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The impact of stress on cytokine and haptoglobin mRNA expression in blood samples from harbour porpoises (*Phocoena phocoena*)

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Cytokines are important cell mediators involved in immune responses. Their expression can be modulated by numerous factors, including stress. The aim of this study was to compare cytokine mRNA expression from harbour porpoises exposed to different environments. Blood samples were taken from two healthy porpoises living in captivity at the Fjord and Belt Centre Kerteminde, Denmark, and from four wild porpoises accidentally caught in Danish waters. Real-time RT-PCR was used to quantify the transcription of interleukin-(IL)-1β, IL-2, -4, -6, -10, tumour necrosis factor-(TNF)-α, transforming growth factor-(TGF)-β and the acute phase protein haptoglobin. This revealed downregulation of the pro-inflammatory cytokines IL-1β, IL-2, IL-6 and TNFα, and a switch towards the T-helper-cell-(Th)2- and Th3-cytokines, IL-4 and TGFβ, in blood samples of the wild-captured animals. This indicated a shift towards immunomodulatory cytokines. In addition, cortisol levels were increased in the wild-caught porpoises. These results are suggestive of stress-induced modulation of the immune responses in the accidentally caught animals. The current study indicates that the expression pattern of these cytokines and the estimation of the Th1- to Th2- and Th3-cytokine mRNA ratios might be a useful indicator to analyse the influence of stress on the immune system in harbour porpoises.

INTRODUCTION

Cytokines are a widespread group of cell mediators essential in the function of the immune system. Cytokine expression is influenced by numerous internal and environmental factors (Iwakabe et al., 1998; Elenkov & Chrousos, 1999; Kidd, 2003; Elenkov et al., 2005). The effect of stress is of particular interest in marine mammals, as human activities such as vessel traffic, fishing, oil spillage, use of sonar systems and creation of underwater noise all elicit stress responses (St Aubin & Dierauf, 2001). Capture and handling is another stressor of particular interest to those who handle animals in captivity or in the wild (St Aubin et al., 1996; Zeneto-Savin et al., 1997; Ortiz & Worthy, 2000).

Stress induced changes to the immune system have been considered as a major cause of an increased risk for a variety of conditions, including infections, neoplasia and immune-mediated disorders. Chronic stress, furthermore, can result in long-term impairment of immune function (Mason, 1991; Iwakabe et al., 1998; Kawamura et al., 2001; Sternberg, 2001; Elenkov et al., 2005; Watari et al., 2005; Wright et al., 2005).

Previous studies to assess the impact of stress on marine mammals measured parameters such as catecholamines, glucocorticoids and mineralocorticoids, but with variable results (St Aubin et al., 1996; Ortiz & Worthy, 2000). As well as stress, the immune system is influenced by injury and inflammation (Lucey et al., 1996; Elenkov & Chrousos, 1999; Kidd, 2003). In marine mammals, diagnosis of infection is often difficult, as they exhibit minimal outward symptoms (King et al., 1996; Zeneto-Savin et al., 1997; Funke et al., 2003).

Cytokines have been used to evaluate disease and stress responses in humans, dogs and mice, particularly alterations in T-helper(Th) cell differentiation and activity (Elenkov & Chrousos, 1999; Brydon et al., 2005; Elenkov et al., 2005). Pro-inflammatory cytokines, such as IL-1β, IL-6 and TNFα, are often produced in the beginning of an immune response, predominantly by macrophages, and can be used to detect subclinical infections (King et al., 1996; Tizard 1996; DiPiro 1997; Funke et al., 2003). Cytokine profiles can also be used to differentiate distinct populations of Th cells (Mosmann & Sad, 1996). Th1 cells produce pro-inflammatory cytokines such as IL-2, IFNγ, IL-12, which promote cell-mediated immunity, whereas Th2 cells primarily secrete cytokines such as IL-4, IL-5, IL-10 and IL-13, which inhibit cell-mediated response and promote humoral immunity (Mosmann & Sad, 1996; Lucey et al., 1996; Elenkov & Chrousos, 1999; Kidd, 2003). Th3 cells are a subset of T-regulatory cells producing the immunosuppressive cytokine TGFβ (Weiner, 2001; Mills, 2004; Wahl et al., 2004).

Acute psychological stress, e.g. mental stress in humans or restraint stress in mice, results in an increase of pro-inflammatory, particularly Th1, cytokines (Søndergaard et al., 2000; Brydon et al., 2005), whereas continuing stress has been associated with Th2 polarization and eventual immunosuppression (Ramirez et al., 1996; Li et al., 1997; Iwakabe et al., 1998; Ramirez, 1998; Elenkov & Chrousos, 1999; Rook, 1999; Elenkov et al 2005).

Table 1. *Primer sequences used for the amplification of the house-keeping gene, cytokine and acute phase protein transcripts, annealing temperature, and sequences used for the selection of the primer pairs.*

Cytokine	Primer sequences (5'–3')		Annealing temperature	Sequences used for primer pair selection (accession numbers, NCBI)
GAPDH	s	GGGGCCATCCACAGTCTTCT	57°C	Published in Beineke et al. (2004)
	as	GCCAAAAGGGTCATCATCTC		
IL-1β	s	TCCAATGTGAAGTGCTGCTG	52°C	Z70047, M35589
	as	ACAGAGCTGGTGGGAGACTT		
IL-2	s	GCACCTACTTCAAGCTCTAC	45°C	AF072871, AF072870, D30710, AB041935, NM_180997
	as	TGTAAATCCAGCAGCAATGA		
IL-4	s	AACGCTGAACATCCTCACAG	48°C	AF346295, AF239917, AF187322, AF104245, AB020732, AF305617, M13982
	as	GTCTTCTACAGGCAGCTCCA		
IL-6	s	GCTTCCAATCTGGGTTCAAT	54°C	L46802, NM_173923
	as	GCTTTGCTGCTAATCTGCAC		
IL-10	s	CCTGGGTTGCCAAGCCCTGTC	57°C	Published in Beineke et al. (2004)
	as	ATGCGCTCTTCACCTGCTCC		
TNFα	s	GGCTGAACACATATGCCAAC	57°C	Z70046, AF348421
	as	TGAAGAGGACCTGGGAGTAGA		
TGFβ	s	TTCCTGCTCCTCATGGCCAC	58°C	L34956, M36271, U97485, X76916, M60316
	as	ACCTTGCTGTACTGCGTGTC		
HP	s	CTGGCAGGCTAAGATGGTTT	54°C	AJ271156, AF492467, NM_017370, BC070299
	as	GTCAGCAGCCATTGTTCATT		

NCBI, National Center for Biotechnology Information.

In veterinary medicine, acute phase proteins (APP) such as haptoglobin (Hp) or C-reactive protein (CRP) are also used as markers for inflammation and stress (Heegaard et al., 1998; Petersen et al., 2002; Hiss et al., 2003; Murata et al., 2004; Vermeire et al., 2004). APP are part of the innate defence system whose expression is stimulated by pro-inflammatory cytokines IL-1, IL-6 and TNFα. In recent reports Hp and CRP were described in declining populations of harbour seals and Steller's sea lions, suggesting that they were a potential marker of health status (Funke et al., 1997; Zeneto-Savin et al., 1997).

Little is known about cytokine expression, APP production and Th subpopulations in harbour porpoises. To get an insight into the potential importance of interleukins for the assessment of the harbour porpoise's immune system, we analysed the expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα, the Th1 cytokine IL-2, and the Th2- and Th3-cytokines IL-4, IL-10 and TGFβ, and the APP Hp in whole blood samples from two captive and four accidentally-caught harbour porpoises by real-time RT-PCR.

MATERIALS AND METHODS

Animals

We analysed blood samples of two harbour porpoises, which had been held in captivity at the Fjord and Belt Centre in Kerteminde, Denmark since their rescue from pound nets set in inner Danish waters in April 1997 (Lockyer et al., 2003). These were a male ('Eigil': Pp1, length 139 cm, weight summer 41 kg, winter 44 kg) and a female ('Freija': Pp2, length 150 cm, weight summer 47 kg, winter 56 kg) housed in an outdoor penned-off area of Kerteminde fjord. Blood samples were taken during routine investigations. The animals remained in the water and were trained to present their tail flukes for sampling. Blood samples taken in June, July and August 2003 were compared with blood samples from four free-ranging porpoises (Pp 3–6), accidentally trapped for more than 24 h in fishing nets in September 2003 near Skagen. It is likely that handling for examination and sampling resulted in further stress. After veterinary investigation and sampling the accidentally-caught animals were released. All four porpoises were males with a length

Table 2. *WBC (×10⁹ l⁻¹), lymphocytes (×10⁹ l⁻¹) and neutrophil granulocytes (×10⁹ l⁻¹) and cortisol levels (μg l⁻¹) in the blood samples of two captive (Pp1, Pp2) harbour porpoises in different months and four by-caught (Pp3–Pp6) harbour porpoises.*

Porpoises		WBC (×10⁹ l⁻¹)	Lymphocytes (×10⁹ l⁻¹)	Neutrophil granulocytes (×10⁹ l⁻¹)	Cortisol (μg l⁻¹)
Pp1	June	2.2	0.2	1.9	7.0
	July	2.4	0.9	1.5	28.6
	August	2.2	0.8	1.4	27.4
Pp2	June	3.7	n.d.	n.d.	43.1
	July	3	1.2	1.8	28.5
	August	2.7	0.9	1.7	21.3
Pp3		3.5	1.2	2.2	n.d.
Pp4		3.8	2.0	1.7	157.2
Pp5		6.3	2.4	3.8	82.6
Pp6		6.5	2.5	3.9	198.5

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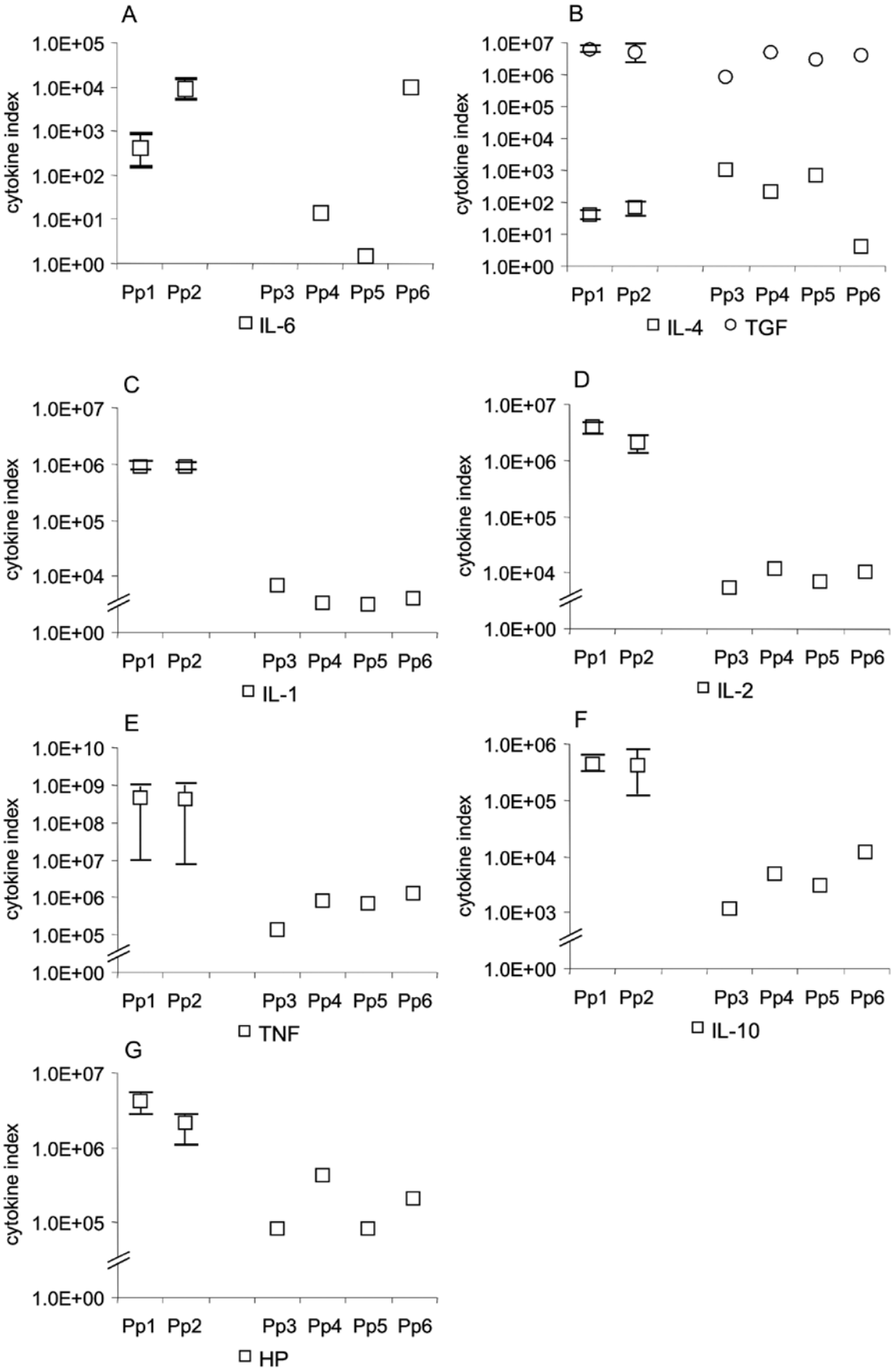

Figure 1. Cytokine index of (A) IL-6; (B) IL-4 and TGFβ; (C) IL-1β; (D) IL-2; (E) TNFα; (F) IL-10; and (G) HP mRNA from the blood samples of two porpoises living in captivity (Pp1, Pp2) and four accidentally-caught animals (Pp3–Pp6). The height of the symbols present cytokine-mRNA amounts calibrated with GAPDH. Symbols for the porpoises living in captivity are the average of June, July and August; the black lines are the minimal or maximal measured values of the appropriate cytokine/APP.

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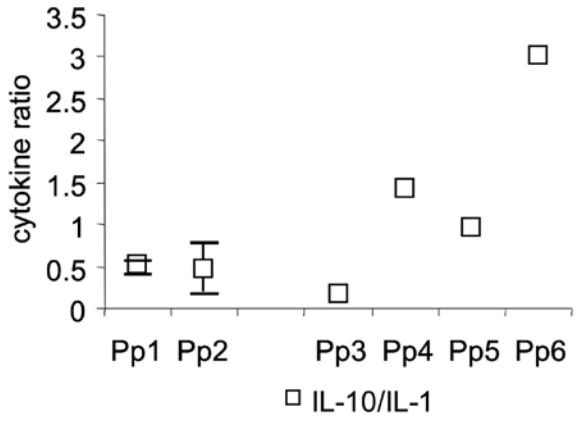

Figure 2. IL-10 to IL-1β mRNA ratios from the blood samples of two porpoises living in captivity (Pp1, Pp2) and four accidentally-caught animals (Pp3–Pp6). The height of the squares presents the ratio of IL 10-mRNA to IL-1β mRNA. Symbols for the porpoises living in captivity are the average of June, July and August ratios; the black lines are the minimal or maximal measured ratios.

of 110–134 cm and a weight of 32–41 kg. Due to the similar length and weight we assumed that the animals were of similar ages, but tooth extraction for precise age estimation was rejected on ethical grounds.

After 12–24 h transportation at room temperature, routine haematology (performed by Dr Joerg Driver, Kleintierpraxis, Reinsbuettel, Germany) and mRNA isolation were performed. Serum was separated for measurement of cortisol levels (performed by Gemeinschaftspraxis Dr Kramer und Kollegen, Geesthacht).

Primer design

Consensual primers for IL-1β, IL-2, IL-4, IL-6, TNFα, TGFβ and Hp were selected using DNAstar™ software (GATC Biotech, Konstanz, Germany) from conserved regions of published sequences (Table 1). Published primers were used for the house-keeping gene GAPDH IL-10 (Beineke et al., 2004).

Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from 300 µL EDTA blood (Ambion blood kit™; Ambion Europe, Huntington, UK) according to the manufacturer's protocol. After DNase treatment (Ambion Europe), 80–100 ng/µL RNA was reverse transcribed with murine reverse transcriptase (RT-PCR Core Kit™; Applied Biosystems, Weiterstadt, Germany). The resulting cDNA served as a template for real-time PCR using the Thermocycler MX4000™ (Stratagene Europe, Amsterdam, Netherlands). For real-time quantification the Brilliant SYBRGreen QPCR Master mix (Stratagene Europe) was used. This contained SYBRGreen I as a fluorescence dye, dNTPs, $MgCl_2$ and a hot start Taq DNA polymerase. The fluorescence response was monitored in a linear fashion as the PCR product was generated over a range of PCR cycles. For each cytokine a standard curve was prepared using a dilution series from 10^9 to 10^2 copies. The PCR started with an initial step at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 1 min, annealing temperature for 30 sec and elongation at 72°C for 1 min. The annealing temperatures are shown in Table 1. The fluorescence was measured at the end of the annealing and at the end of the dissociation program at a wavelength of 530 nm. To exclude measurement of non-specific PCR products and primer dimers, and to determine true amplification, each PCR was followed by a dissociation program for 1 min at 95°C, followed by 41 cycles during which the temperature was increased in each cycle, starting at 55°C and ending at 95°C. Only PCR reactions with one well-defined peak were used for analysis. All reactions were performed in duplicate and two separate PCR reactions were performed. GAPDH was used as the control gene. To calculate the cytokine/APP expression GAPDH was used as calibration compound. Cytokine/APP expression index (CI) was calculated as follows:

$$CI = \frac{\text{Number of cytokine copies}}{\text{Number of GAPDH copies}} \times 10^8$$

To verify the true amplification of the cytokines, the PCR-products were sequenced (Seqlab Laboratories, Göttingen) and sequences were published in GenBank (NCBI accession numbers—GAPDH: AY919329, IL-1β: AY919330, IL-2: AY919341, IL-4: AY919339 IL-6: AY919332, IL-10: AY919333, TNFα: AY919337, TGFβ: AY919334, Hp: AY919335).

RESULTS

The number of animals were too small for statistical analysis. Total WBC counts ranged from 2.2–3.7×10^9 l^{-1} in the porpoises living in captivity and from 3.5–6.5×10^9 l^{-1} in the captured animals. Lymphocyte counts ranged from 0.2–1.2×10^9 l^{-1} in the captive animals and from 1.2–2.5×10^9 l^{-1} in the captured porpoises. Neutrophil counts in the respective groups were 1.4–1.9×10^9 l^{-1} and 1.7–3.9×10^9 l^{-1} (Table 2). Porpoises Pp5 and Pp6 showed obviously higher WBC, lymphocytes and neutrophil numbers than the others (Table 2). Serum cortisol levels of the captured porpoises ranged from 82.6–198.5 µg l^{-1} and appeared to be higher than those in the porpoises living in captivity, which were 7.0–43.1 µg l^{-1} (Table 2).

Transcripts of the house-keeping gene GAPDH (data not shown) and of the cytokines were amplified in each blood sample, except for IL-6 where no PCR product was found in the sample derived from Pp3 (Figure 1A). Reduced amounts of IL-6 mRNA were detected in Pp4 and Pp5, whereas Pp6 revealed similar IL-6 mRNA levels as Pp1 and Pp2 (Figure 1A). TGFβ mRNA expression was similar in blood samples from all porpoises (Figure 1B). IL-4 mRNA were slightly elevated in Pp3, Pp4 and Pp5, and reduced in Pp6, compared to the captive porpoises Pp1 and Pp2 (Figure 1B). Apparently lower levels of mRNA for IL-1β, IL-2, IL-10, TNFα and Hp were detected in the blood samples from the captured animals compared with the captive animals (Figure 1C–G). IL-10 to IL-1β mRNA ratios were increased in the captured animals (except for Pp3) in comparison with the captive animals (Figure 2).

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DISCUSSION

Captured porpoises exhibited apparently higher total WBC lymphocytes and neutrophil counts compared to porpoises living in captivity. Increased WBC counts may indicate an infection, but increased levels of WBC, lymphocytes and neutrophils in free-ranging marine mammals compared with captive animals could also be stress-induced. The slightly elevated values of the captured porpoises were, nevertheless, still within normal ranges (Bossart et al., 2001). Cortisol levels, which are used to assess stress responses, were also increased in the captured wild animals compared to those living in captivity (Bossart et al., 2001). It is possible that the porpoises could have been exhibiting circadian variation in cortisol levels, but circadian changes are generally less variable than the increased levels noticed in the captured animals, which were 2–6 times higher than in captive porpoises (Bossart et al., 2001; Oki & Atkinson, 2004).

Compared to the captive animals, lower amounts of mRNA for IL-1β, IL-2, IL-10, TNFα and Hp mRNA were detected in the blood samples of all the captured animals, whereas TGFβ and IL-4 mRNA were only reduced in single animals. Cytokine expression is influenced by several factors. The investigated animals were of similar size and weight, suggesting a similar age, and samples were taken in the same season. It is not possible to rule out the potential impact of nutrition, environment and activity completely. The porpoises live in the same sea, but free-ranging animals live in different conditions with more exercise than animals in captivity. Additionally the captured animals were released back into the wild after investigation and no further information is available. The high WBC counts in two of the wild caught porpoises would suggest an infection but this was not supported by a concomitant increase in pro-inflammatory cytokines. Assuming that the porpoises in captivity were healthy, the reduced cytokine transcription in the captured animals is more suggestive of stress-mediated immunosuppression. Ramirez (1998) described an inhibition of cytokine synthesis caused by the continuous presence of glucocorticoids, which is similar to our results. Furthermore, high psychological stress resulted in lower levels of IL-1β and IL-8 in wound fluid from humans, and in patients with septic shock, lower levels of intracellular TNFα in leukocytes were found (Broadbent et al., 2003; Fumeaux et al., 2004). In contrast to our results, however, acute psychological stress, e.g. mental stress in humans or restraint stress in mice, has been associated with an increase of pro-inflammatory cytokines (Brydon et al., 2005; Elenkov et al., 2005). In the blood sample of one accidentally-caught porpoise (Pp6) elevated IL-6 and IL-10 mRNA, and cortisol were found compared with the other captured animals. As glucocorticoids can induce IL-10 expression, elevated IL-6 and IL-10 mRNA levels might indicate a more acute stress situation for that animal in comparison with the others. However, activation of the immune system associated with a subclinical infection is also possible; IL-6 is a possible precursor of inflammatory reactions and marker for subclinical infections in marine mammals (King et al., 1996, Funke et al., 2003).

The ratio of IL-10 to IL-1β is used as an indicator of immune system function (Pachot et al., 2005). The study found increased IL-10 to IL-1β ratios in the captured animals (except Pp3) in comparison with the captive animals. Human patients who did not survive septic shock, showed an increased IL-10 to IL-1β ratio compared to survivors (Pachot et al., 2005). Remarkably, Pp6, which had the highest IL10 to IL1β ratio, also showed the highest IL-6 mRNA and cortisol levels, which corresponds to the findings of Pachot et al. (2005).

Increased or similar IL-4 and TGFβ mRNA levels (Th2- and Th3-cytokines) were found in the accidentally-caught porpoises compared to the porpoises from the Fjord and Belt Centre, again except for Pp6 which showed a lower value for IL-4. In addition, IL-2 (a Th1-cytokine) was reduced in the captured animals. This could indicate a predominance of anti-inflammatory cytokines and a shift towards Th2 and Th3 immune responses in the captured animals. Stress has been shown to suppress Th1-cytokine expression and induce a Th2-shift with an increase of anti-inflammatory cytokines (Li et al., 1997; Iwakabe et al., 1998; Ramirez, 1998; Elenkov & Chroussos, 1999; Sternberg, 2001; Elenkov et al., 2005). Restraint stress strongly inhibited IFNγ and IL-2 production of stimulated and immunized mice spleen cells, but did not affect IL-4 production (Li et al., 1997; Iwakabe et al., 1998). Ramirez (1998) found that after prolonged incubation with glucocorticoids, the immune system shifted towards Th2-type reactions. Th2 cytokine polarization has also been associated with incomplete clearance of pathogens, progression of infection and chronic infections (Mosmann & Sad, 1996; Lucey et al., 1996). However, early and sustained anti-inflammatory profiles are associated with immunoparalysis (Fumeaux et al., 2004; Pachot et al., 2005). As no following examinations of the captured porpoises were possible, immunosuppression could not be ruled out.

Interestingly, transcripts for the acute phase protein haptoglobin (Hp) were found in blood samples of all animals, although liver cells are described as the main acute phase protein source (Murata et al., 2004). It is likely, therefore, that white blood cells also produce Hp. A recent paper described neutrophil production of Hp (Theilgaard-Mönch et al., 2006), although neutrophil counts and Hp levels did not correlate in the present study. Hp has been used as a marker for infections and stress situations (Hiss et al., 2003; Petersen et al., 2002), and was found to be elevated in declining populations of harbour seals (Zeneto-Savin et al., 1997). Whether Hp is a main acute phase protein for harbour porpoises is not known. As Hp expression was lower in the captured animals, Hp mRNA levels might not act as a marker for infection or stress situations in these porpoises, although the relevance of Hp protein as a stress marker could not be ruled out.

Our results demonstrated higher cortisol values, reduced pro-inflammatory cytokine transcription and a shift towards Th2 and Th3 immune response in blood samples of harbour porpoises trapped for more than 24 h in fishing nets. Although we cannot exclude infection, different environment, nutrition, or activity as influence factors on cytokine expression completely, we suggest that an ongoing stress situation was responsible for this cytokine pattern, which may result in immunosuppression. These are, however, only preliminary results, as we obtained only one blood sample

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per animal, and continued observation and successive sampling of the animals during and after stress situations was not possible. The stress impact on accidentally-caught harbour porpoises could have severe consequences for these animals (Mason, 1991; Iwakabe et al., 1998; Kawamura et al., 2001; Sternberg, 2001; Elenkov et al., 2005; Watari et al., 2005; Wright et al., 2005). To confirm these preliminary results, and to get more information about possible effects from different stress situations, such as fishing, vessel traffic, noise and oil spilling, further investigation of animals living in different regions and exposed to diverse environments are necessary.

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