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Cytokine and acute phase protein expression in blood samples of harbor seal pups

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Abstract:

Cytokines as mediators of the immune response are useful parameters to assess the immune status of mammals.. Especially in newborn animals the developing immune system is important for survival. However, only little information is available for pinnipeds. Therefore, cytokine expression in blood samples of 14 harbor seal pups, collected at the German North Sea coast and rehabilitated in the Seal Centre Friedrichskoog, were analyzed. Six animals in 2004 and eight in 2005 were investigated immediately after arrival and after 2-3 month just prior to their release into the North Sea. The mRNA expression of cytokines interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, interferon (IFN)γ, and transforming growth factor (TGF)β, as well as of acute phase proteins haptoglobin (HP), heat shock protein (HSP) 70 and metallothionein (MT) 2 were analyzed using real time RT-PCR. The cytokine expression in pups varied between animals and years, but interesting trends were obvious. Higher levels of pro-inflammatory cytokines IL-1β, IL-6, IL-8, and IL-12 were found at admission, consistent with an activated immune system, whereas the anti-inflammatory cytokine IL-4 was increased before release, suggesting recovery from infections and a mature immune system. This study indicates that cytokines detection is highly sensitive for the status of the immune system of harbor seals.

Keywords: harbor seal, *Phoca vitulina*, pups, real-time RT-PCR, cytokine, interleukin, acute phase protein, haptoglobin, heat shock protein, metallothionein

Introduction:

Cytokines are important mediators of the immune system (Di Piro, 1997; Elenkov and Chrousos, 1999; Kidd, 2003; Lucey et al., 1996; Mosmann and Sad, 1996). Pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, IL-12, and tumour necrosis factor (TNF)α, and anti-inflammatory cytokines such IL-4 and IL-10 are differentiated (Elenkov and Chrousos, 1999; Kidd, 2003; Mosmann and Sad, 1996). Furthermore, for medical purposes cytokine expression is used to differentiate lymphocyte subpopulations. T helper cells-1 (Th-1) primarily secret IL-2, and interferon (IFN)γ, whereas T helper cells-2 (Th-2) produce IL-4, IL-5, IL-10, IL-13, and transforming growth factor (TGF)β (Elenkov and Chrousos, 1999; Kidd, 2003).

Pro-inflammatory cytokines, IL-1β, IL-6 and TNFα, stimulate acute phase protein (APP) expression, such as haptoglobin (HP), heat shock protein (HSP) 70 and metallothionein (MT) 2. Liver is the primary site of APP synthesis, but expression has also been reported in macrophages or lymphocytes (Allan et al., 2000; Kostro et al., 2001). APPs play an active role in modulating immune responses and are used as marker for infection and stress (Kostro et al., 2002; Murata et al., 2004; Petersen et al., 2004).HP inhibits the production of pro-inflammatory, but not anti-inflammatory cytokines in human monocytes and reduces respiratory burst activity of human neutrophils (Oh et al., 1990; Arredouani et al., 2005). MT 2 participates in acute phase response, detoxification of heavy metals and scavenging of free radicals (Bremner and Beattie, 1990; Davis and Cousins, 2000). The APP HSP)70 is highly conserved and known to be involved in immune responses to several bacterial, viral and parasitic pathogens (reviewed by Young, 1990; Njemini et al., 2002).

These proteins are useful to assess the immune status in mammals. Several studies investigated cytokine-like activities or cytokine/APP expression in whales or dolphins (Beineke et al., 2004, 2006, Denis and Archambault, 2001; Fonfara et al., 2007a, b; Funke et al., 2003; Inoue et al., 1999a, b, c, 2001; Ness et al., 1998; Shoji et al., 2001; St-Laurent et al., 2000). For harbor (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) IL-1-like activity in leukocytes is reported (King et al., 1995). IL-2 was investigated in northern elephant seals (*Mirounga angustirostris;* Shoda et al., 1998), grey seals (St-Laurent et al., 1999) and harbor seals (Di Molfetto-Landon et al., 1995). IL-2 and IL-4 was used to characterize metal-specific hypersensitivities in a grey seal (Kakuschke et al., 2006). IL-6-like activities and sequencing of IL-6 cDNA fragments was performed for harbor and grey seals (King et al., 1993, 1996). MT expression in peripheral blood leukocytes of grey seals was reported by Pillet et al. (2002). To the author's knowledge, reports of three cytokines (IL-1, IL-2, and IL-6) exist for harbor seals. Analysis of a wide range of cytokines/APPs in this species is missing.

Furthermore, few studies only investigated newborn seals, although development of the immune system is essential for survival. At birth the immune system of marine mammals is not completely developed, reflected by low immunoglobulin levels. As temporary protection, pups receive maternal antibodies with colostrum (King et al., 1998; Ross et al., 1993, 1994). Incomplete development of the immune system in newborn grey seals was shown by reduced phagocytic activity of macrophages and reduced lymphocyte transformation (Lalancette et al., 2003). However, newborn harbor seals revealed strong mitogen-induced lymphocyte proliferation , consistent with developed T-lymphocyte function (Ross et al., 1993, 1994; Kakuschke et al., 2008), but increased susceptibility to toxic effects of

metals (Kakuschke et al., 2008). This result supports the conclusion that the harbor seal newborns have a relative immunocompetence as reflection of an evolutionary adaptation to its short nursing period and limited maternal care (Ross et al., 1994).

In this study quantitative PCR for the detection several cytokines and APP was established to investigate expression pattern in newborn harbor seals, the development of the immune system in first months of life and to obtain possible markers for the immune status. To the authors knowledge this is the first report of amplification of harbor seal GAPDH, IL-4, IL-8, IL-10, IL-12, IFNγ, TGFβ, MT and HSP70 with RT-PCR.

Materials and Methods:

Animals: Six and eight abandoned harbor seal pups were collected during birth period from June until July at the coast lines of the German North Sea in 2004 and 2005, respectively. . Pups, which were likely to survive, were rehabilitated in the Seal Centre Friedrichskoog. A veterinary examination, including weight, length, body condition, body temperature, physical examination and blood sampling of the animals was performed immediately after collecting the seals. EDTA blood was taken from the epidural vertebral vein for haematology and RNA isolation for this study. A second blood sample was taken just before release of the seals back into the North Sea. At that time point they animals have gained a weight of at least 25 kg and revealed no clinical signs of disease. Blood samples were processed within one hour after sampling. Haematology was performed by Dr. Driver (Kleintierpraxis, Reinsbuettel, Germany). White blood cell count of all animals was

within the normal range (6-12*10^3 mm^{-3}*;* Siebert et al., 2006*)*. Details of the animals investigated are listed in Table 1.

Table 1:

Animal number, sex, dates (of admission and release; dd/mm/yy), age (at admission and release), white blood cell count (at admission and release; WBC), weight, length and body temperature of pups used for cytokine/acute phase protein detection.

Animal	Sex	Admission						Release		
		Admission date	Age (days)	WBC ($x10^9$/L)**	Weight (kg)	Length (cm)	Body temperature (°C)	Release date	Age (days)	WBC ($x10^9$/L)**
Pv 1	F	21/06/04	7	9.7	8.9	58	37.0	26/08/04	73	10.2
Pv 2	F	21/06/04	7	8.8	8.6	57	37.2	14/09/04	92	12
Pv 3	F	21/06/04	7	6.5	8.9	57	36.6	26/08/04	73	6.3
Pv 4	F	21/06/04	7	8.6	10.5	59	37.4	14/09/04	92	9.1
Pv 5	M	21/06/04	10	8.1	10.9	65	37.0	26/08/04	76	7.5
Pv 6	M	21/06/04	7	9.7	8.7	54	36.4	26/08/04	73	9.8
Pv 7	F	07/06/05	3	4.7	7.2	54	36.6	04/09/05	92	9.9
Pv 8	F	07/06/05	3	9.9	9.5	60	36.8	25/08/05	82	7.2
Pv 9	F	16/06/05	7	8.8	8.7	53	38.2	25/08/05	77	11.3
Pv 10	F	22/06/05	5	7.2	10.1	49	38.8	25/08/05	69	7.5
Pv 11	F	23/06/05	7	6.8	8.4	52	37.8	18/09/05*	94	8.6
Pv 12	F	25/06/05	7	5.1	7.7	48	37.5	04/09/05	78	8.8
Pv 13	F	01/07/05	6-7	6.6	9.3	56	37.7	04/09/05	71-72	7.5
Pv 14	F	03/07/05	10-12	12.1	8.6	53	37.5	04/09/05	73-75	9.7

Pv: *Phoca vitulina*; F: female; M: male; WBC: white blood cells.

*Pv 11 stayed in the Seal Station until 21/11/2005. Blood sample taken at the 18/09/2005 was for routine re-investigation.

** Normal range of WBC for harbor seals 6-12*10^3 mm^{-3} (Siebert et al., 2006).

Primer design:

Primers for the detection of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-12, IFNγ and HP, HSP, MT2 were selected from conserved nucleotide sequences of published sequences. Sequences of different species (depending on availability bovine, ovine, canine, mice, equine,) were compared choosing regions of great homology. Primer sequences, annealing temperatures, and length of amplicons are listed in Table 2. For the house-keeping gene GAPDH, for IL-10 and TGFβ published primer pairs for harbor porpoises (*Phocoena phocoena*) from Beineke et al. (2004) were used.

Table 2:

Primer sequences used for the amplification of the house keeping gene, cytokine and acute phase protein transcripts. Base pair length, annealing temperature and GeneBank accession numbers for seal nucleotide sequences of this study are listed.

Gen	Primer sequences (5'-3')		Length of amplicon (bp)	Annealing temperature	Accession number
	s	as			
House-keeping gene					
GAPDH	GGG GCC ATC CAC AGT CTT CT	GCC AAA AGG GTC ATC ATC TC	185	57°C	AY919320
Cytokines					
IL-1β	GGC ATT TCG TGT CAG TCA TT	GCT CTT CAG GTC ATC CTC CT	90	52°C	AY919321
IL-2	CTT GCA TCG CAC TGA CTC TT	GCT CCA ACT GTT GCT GTG TT	89	55°C	AY919322
IL-4	CCT CCC AAC TGA TTC CAA CT	GAC AAA GGT GCT GGT GAG TG	74	50°C	AY919338
IL-6	TGG TGA TGG CTA CTG CTT TC	GGA GAG GTG AGT GGT GGT CT	61	54°C	AY919323
IL-8	ACA CAC TCC ACA CCT TTC CA	AGG CAC ACC TCA TTT CCA TT	149	50°C	AY919324
IL-10	CCT GGG TTG CCA AGC CCT GTC	ATG CGC TCT TCA CCT GCT CC	206	57°C	AY919326
IL-12	TTC TGG ACG TTT CAC ATG CT	CAC TCT GAC CCT CTC TGC TG	102	53°C	AY919327
IFNγ	GCA AGG CGA TAA ATG AAC TC	TGC GGC CTC GAA ACA GAT	98	50°C	DQ118388
TGFβ	TTC CTG CTC CTC ATG GCC AC	GCA GGA GCG CAC GAT CAT GT	392	58°C	AY919328
Acute phase proteins					
HP	CTG GCA GGC TAA GAT GGT TT	GTC AGC AGC CAT TGT TCA TT	75	54°C	AY919335*
HSP70	GGG GCT GAA CGT GCT GAG G	CCG CTT GTT CTG GCT GAT GTC	280	60°C	DQ118386
MT2	AAG GGC GCA TCG GAC AAG T	GAC CCA AAA CAA AAA GCA CCA A	91	55°C	AY919325

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; IL: Interleukin; INF: Interferon; TGF: Transforming growth factor; HP: Haptoglobin; HSP70: Heat-shock-protein 70; MT: Metallothionein; s: sense; as: antisense; bp: base pair.

* HP primer pairs published for harbor porpoises were used.

Reverse Transcriptase-Polymerase Chain Reaction:

Total RNA was isolated from 300µl EDTA blood by Ambion blood kit (Ambion Europe, Huntington, Cambridgeshire, United Kingdom). RNA isolation was performed according to the manufacturer's protocol. After DNase treatment (Ambion Europe, Huntington, Cambridgeshire, United Kingdom), 80-100ng/µl RNA was reverse transcribed with murine reverse transcriptase (RT-PCR Core Kit, Applied Biosystems; Weiterstadt, Germany) and 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, Amsterdam, Netherlands) was used. It 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 and APP a dilution series from 10^9 copies to 10^2 copies was prepared which

served as a standard curve. 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 2. The fluorescence was measured at the end of the annealing and at the end of the dissociation program at a wavelength of 530nm. To exclude measurement of non-specific PCR products and primer dimers and to determine a true amplification the PCR was followed by a dissociation program for 1 min at 95°C, succeeded by 41 cycles during which the temperature was enhanced each cycle, starting at 55°C and ending at 95°C. Only PCR reactions with one well defined peak were used for analysis. All PCR reactions were performed in duplicates. The constitutively expressed house-keeping gene GAPDH was used as control gene and to normalize cytokine and APP expression. Cytokine and APP expression index (EI) was calculated as follows:

$$EI = \frac{\text{Number of cytokine copies}}{\text{Number of GAPDH copies}} \times 10^6$$

To verify the true amplification of cytokines and APPs, the PCR-products were sequenced (Seqlab Laboratories, Göttingen) and sequences were published in GenBank (NCBI, Table 2).

Results:

Blood samples of six and eight animals were investigated in 2004 and 2005, respectively. GAPDH as house keeping gene was amplified in all blood samples. Interestingly, mRNA of cytokines and APP were present in blood samples of all

animals. Sequencing of PCR products showed amplification of the appropriate cytokine, which supports the use of this method with seal blood.

Cytokine expression:

In 2004, expression of IL-1β, IL-6, IL-8 and IL-12 mRNA decreased between admission and release for the majority of pups (Fig. 1). An exception was Pv 4, wherean increase of IL-1β, IL-6, and IL-8 was present. Pv4 revealed also higher mRNA amounts in comparison to other seals of this study. Additionally, APP mRNA expression increased over the time period of investigation (see also below).

For IL-4 an increase in mRNA expression was detected in most animals (Fig. 1). IL-10 mRNA expression increased in two pups (Pv 2, Pv 3), whereas four pups (Pv 1, Pv 4, Pv 5, Pv 6) revealed a reduced levels comparing admission and release (Fig. 1).

IL-2, and IFNγ mRNA expression increased in most animals during the time of investigation (Fig. 1). Exceptions were IFNγ mRNA levels in Pv 3, and IL-2 in Pv 2, where lower levels at release were present.

The TGFβ mRNA expression decreased between admission and release in blood samples of three pups (Pv 1, Pv 3, Pv 5), and increased in three other pups (Pv 2, Pv 4, Pv 6) (Fig. 1).

In 2005, cytokine expression was highly variable between the animals. Comparing expression of IL-1β, IL-2, IL-4, IL-6, IL-8, and IFNγ at arrival and before release a wide range of cytokine levels were present at arrival, whereas variability between single animals was reduced at release (Fig. 3).

IL-1β, IL-6, IL-8 and IL-12 mRNA expression decreased in most pups between admission and release, comparable to 2004 (Fig. 3). In detail, for IL-1β decreasing levels were found in four of eight pups (Pv 8, 9, 11, 12). For IL-6 five of eight pups (Pv 7, 8, 9, 11, 13), and for IL-8 and IL-12 four of eight pups (Pv 8, 9, 11, 13) revealed reduced mRNA levels at release.

IL-4 mRNA amounts increased in five of eight pups (Pv 9, 10, 11, 12, 14) (Fig. 3). IL-10 mRNA levels were low in comparison to 2004, and increased from admission to release in blood samples of four pups (Pv 7, 9, 10, 14).Two pups (Pv 8, 11) showed an increase and two pups only minor differences (Pv 12, 13) in IL-10 mRNA expression (Fig. 3).

IL-2, and IFNγ mRNA increased in the majority of pups (Fig. 3). In detail, six of eight pups (Pv 9, 10, 11, 12, 13, 14) revealed an increase in IL-2, and five of eight pups (Pv 9, 10, 11, 12, 14) showed elevated IFNγ mRNA amounts.

TGFβ mRNA expression decreased during rehabilitation in two pups (Pv 8, 11), and increased or were unchanged in six other pups (Pv 7, 9, 10, 12, 13, 14) (Fig. 3).

APP expression:

In 2004, mRNA expression of the APPs HP and HSP increased between admission and release (except for Pv 1). MT2 mRNA increased in three pups and decreased in three other pups. As mentioned above, Pv 4 showed an increase in HP, HST and MT2 mRNA and, for HSP and MT2 higher values than other pups at release.

In 2005, higher HP mRNA levels were detected for several pups in comparison to 2004. Furthermore, expression was highly variable between animals. HSP mRNA

decreased during rehabilitation in majority of pups, which was in contrast to results of 2004.. MT2 mRNA expression revealed less variability between animals. Majority of animals showed lower MT2 mRNA amounts in comparison to animals investigated in 2004. However, a ca reduction of MT2 mRNA was obvious.

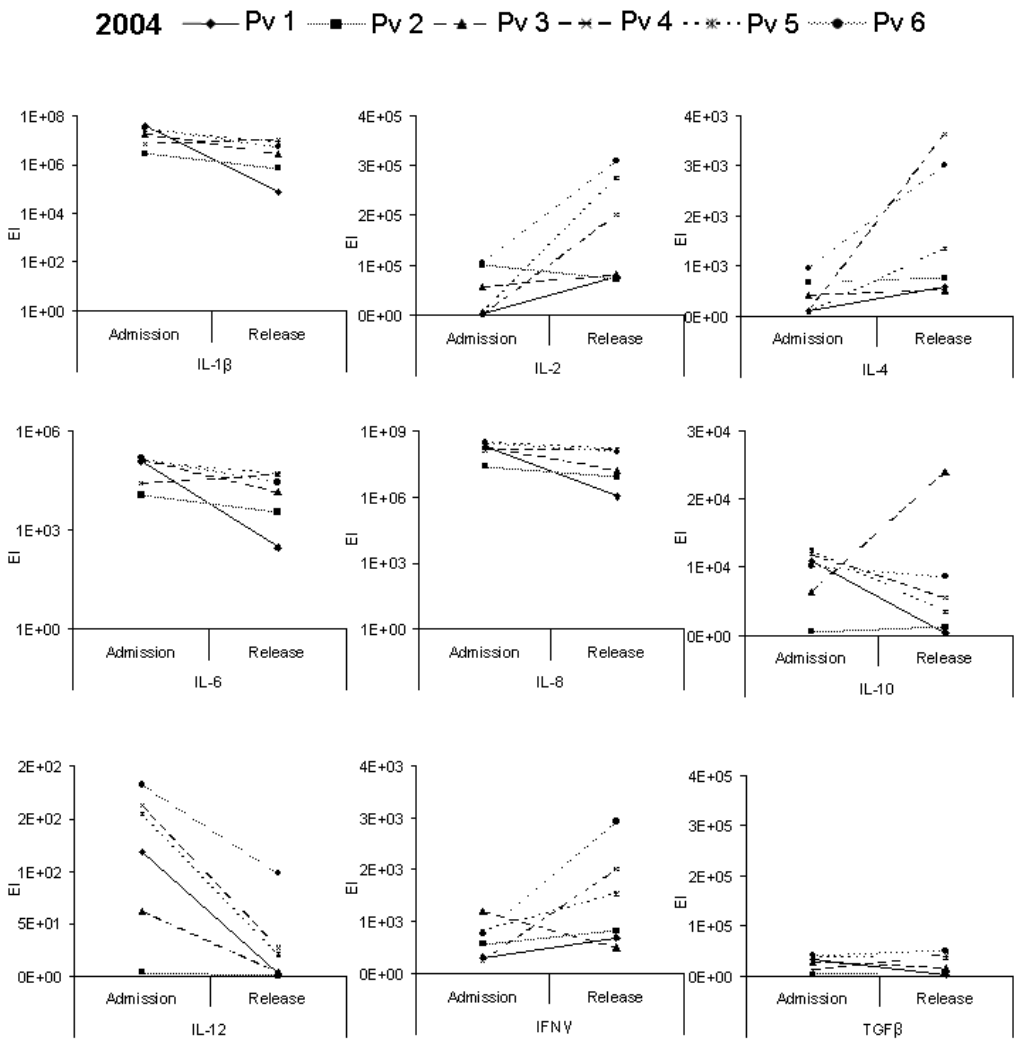

Figure 1: Expression index (EI) of IFNγ, IL-2, -4, -1β, -6, -8, -10, -12, and TGFβ in blood samples of six harbor seal pups (Pv 1 - Pv 6) taken at admission and before release in 2004.

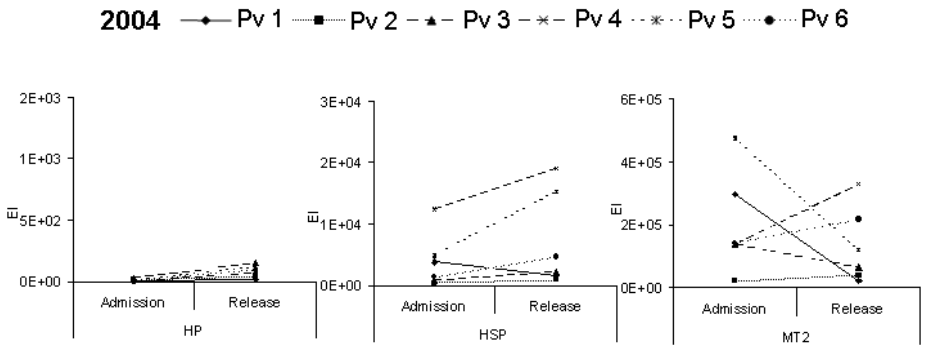

Figure 2: Expression index (EI) of haptoglobin (HP), heat shock protein 70 (HSP) and metallothionein 2 (MT2) in blood samples of six harbor seal pups (Pv 1 - Pv 6) taken at admission and before release in 2004.

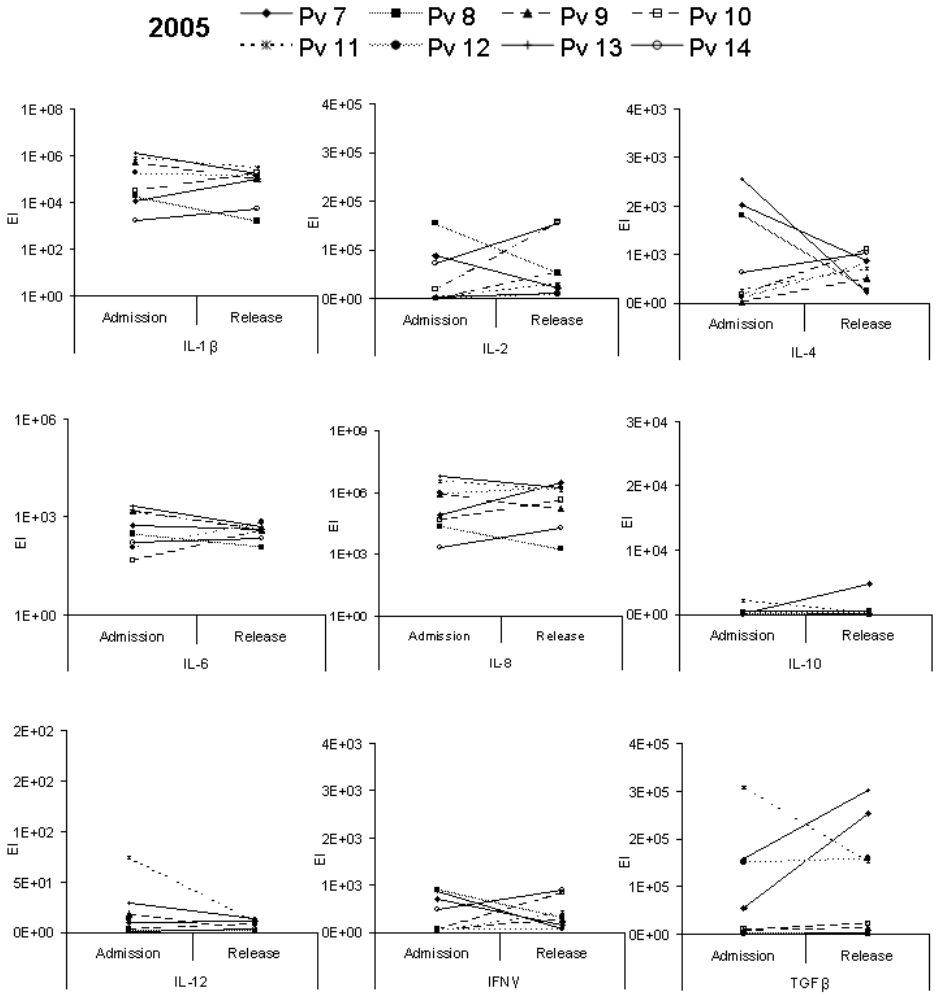

Figure 3: Expression index (EI) of IFNγ, IL-2, -4, -1β, -6, -8, -10, -12, and TGFβ in blood samples of eight harbor seal pups (Pv 7 - Pv 14) taken at admission and before release in 2005.

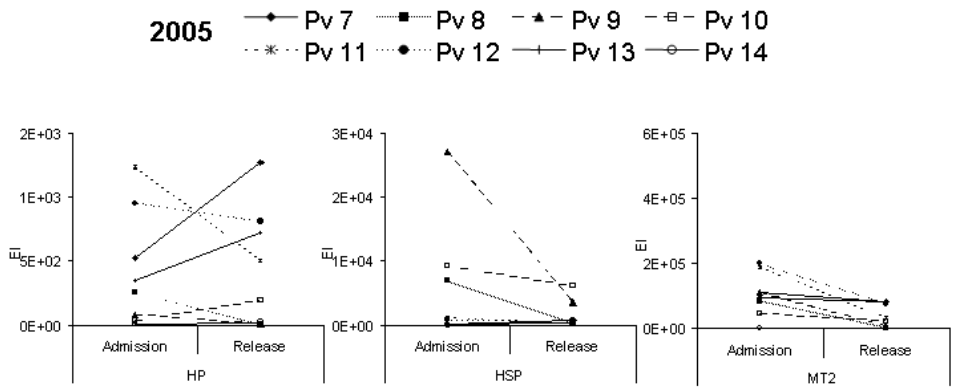

Figure 4: Expression index (EI) of haptoglobin (HP), heat shock protein 70 (HSP) and metallothionein 2 (MT2) in blood samples of eight harbor seal pups (Pv 7 - Pv 14) taken at admission and before release in 2005.

Discussion:

A developing immune system of harbor seal pups is crucial for their survival. mRNA expression of cytokines and APP, as mediators of the immune system, was analysed in blood samples of harbor seal pups at admission in the seal station and before release into the wild. Individual variations were present, , but trends were obvious. Single animals with obvious changes in expression could be identified. Unfortunately follow-up is not possible. Therefore progression of these animals after rehabilitation is not known. Amplification and sequencing of the house-keeping gene GAPDH, cytokines and APPs showed the use of this method with seal blood.

In 2004 cytokine mRNA expression pattern showed similar trends in all seal pups. These animals were collected at two consecutive days, suggesting similar environmental conditions (e.g. weather), and were of similar weight and age . In 2005 results revealed higher variability. These animals were collected and

sampled over one month and varied in age and weight. Differences between animals are likely due to individual regulation and several impact factors on cytokine expression (Di Piro, 1997; Elenkov and Chrousos, 1999; Elenkov et al., 2005; Iwakabe et al., 1998; Kidd, 2003; Lucey et al., 1996).

Most pups revealed increased pro-inflammatory cytokine levels at admission in the Seal centre. These values decreased during rehabilitation, whereas anti-inflammatory cytokines, IL-4 and IL-10, increased during this time period. Baseline levels of cytokine expression are not known in harbor seals. Therefore, the differentiation of an increase or reduction into physiological levels or changes consistent with pathological conditions is not possible. However, the alterations over time were obvious. In harbor porpoises an increase of pro-inflammatory cytokines during suspected infection was present (Fonfara et al., 2007b). Bacterial infection and malnutrition resulted in increased IL-1, IL-6, IL-8, and CPR levels (Enwonwu et al. 2005; Yeung et al. 2004). Therefore, increased levels of IL1-, IL-6, IL-8, and IL-12 found in pups at admission might be consistent with poor body condition and infection. Although the infection was not confirmed by WBC, altered cytokine expression is likely to be present before changes in WBC occur.

IL-2, IL-4, and IFNγ mRNA expression increased during rehabilitation, in particular in animals sampled in 2004. Similar results were reported by Härtel et al. (2005), who found an age dependent upregulation of IL-2, IL-4, and IFNγ. This might reflect physiological maturation of humoral and cellular immune response. Clinical implications or physiological variation was suspected for subjects who failed to produce IL-2, IL-4, or TNFα which is (Härtel et al., 2005), similar to the results presented here. During rehabilitation of pups recovery from infection and development of the mmune system into a mature immune system will have an

impact on cytokine expression (Hall, 1998; King, et al., 1998; Lalancette et al., 2003; Marchant and Goldman, 2005; Ross et al., 1993, 1994). A separation of is not possible.

It was not possible to differentiate Th-cell subpopulations in the seal pup blood samples. IL-2 and IFNγ as Th1 cytokines and IL-4 as Th2 cytokine were combined expressed. No differentiation of Th subpopulations or activation of both subpopulations is possible (Bremner and Beattie, 1990; Kidd, 2003; Mosmann and Sad, 1996).

The APP mRNA expression pattern was highly variable. However, in 2004 the expression of HP and HSP was associated with IL-2, IL-4 and IFNγ. HP as marker for infection and stress (Eckersall and Conner, 1988; Heegaard et al., 1998; Murata et al., 2004; Petersen et al., 2004; Zenteno-Savin et al., 1997) and HSP, which is involved in immune responses to several bacterial, viral and parasitic pathogens (reviewed by Young, 1990; Njemini et al., 2002), might suggest progression of infection. However, as baseline levels are not and HP levels in pup blood samples of 2004 were lower than HP mRNA levels in blood samples of animals found in 2005an increase into physiological levels might be possible. this was not confirmed by cytokine expression, WBC or clinical examination of the animals.

In 2005, for the most animals, the APP mRNA blood levels decreased during rehabilitation, whichmight be consistent with higher stress level or pathogen impact at admission and/or progressed immune responses at release (Cousins and Leinart, 1988; Daffada and Young, 1999; Heegaard et al., 1998; Murata et al., 2004; Petersen et al., 2004; Sato et al., 1994).

As a follow-up of animals is not possible, limited amount of information is available and the small sample number, It is not possible to draw conclusions and interpretation is speculative only.

This study revealed that cytokine / APP expression in pups varies between animals and years. It is likely that individual animals differ in their health status, development of their immune system and ability to cope with stress. However, our results suggest an activated immune system of animals at arrival at the seal centre and identification of animals with immune suppression. Limitations of the study are the small number of animals, different collecting periods in both years and lack of information about cytokine and APP expressions during rehabilitation. It was not possible to separate the effect of infection/stress and physiologic development of the immune system. To detect age related variations, comparison with cytokine and APP expressions of adult seals would be of interest.

Nevertheless, this preliminary study showed that cytokine detection is highly sensitive to get information about the status of the immune system of seal pups. The pro-inflammatory cytokines IL-1β, IL-6, IL-8, and IL-12 as well as the anti-inflammatory cytokine IL-4 seem to be useful diagnostic parameter for the assessment of the immune status of harbor seals.

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