female	19 April 1999	115	31	3 Feb. 2000*	124	37
Sif	Female	23 July 2004	106	19	25 Oct. 2005	130	35

*Nuka died from a *Staphylococcus aureus* septicaemia on 22 February 2000 (Siebert et al., 2002a)

Statistical Analysis—The degree of significance was taken at 5%. One way or two ways ANOVA, as well as pair-wise comparisons with the Fisher PLSD (Protected Least Significant Difference) test were used for comparison.

Results

Individual Differences in Cortisol Levels

Individual variation in cortisol levels are shown in Figure 1a-d and summary data are given in Table 2. The four porpoises exhibited quite high variations in cortisol levels, especially in the first period of acclimation. From time to time, they also exhibited values outside their usual variation range, with the extreme observed in Eigil 5 wks after it arrived from the wild with a level of 793 µg/l (14 May 1997) compared to an average of 49 µg/l.

The data from the first month of acclimation illustrated the specific response of the individual to its arrival in human care, and the values typically outside the usual individual variation range likely represented an acute response to an unknown factor. Therefore, we chose not to include these values (outliers) in an analysis, looking instead at the long-term adaptation to handling and the effect of different handling modes on the stress response.

Individual Variation in Cortisol Level Under OWR Sampling

Cortisol levels differed between the four porpoises (Figure 2; one-way ANOVA: df = 3; F-value = 10.998; $p < 0.0001$). Nuka had significantly higher levels than the three other porpoises (Pairwise comparisons with Fisher PLSD: $p < 0.0008$ in all cases), and Freja had significantly higher levels than Eigil ($p = 0.0047$).

Effect of Sampling Mode on Cortisol Levels: OWR vs VHB Sampling

In Eigil and Freja, cortisol levels dropped dramatically when blood was collected by VHB sampling at poolside compared with levels observed with OWR sampling for the same timeframe (Figures 1a & b). This resulted in a three-fold average decrease in cortisol levels (Figure 3). OWR sampling induced significantly higher cortisol levels than VHB sampling in spite of differences between the two porpoises (two-way ANOVA analysis; Table 3).

The only VHB blood sample for Sif that was available for analysis also exhibited a cortisol level half the average of those obtained with the OWR sampling.

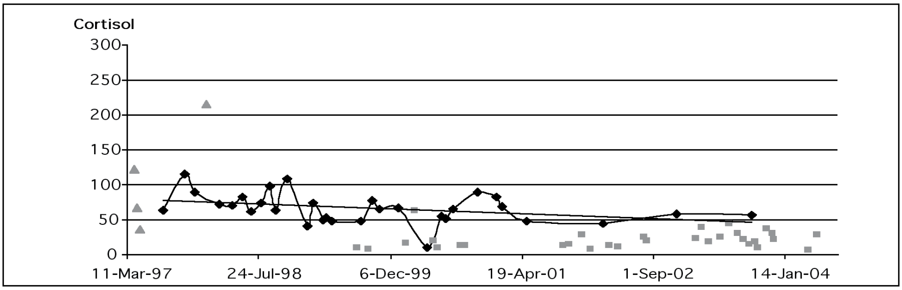
Discussion

The dynamic response of cortisol causes it to exhibit transient changes within minutes of an acute stress situation (see Kirby, 1990). The extent of the stress response to capture and handling, as reflected by the circulating cortisol level, varied quite a bit for each porpoise and differed significantly between the four individuals, with Nuka and Freja exhibiting higher levels than the two others. Such individual variations have been observed in other odontocete species (Orlov et al., 1991; St. Aubin et al., 1996). Wide individual variations have also been noted in point samplings of wild porpoises (Koopman et al., 1995; Buholzer et al., 2004; Teilmann et al., 2004).

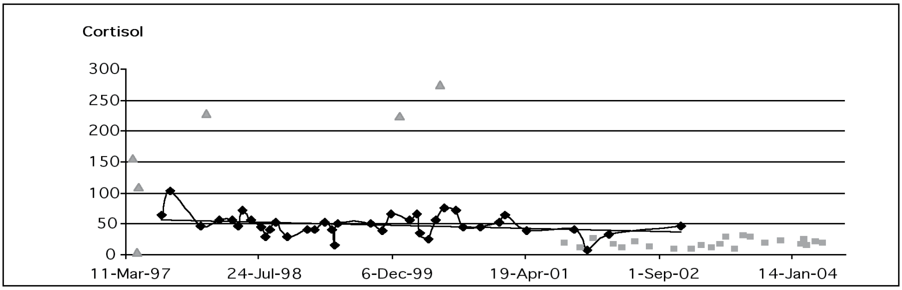
Besides the individual stress response, several other factors, such as diurnal and seasonal variations, can contribute to the wide intra-individual variation observed in cortisol levels. As with other hormones, cortisol has been shown to fluctuate diurnally and seasonally in several mammal species (Turner, 1984; Orlov et al., 1988, 1991; Ekkel et al., 1996; Gardiner & Hall, 1997; Suzuki et al., 2003). Increased cortisol levels have also been associated with the breeding season in some land mammals and pinnipeds (e.g., wolves [*Canis lupus*], MacLeod et al., 1996; Weddell seals [*Leptonychotes weddellii*], Bartsh et al., 1992; wild harbour seals (*Phoca vitulina*), Gardiner & Hall, 1997). In toothed whales, Orlov et al. (1991) observed seasonal changes in cortisol levels in captive white whales and bottlenose dolphins (age composition not specified), although St. Aubin et al. (1996) found no relationship with age or season in neither wild nor semi-domesticated bottlenose dolphins.

The health status of the animals could also influence the level of cortisol. Exploratory analysis showed that there was no correlation between WBC and cortisol level in any of the four porpoises, however. Exploring the effect of other disease-related parameters would be interesting.

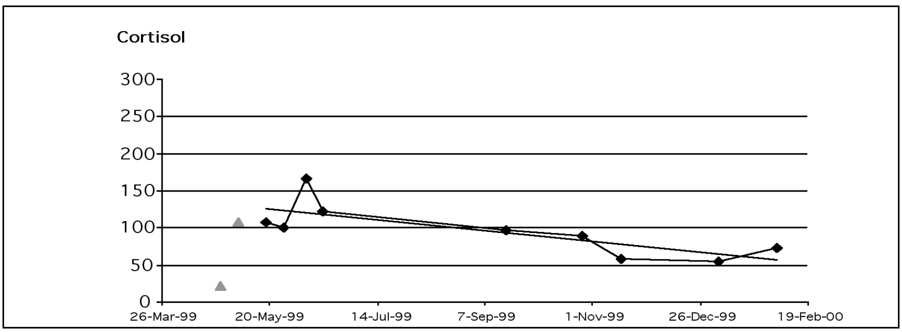
Bearing in mind that an assessment of stress based on a number of biochemical and hematological variables would be preferable to the use of a single measurement (Moberg, 1987; Hattingh, 1988; Dierauf, 1990; Kock et al., 1990; St. Aubin & Dierauf, 2001) and that stress response may not include elevated blood cortisol levels (Moberg, 1987), the significant difference in cortisol levels under VHB and OWR sampling observed in this study seem to indicate that cortisol levels can provide a useful index of acute stress in harbour porpoises. Obtaining the true baseline, or resting level, of cortisol through blood sampling is impossible since sampling blood is, by definition, invasive. The levels observed with VHB sampling might yield data that are as close to resting as can be expected in



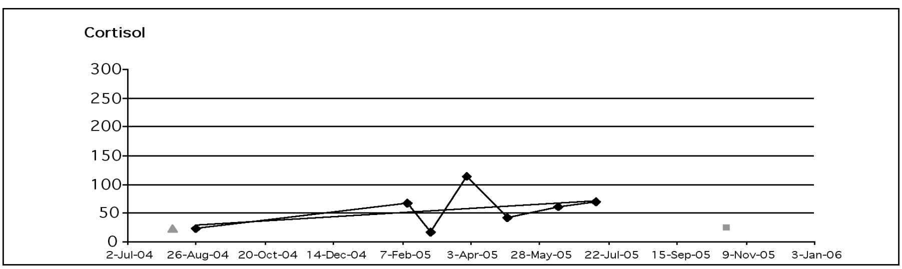
a: Freja (OWR trend, y = -0,0165x + 665; R^2 = 0,155)



b: Eigil (OWR trend, y = -0,0102x + 421,12; R^2 = 0,079)



c: Nuka (OWR trend, y = -0,2658x + 9774,3; R^2 = 0,606)



d: Sif (OWR trend, y = 0,1357x - 5159,7; R^2 = 0,187)

Figure 1a-d. Level of cortisol (µg/l) under different sampling modes in four harbour porpoises in human care; all data are included with the exception of the extreme cortisol value of 793 µg/l exhibited by Eigil in May 1997 under OWR.

Outliers (▲), data from the first month of acclimation and data outside the "normal" variation range; OWR (◆), data from blood sampling out of the water on a restrained animal, with the linear trend line given for information; VHB (■), data from blood sampling at poolside under voluntary husbandry behaviour.

Table 2. Serum cortisol levels in four porpoises kept in human care; medians and 25 to 75% (narrow range estimator) quartiles around the median are given for comparison with other studies.

Cortisol µg/l	N	Mean (SD)	Median	Max	Min	25-75%
Freja OWR	35	71.5 (33.7)	65.7	216.0	11.1	53.1-79.6
Freja VHB	31	20.2 (12.4)	17.8	61.7	5.1	11.6-26.0
Eigil OWR	44	64.9 (55.1)	50.9	274.0	3.0	40.0-65.2
Eigil VHB	23	16.6 (6.8)	16.5	28.6	7.0	10.5-20.5
Nuka OWR	11	90.7 (38.5)	96.1	166.0	22.5	65.3-108.3
Sif OWR	8	51.4 (33.6)	51.8	114.2	16.3	21.9-68.1
Sif VHB	1	23.1	--	--	--	--

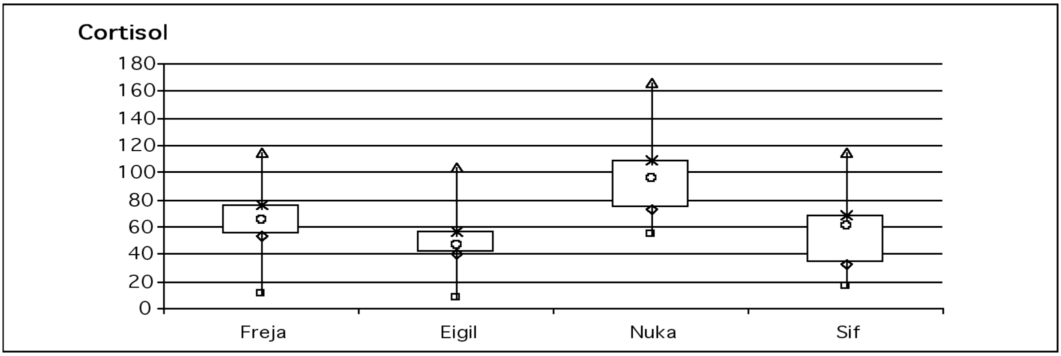


Figure 2. Box Plot of cortisol levels (µg/l) obtained under OWR sampling (outliers excluded) for the four porpoises

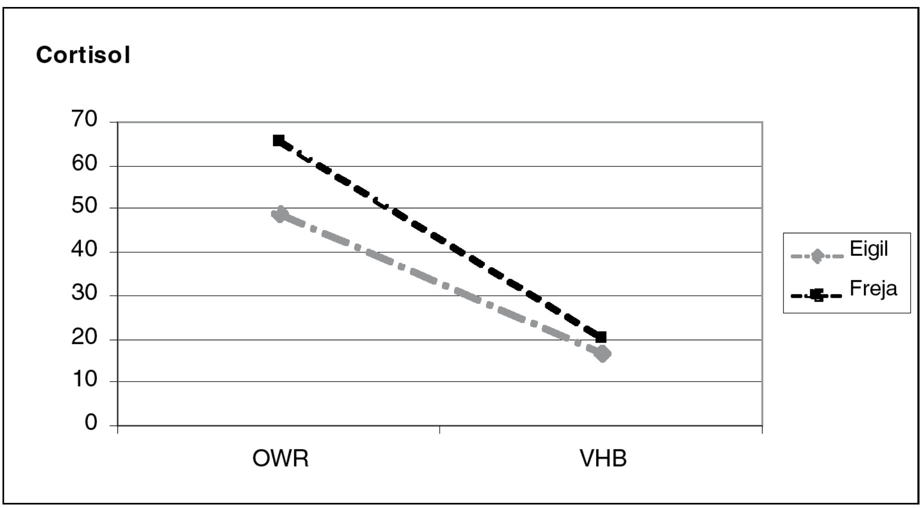


Figure 3. Average cortisol levels (µg/l) obtained under OWR and VHB sampling for Eigil and Freja (outliers excluded)

human care. For use as a management tool, allowing for stress control of animals on a frequent basis (e.g., during experiments), cortisol should ideally be measured in a medium that does not necessitate any invasive procedure like expelled air or saliva.

Based on cortisol levels as single stress index, the porpoises showed only a little habituation to a regular and frequent handling over several years, even if the procedure was frequent, kept similar, and performed by people taking care of the porpoises daily, with Sif apparently exhibiting an increase over her few months in captivity. The dramatic three-fold decrease of cortisol level observed under VHB sampling, after more than

Table 3 a-b. Results of the two-way ANOVA analysis for testing whether the animal or the mode of sampling affected the level of cortisol and whether there was an interaction between the two

a. Means table for cortisol

	N	Mean	SD	SE
Eigil / OWR	38	49.047	17.544	2.846
Eigil / VHB	23	16.580	6.765	1.411
Freja / OWR	31	65.426	22.523	3.982
Freja / VHB	31	20.196	12.350	2.218

b. Results of the two-way ANOVA analysis

	df	F-Value	P-Value
Animal	1	11.006	0.0012
Mode	1	166.202	< 0.0001
Animal*Mode	1	4.485	0.0363

two and four years of regular handling for Freja and Eigil, respectively, clearly points to the significance of the stress occasioned to the porpoises by removing them out of the water. Thomson & Geraci (1986) also observed this type of dramatic increase in cortisol with handling in bottlenose dolphins and noted that a stress response was evoked even under the calmest conditions of capture they could employ. In 42 naive wild porpoises handled for satellite tagging (Eskesen et al., submitted), the average level of cortisol (165 µg/l, range 338.6 µg/l to 62.0 µg/l) is more than double that of the levels observed here under the OWR sampling.

Regular and frequent handling over several years did not suppress a significant stress response in the porpoises. Although three of the porpoises showed some habituation to handling over the course of the study, the circulating cortisol levels obtained under VHB sampling showed a dramatic three-fold decrease compared to levels obtained under OWR sampling. This sensitivity to handling shown by porpoises, as well as bottlenose dolphins and likely other marine mammals, points dramatically to the advantage of using VHB for limiting stress in husbandry practices. Preventing stress from arising in a given situation is impossible, but limiting the number of stressing situations is within our capabilities by prioritising training for voluntary husbandry behaviours.

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