

# Evaluation of Blood Tests to assess the Health Status of Harbor Seals (*Phoca vitulina vitulina*) of the German North Sea



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**Ilka Hasselmeier**

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Referent: Prof. Dr. D. Adlung

Koreferent: Prof. Dr. Colijn

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## **General Introduction**

The harbor seal belongs to one of the two marine mammal groups that occur in the North Sea: pinnipedia and cetacea. In the sub-order pinnipedia it represents one of the seven species of the genus phoca. Harbor seals stand at the end of the food web and are therefore a valuable indicator for the functioning of the marine ecosystem. Contrary to cetaceans seals live amphibious and are more easily accessible. Accordingly, the possibility of examining their health status should be of vital importance and interest to numerous institutions.

Assessing the health status of a population is essential in order to maintain adequate managing plans. Basic tests and resulting values are important features for this health assessment. The main focus of the present research was the evaluation of different blood tests for potential routine application in the quick assessment of the health status of different harbor seal (*Phoca vitulina*) study groups (free-ranging, rehabilitated, captive). The aim was to establish baseline data for use in medical examinations and diagnoses. Never before has a single harbor seal population been examined in regard to comparing free-ranging, rehabilitated and captive animals.

This doctorate thesis gives examples of four different tests performed on fresh harbor seal blood: differential hemogram, phagocytosis, oxidative burst and serum electrophoresis. For harbor seals only limited or no results, respectively, have been published yet. The results of these tests reflect the health status of the animals in different ways and complement one another. The three different groups of harbor seals investigated, raise several questions that are dealt with in all four tests:

- 1.) What do the applied assays say about the current but also long-term health status of harbor seals?
- 2.) Are the assays applicable for routine handling?
- 3.) Do the free-ranging (adult) animals differ from the ones living in captivity?
- 4.) Do young harbor seals differ from adults?
- 5.) Do the rehabilitated pups differ from pups born in captivity?

For a better understanding of the subject matter a short description of the biology of harbor seals and general information about the immune system is presented in Chapter 1, following this introduction.



Due to repetition in the tests, general information about the animal groups, location and handling, as well as some implements are also comprised in a second chapter.

In Chapter 3 the differential hemogram is discussed. It is an established tool for assessing the health status in humans as well as in animals. Baseline values are needed for reference. Data dating back to 1997 were taken into consideration in all three seal groups (free-ranging, rehabilitated, captive), which ensured a considerable sample size. Sub-groups that had never been looked at before could be differentiated. Pups, yearlings, and adult free-ranging animals were compared between each other as well as with the other groups. Differences not known before have been found, results of other studies partly refuted.

Chapter 4 addresses phagocytosis of neutrophils and monocytes. Phagocytosis is the most important and initial defense against many pathogens in mammals. For the first time a test kit developed for human application has been used on harbor seal blood, and results of the three above mentioned groups compared.

In Chapter 5 the oxidative or respiratory burst of neutrophils is tested. The oxidative burst investigates the reaction of neutrophils after phagocytizing pathogens. After ingestion a large increase in oxygen uptake by neutrophils is observed. Again, a test kit developed for human medicine has been applied.

Finally, Chapter 6 completes the analysis of the immune system of the three harbor seal groups. Serum protein electrophoresis (SPEP) as a marker for the immune system has long been used in human medicine. Indications for identification of the protein fractions are especially all forms of hyper- or hypoproteinemia, which are caused by numerous diseases.

A final conclusion summarizes chapters 3 to 6 and gives recommendations for future projects.

In the attached appendices detailed information about facts and function of blood components and general overviews of phagocytosis and burst processes are presented.

## Chapter 1

### **Biology of Harbor Seals (*Phoca vitulina*)**

#### **1.1 Taxonomy**

There are 34 species of pinnipeds (suborder pinnipedia) worldwide, all of which are assigned to 3 families of the mammalian order carnivora: the otariidae, phocidae and odobenidae (Jefferson *et al.* 1993). The subject of this thesis, *Phoca vitulina vitulina*, is found within the seven species of the Genus Phoca (King 1983).

In German waters several species can be found, but only the harbor (or common) and the gray seal can be called domestic. These two species stay in the North Sea year-round.

#### **1.2 Habitus**

Pinnipeds are highly specialized aquatic carnivores that live in a diversity of marine habitats, and some in freshwater as well. One unifying feature of the group is that all must return to a solid substrate, such as land or ice, to bear their pups. In general, phocids are more capable divers and breath-holders, although there is overlap in the capabilities of some otariids and phocids (Jefferson *et al.* 1993).

All pinnipeds have fur, but phocids exclusively use their blubber for thermoregulation (King 1983). They possess 2 sets of limbs (fore- and hindflippers), and long whiskers, which play an important role in locating fish. Harbor seal pups are usually born in the “adult” fur, which makes them able to swim just a few minutes after birth.

The fur of harbor seals shows many fine dots, ring-like markings, and/or some blotches that vary in color (Fig. 1). The pattern of the markings returns after each molting and is the only positive distinctive feature in harbor seals. Harbor seal pelage shows only one kind of hair: the guard hair for the outer protection that contains the pigments for the coloring (Jefferson *et al.* 1993).



Figure 1: Harbor seals (*Phoca vitulina*) with varying coloration of fur.

The body is rather plump but fusiform and is perfectly adapted to the medium water. The external ear openings are relatively large and conspicuous. The skeleton of the harbor seals is, compared to the land carnivores, characterized by a sturdy construction of the cervical spine, front limbs and thorax, but at the same time by an asthenic skeleton of the hind body. Due to lack of anticlinal vertebrae the spine is extremely maneuverable (King 1983, Jefferson *et al.* 1993).

The number of teeth is reduced and the dentition is, again compared to land living carnivores, almost homodont. The carnassials are not discernable anymore and the pre-molars and molars are almost identical (Fig. 2).



Figure 2: Harbor seal skull.

The harbor seal is a food opportunist and the fish caught is in accordance with the most available species (Sievers 1989, Pierce and Boyle 1991, Schwarz and Heidemann 1994,

Krause 1999, Bowen *et al.* 2002). The adults seldom feed on molluscs and crustaceans. Overall they seem to prefer benthic and demersal fish. Seasonal changes in the proportion of prey uptake confirm the opportunistic behavior (Behrends 1985, Krause 1999). They find their prey primarily with help of their vibrissae, which are able to locate fish or water turbulences caused by fish movements (Dehnhardt *et al.* 1998, 2001).

### 1.3 Distribution

Harbor seals are shore living animals found principally, although not exclusively, in estuaries and in areas where sandbanks are exposed at low tide. They are also found on shingle beaches and on rocky shores that shelve gradually into the water and are easy to access (King 1983). Harbor seals are one of the most widespread of the pinnipeds. They are confined to the Northern Hemisphere, from temperate to polar regions. At least 4 subspecies are recognized: *P. vitulina vitulina* in the eastern Atlantic from northern Portugal to the Arctic, including Iceland and Greenland; *P. v. concolor* in the western Atlantic from the mid-Atlantic United States to the Canadian Arctic; *P. v. richardsi* in the eastern Pacific from central Baja California, Mexico, to the eastern Aleutian Islands, and *P. v. stejnegeri* in the western Pacific from Japan to the Kamchatka Peninsula (Jefferson *et al.* 1993).

Telemetric studies have shown that the harbor seals living at the German coast swim to offshore areas for foraging and return a few days later to their haul-out sites (mostly sandbanks) (Fig. 3; MINOS 2005, Liebsch Diss. 2006).

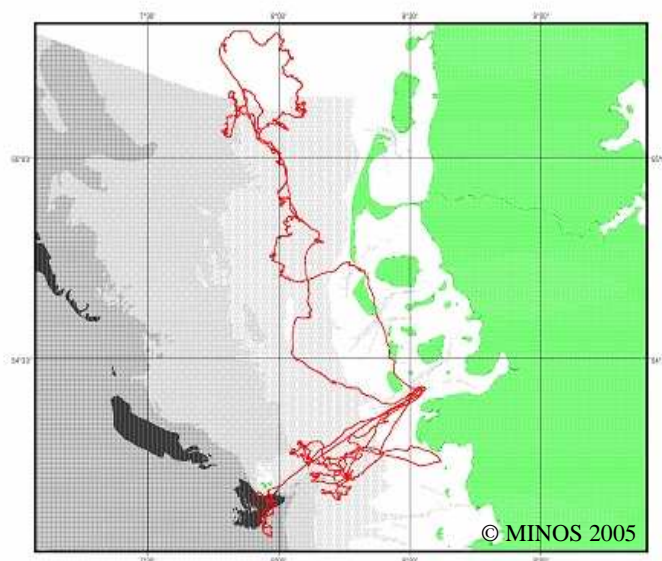


Figure 3: Example of 6 foraging trips of 3 harbor seals, equipped at the Lorenzenplate, Germany (MINOS 2005).

### 1.3.1 Survey of the harbor seal population of the European Wadden Sea

Since 1975 the complete Wadden Sea is surveyed on a specific route by a low flying plane (~ 150m) several times a year (Fig. 4). Since the early 1990ies these flights are coordinated with Denmark and The Netherlands by the Common Wadden Sea Secretariat. During pupping season, differentiation between juvenile/adult animals and pups is possible.



Figure 4: Aerial survey picture from 150m above sea level (ASL).

The development of the harbor seal population in the European Wadden Sea is shown in Figure 5. Worldwide the stock was calculated by Bonner (1989) to be about 300,000 – 400,000 individuals. In the mid 1970ies a law was ratified in Germany prohibiting the hunt on harbor seals in the German Wadden Sea. In the following five years the population remained stable. Only since about 1979 the number of animals continuously increased until 1988 when the first major Phocine Distemper Virus (PDV) epidemic carried off about 50-60% of the trilateral Wadden Sea stock (The Netherlands, Germany, Denmark). The following year, though, the number increased again and found its peak to date with approximately 21,000 animals in the Wadden Sea in 2002 (Trilateral Seal Expert Group 2003).

A second PDV epidemic in 2002 killed about 50% of the Wadden Sea populations (Jensen *et al.* 2002, Müller *et al.* 2004, Essink *et al.* 2005, Harkönen *et al.* 2006). Similar to the pattern observed between 1989 and 2002 the annual growth ratio was very high in 2003 (18.5%) following the epizootic. Today the population has recovered to a grand total of about 15,500 in the Wadden Sea and a maximum of 7,160 harbor seals in Germany (Fig. 5).

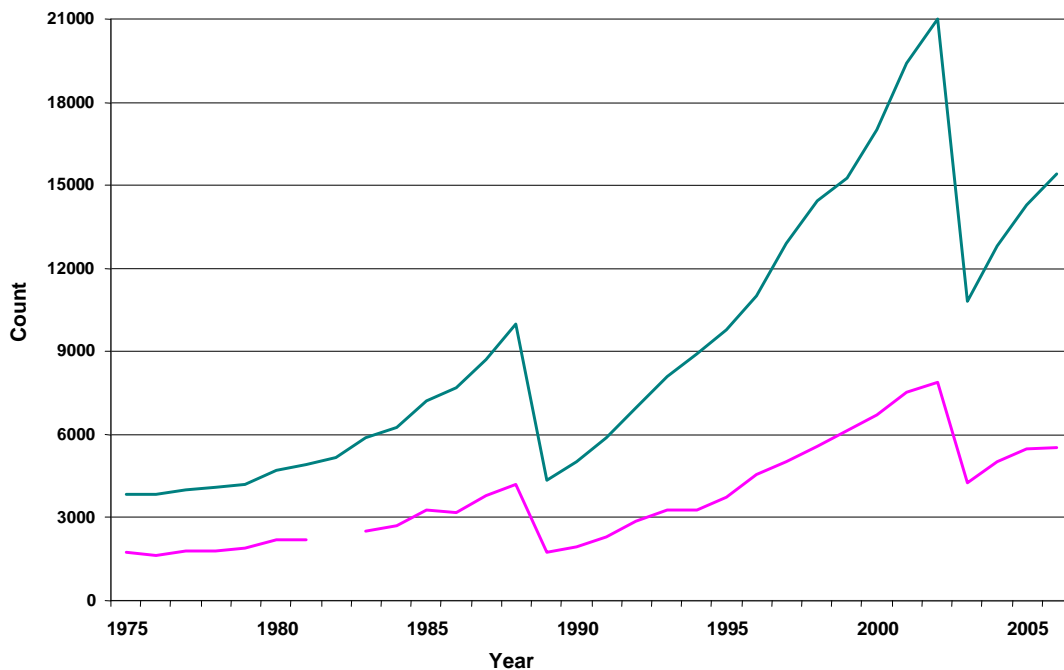


Figure 5: Population graphs of harbor seals in the Wadden Sea (blue) and Schleswig-Holstein, Germany (pink). 1975-2002 max. numbers of counts are shown; since 2003 max. numbers in August are depicted.

Next to the harbor porpoise (*Phocoena phocoena*) and gray seal (*Halichoerus grypus*) the harbor seal is one of the main representatives of the marine mammals in the ecosystem of the North Sea. Besides the sea, seals use other environments like sandbanks and beaches in neighborhood to humans. Due to their neighborly natural habitat to humans the seals are exposed to a variety of anthropogenic influences, like pollutants, ship traffic, recreational and construction disturbances. Species with overlapping habitats require special attention. The protection and conservation of the Wadden Sea is therefore in the main focus of the adjacent countries. Since 1978 The Netherlands, Germany and Denmark have joined forces for its protection and have adopted the “Trilateral Monitoring and Assessment Program” (TMAP). In this agreement the seal population, next to other biological parameters, is supposed to serve as bioindicator of the ecosystem. For further protection of seals another agreement was adopted (“Abkommen zum Schutz der Seehunde im Wattenmeer”, Seal Agreement)

between The Netherlands, Germany and Denmark in 1991. The convention comprehends terms of research and monitoring, withdrawal from nature and protection of habitat. These terms were specified in the Seal Management Plan for the Wadden Sea Seal Population 1991 – 1995, followed by the revised version 1996 – 2006.

Next to demographic criteria the management plan uses qualitative parameters like reproduction, mortality and health status to assess the seal population. The assessment of the health status, however, requires immunological, physiological, toxicological, patho-histological, microbiological, virological, and parasitological studies. The present study therefore is a contribution to the implementation of the management plan.

#### **1.4 Immune system of harbor seals**

The immune system is an early indicator for the health status of any mammal. It is primarily a series of defense mechanisms that functions to protect the body against the potential harmful effects of foreign micro-organisms. In recent years, there have been rapid advances in the field of immunology. With these advances new methods have come in order to prevent and treat infectious diseases (Tizard 2000, Janeway *et al.* 2002). Although marine mammal immunology is a relatively recent field of scientific endeavor, it is already possible to perform reliable and pertinent studies to address specific aspects of health and disease in these species. Immune system monitoring and serological diagnostic assays have clear roles in the management of disease in individual marine mammals (Dierauf & Gulland 2001).

The immune system has been classically divided into the innate and adaptive (or acquired) immune responses. Whereas the innate immune system is static relative to the quantity and quality of a response, the adaptive response gains quantity and quality (immunological memory) upon repeated exposure to a pathogen. Although these divisions are descriptively useful it is important to realize that successful host defense responses rely on close interaction between these two mechanisms (Tizard 2000, Janeway *et al.* 2002).

### 1.4.1 Innate immunity and inflammatory response

A normal, healthy mammal is exposed to a vast number of potentially pathogenic micro-organisms each day (Dierauf & Gulland 2001). Since clinical infectious diseases are relatively uncommon in healthy individuals, defense against these organisms must be a constant process. The majority of micro-organisms are repelled by innate host defenses that include non-immunological anatomical and physiological barriers (e.g., mucociliary blanket), antimicrobial factors (e.g., complement, lysozyme, lactoferrin, defensins, and reactive oxygen and nitrogen intermediates), and immunological effector cells (e.g., neutrophils, eosinophils, macrophages, and natural killer (NK) cells). Many of these immune mechanisms act immediately following microbial invasion, particularly against those pathogens possessing identifiable structures such as lipo-polysaccharide (LPS), present on gram-negative bacteria, or double-stranded viral RNA. To be effective, the mammalian immune system possesses molecules capable of recognizing and neutralizing an enormous repertoire of infectious agents. Recognition is one of the key steps in the stimulation of early-induced immune responses that function to keep the infection under control, while the antigen-specific cells of the adaptive immune response are recruited and activated (Tizard 2000, Janeway *et al.* 2002).

Occasionally, the nature or extent of the localized inflammation may be severe enough to evoke a number of systemic inflammatory processes, called the *acute-phase response*, which serves to produce inflammatory mediators and recruit more inflammatory cells to the site of infection (Roitt *et al.* 1993, Funke *et al.* 1997, Zenteno-Savin *et al.* 1997). The most important effector cells in these early phases of the immune response are phagocytes (migrating neutrophils and monocytes, and tissue macrophages). These not only trap, engulf, and destroy microbes, but also secrete cytokines that initiate the systematic acute-phase response and recruit additional leukocytes to magnify the local inflammatory response. The recruitment of cells involves chemotaxis and an increase in vascular endothelial cells and immune cell-adhesion molecule expressions. These factors, in conjunction with an increased local blood flow and increased vascular permeability, lead to an accumulation of leukocytes, immunoglobulins, and other blood proteins in the infected tissue (Roitt *et al.* 1993, Tizard 2000, Janeway *et al.* 2002, Funke *et al.* 1997, Zenteno-Savin *et al.* 1997).



### 1.4.2 Adaptive immune response

If a pathogen evades or overwhelms the innate defense mechanisms of the host, causing the foreign antigen to persist beyond the first several days of infection, an adaptive immune response is initiated. In contrast to the innate immune responses, the adaptive response produces effector cells (B- and T-lymphocytes) and molecules (immunoglobulins), which are highly specific to the antigens of the invading microbe. In addition, the antigen-specific lymphocytes of the adaptive immune response are capable of swift clonal expansion and of a more rapid and effective immune response on subsequent exposures to the pathogen (immunological memory) (Roitt *et al.* 1993, Tizard 2000, Janeway *et al.* 2002).

The trigger for the adaptive immune response, the activation and proliferation of lymphocytes, takes place in organized lymphoid tissues. The three major portals by which an invading pathogen can enter the host are namely, via a mucosal surface (respiratory tract, gastrointestinal tract), through the skin, or by direct inoculation into the bloodstream. At each of these portals are organized lymphoid tissues (mucosal-associated lymphoid tissue, regional lymph nodes, and spleen, respectively), which provide the organized microenvironment in which the intricate events of the adaptive immune response are closely coordinated. Microscopic investigations of the marine mammal system reveal that the morphology of the lymphoid organs is similar to terrestrial mammals, but with a few adaptive attributes, like thymic cysts, “anal tonsils”, and a complex laryngeal gland (Romano *et al.* 1993 and 1994, Cowan and Smith 1995 and 1999, Cowan 1999, Smith *et al.* 1999, Wünschmann *et al.* 1999). At these lymphoid sites, pathogens are trapped and engulfed by phagocytic cells.

Some of the lymphoid cells are specialized for processing microbial antigens into small peptides, and presenting these peptides in association with highly polymorphic glycoproteins, called major histocompatibility (MHC) proteins, on their cell surface. The ability of the immune system to recognize and respond to such a vast array of foreign proteins is determined to a large degree by the number and structural diversity of the MHC molecules present in an individual. The polymorphic nature of these MHC proteins ensures maintenance of the host’s immunological vigor by minimizing the ability of a pathogen to avoid presentation by selective mutation. It is speculated that genetically restricted species, such as those that have been subjected to a “population bottleneck”, will lack MHC diversity (Gyllensten *et al.* 1990, Slade 1992, Murray and White 1998, Hoelzel *et al.* 1999, Zhong *et al.* 1999). The immunogenic peptides of the invading pathogens bound to the cell surface MHC

molecules are recognized by the highly specific receptors of T-helper lymphocytes, which by specific patterns of cytokine secretion stimulate either B lymphocyte expansion and antibody production (humoral immunity) or activation of macrophages (delayed-type hypersensitivity), and expansion and activation of cytotoxic T-lymphocytes. The subsets of lymphocytes with these polarized patterns of cytokine production are T-helper1 and T-helper2 cells, respectively (Roitt *et al.* 1993, Janeway *et al.* 2002).

### 1.4.3 Cellular immunity

Classical differential white blood cell counts can morphologically distinguish and enumerate major leukocyte subpopulations into lymphocytes, monocytes, eosinophils, and neutrophils. These cells, although ultimately derived from the same progenitor bone marrow stem cell population, make different functional contributions to the immune system. There is a wide range of immunological techniques that can be used to evaluate the cellular immune system. Broadly speaking, these assays can be divided into those that measure the phenotypic qualities of leukocytes (lymphocyte subpopulations and the cell-surface density of adhesion proteins) and those that assess functional aspects of the cell. Recently, a major use of these assays in marine mammals has been to examine immunological dysfunction arising from the presence of environmental pollutants (De Swart *et al.* 1995 & 1996, Lahvis *et al.* 1995, Ross *et al.* 1995, Kakuschke *et al.* 2005). Furthermore, since the immune system is acutely sensitive, these methods have the potential to measure the influence of many internal and external stresses that affect marine mammals.

The peripheral blood represents the most convenient sampling window for the assessment of the cellular immune system. In many circumstances, cells can also be isolated from tissues, such as spleen and lymph nodes collected during post-mortem examination, and can be subsequently used in phenotypic assays and/or *in vitro* functional testing.

Due to the importance of the neutrophil granulocytes in this study a more specific description is attached as Appendix A.

#### 1.4.4 Humoral immunity

Immunoglobulins (antibodies) are soluble, antigen-specific effector molecules of the adaptive immune response. These proteins are produced by B-lymphocytes of the humoral immune system. Distinct classes of immunoglobulin molecules (IgG, IgM, IgA, and, of lesser importance in the peripheral circulation, IgE and IgD) have been identified in most mammalian species. Because of their relatively high concentration in serum, purification and characterization of these proteins are often the first tasks undertaken by comparative immunologists. To date, several studies have characterized immunoglobulin molecules with characteristic heavy and light chain components, using sera collected from a selected number of cetacean and pinniped species (Nash and Mach 1971, Cavagnolo and Vedros 1978, Carter *et al.* 1990). Binding of immunoglobulin proteins to unique determinants (epitopes) on foreign proteins is an important mechanism by which pathogens are targeted for subsequent elimination from the body. By measuring changes in the circulating levels of antigen-specific immunoglobulin, exposure to infectious agents can be documented. This can be used in epidemiological studies of infectious disease and to enhance the management and prevention of disease outbreaks by identifying naïve unexposed animals. It must be emphasized, however, that pathogen-specific antibody levels do not necessarily confirm the presence of an active pathogen (Tizard 2000, Janeway *et al.* 2002).

## Chapter 2

### General Material & Methods

#### 2.1 Animals, Location and Handling

During March/April (spring), August/September (summer), and October-December (winter), 1997-2004, free-ranging seals were caught with a net (3m x 200m), transferred into tube nets, and restrained manually ( $\bar{x}$  = 45 min) for blood collection and measurements. Three age groups were differentiated: animals born the same year of the catch (pups), seals born in the previous year of the catch (yearlings) and animals at least 2 years old (older).

Lest pregnant females were disturbed, no animals were caught during pupping season, which usually starts at end of May and lasts until late June. No values of pups were available for the first catching season (March/April, w\_pups\_spr). In some cases the count of values was very limited, e.g. the numeric counts of lymphocytes, monocytes and granulocytes in the summer (August/September) of “w\_pups\_su” and “w\_yearlings\_su”.

Seals are known to travel hundreds of kilometers irrespectively of seasons (MINOS 2005, Reijnders *et al.* 2005). Due to the close geographical proximity and no significant differences ( $p < 0.05$ ) between free-ranging animals from the sandbank Lorenzenplate, Germany, and the island Rømø, Denmark, they were pooled (Fig. 1).

Seal pups are sometimes left behind by their mothers due to numerous reasons (e.g., storms, death of mother, and disturbance by humans). Harbor seal pups found along the coast of Schleswig-Holstein were brought to the Seal Center Friedrichskoog for rehabilitation. Hemograms of pups were examined when first admitted into the Seal Center Friedrichskoog and shortly before release. If animals displayed clinical symptoms (e.g., diarrhea, vomiting, or slackness) additional hemograms were processed. After 2-3 months the pups were usually released into the wild again. Occasionally, pups became permanent residents due to lasting health or behavior problems.

The eight animals held at the Seal Center were also confined into tube nets for blood withdrawal. No anesthesia was used. The captive seals were restrained about as long as the free-ranging. They have not yet been trained to blood withdrawal.

Blood was collected from three different groups of harbor seals, including free-ranging animals from the Lorenzenplate and Rømø (n = 170), rehabilitated pups at the Seal Center Friedrichskoog, Germany (n = 236), and captive animals from the Seal Center (n = 8) (Fig. 1). In total, 793 differential hemograms of harbor seals were measured between 1997 and 2004. 170 of free-ranging animals, 517 repeat samples of the pups, and 106 repeat samples of eight different captive animals of varying ages were collected. The captive seals were tested for health checks several times over the years. Due to the dependency of the sample values, repeat measures analysis was used. Not all of the hemograms were suitable for analysis and not every blood value could be measured for every animal. As pupping season of harbor seals in the German North Sea occurs at the end of May, there were no pups available in the first catching season (spring).

Blood was always collected from the epidural vertebral vein (Fig. 2) with a needle (1.2 x 100mm) and syringe and immediately transferred into tubes containing either ethylenediaminetetraacetic acid (EDTA) for differential hemograms, coagulating substances for serum extraction or NH<sub>4</sub>-heparin for Phago- and Bursttest (Sarstedt Aktiengesellschaft & Co., 51588 Nümbrecht, Germany). The tubes were carefully shaken and kept at room temperature until further handling. Blood samples were processed within 1 to 12 hours.

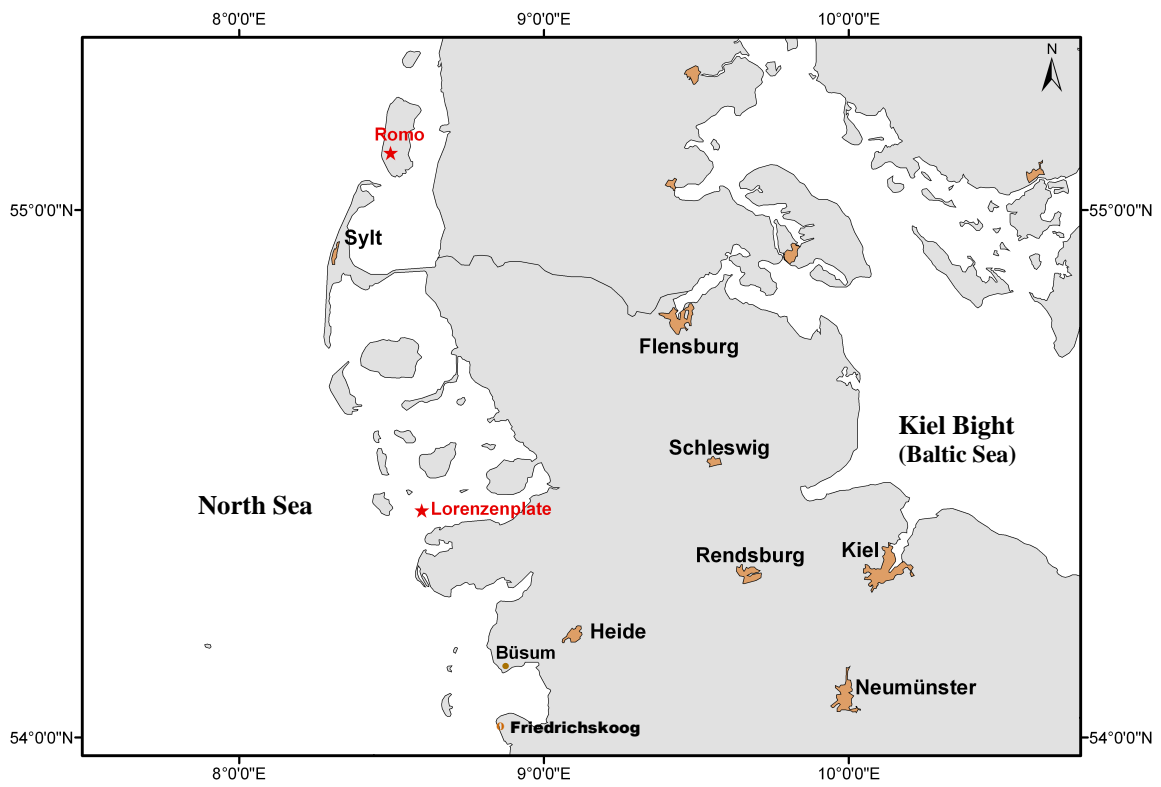


Fig. 1: Study area: State of Schleswig-Holstein, Germany, and the island Rømø, Denmark, with the two locations of the seal catches marked (\*).

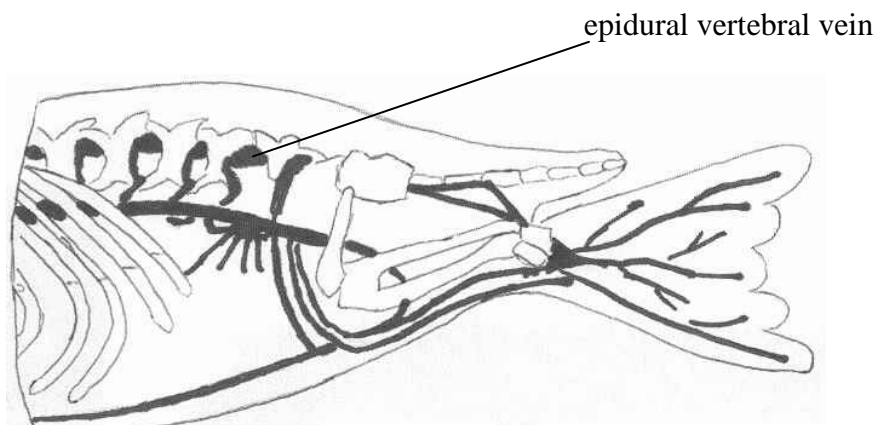


Fig. 2: Epidural vertebral vein, used for blood collection of harbor seals (*Phoca vitulina*)

## 2.2 Flow Cytometry

The flow cytometer used in this study was a FACS Calibur™ by Becton Dickinson Biosciences (San José, CA, USA). The FACS Calibur™ is a four-color dual laser, desktop analysis device (Fig. 3). It is a flow cytometer easy to handle, but at the same time is able to use prevalent coloring antibodies due to two laser lines. By an additional diode-laser fluorochromes can be used, which are not detectable by the common 488nm (blue) but lie at 635nm (red).

In this study the flow cytometer was used primarily for the phagocytosis and respiratory burst tests (Chapter 4, 5). Additionally differential hemogram acquisition was also performed with the FACS Calibur™ (Chapter 3). Only the blue-green excitation light (488nm argon-ion laser) was necessary for all three applications.

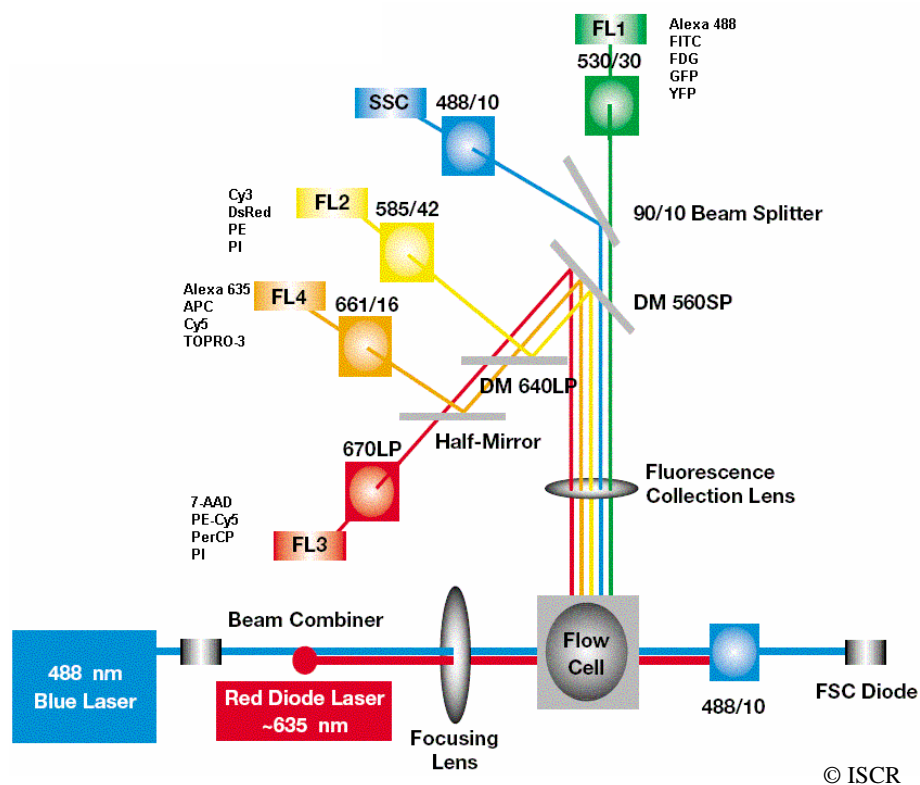


Figure 4: Sketch of a FACS Calibur™ with commonly used fluorochromes.

### 2.3 Differential Hemogram

During the years, three different laboratories analyzed the blood for differential hemograms. Both the *Veterinary Laboratory* in Geesthacht and the *Veterinary Laboratory Ingolstadt GmbH* used a *Cell-Dyn 3500* (Abbott). Since 2001 the private veterinary clinic of Dr. Jörg Driver has analyzed the hemograms. Dr. Driver used a *scil Vet abc* (Centre de distribution de médicaments vétérinaires, CDMV). During the first phase of using the *scil Vet abc*, the hemograms were simultaneously measured at the laboratory in Geesthacht, in order to check for comparability. In all three laboratories the differential blood count was done manually. Red and white blood cells and thrombocytes are measured by electrical impedance; hemoglobin is measured by spectrophotometry. Special "Smart Cards" (chip cards with reference values) for harbor seals were developed by the company.

### 2.4 Electrophoresis

In this study the use of cellulose acetate was preferred to gel, to be able to manually adjust the appliance for best possible results. An *Elphoscan Mini Plus* by Eltest (Bornheimer Str.33, 53111 Bonn, Germany) was used, which was located at the WestKüstenKlinikum (WKK) Heide. After several tests a voltage of 115, 20 mA and a separation time of 18 min proved to be the optimum. For reference values of the protein fractions, TSP was also measured by the laboratory of the WKK Heide.



## Chapter 3

### **Differential Hemogram of Harbor Seals (*Phoca vitulina vitulina*)**

#### **3.1 Introduction**

Acquisition of the (differential) hemogram is a common procedure used to investigate the health status of humans and animals. It provides much information on the physiologic condition of (marine) mammals but it is essential to have reference values in order to evaluate their health status. Next to sea lions (*Zalophus californianus*), the harbor seal is one of the most common pinnipeds held in captivity (Andrews *et al.* 1997). Because captive animals are dependent on human care, reference values are needed to provide the best possible medical assistance. Due to different living conditions (e.g. water, food) the hematology could be different from free-ranging animals. A shortcoming of many marine mammal hematology studies, especially those of wild animals, is insufficient knowledge of their life history. Pinnipeds are known to show clinical symptoms only at a very late stage. Deviations of hematologic values from baseline values may therefore be used to assess treatment or the need for more intensive testing of a suspected problem.

Several papers have been published regarding hematologic values of harbor seals (*Phoca vitulina*). However, previous studies were either comprised of one group of seals (free-ranging, rehabilitated or captive) or small sample sizes (e.g. McConnell and Vaughan 1983, Roletto 1993, Morgan *et al.* 1998, Bossart *et al.* 2001, Lander *et al.* 2003). Resulting values can therefore hardly be considered as reference values. Results of former studies are also difficult to compare with one another and the reliability for a different group is debatable.

Since 1999 the State of Schleswig-Holstein has funded a monitoring project to examine live harbor seals in the German North Sea. On this account, reference values of blood constituents for free-ranging, rehabilitated, and captive harbor seals could be compared in this study. The hemograms presented will be important for routine health examinations of harbor seals of different ages and living conditions.

The goal of the present thesis was to increase the sample size and compare harbor seals of one population of different living conditions. The study was primarily concerned with blood values that potentially indicate diseases: white blood cells (WBC) and their derivatives (lymphocytes, neutrophils, eosinophils, monocytes), red blood cells (RBC), hemoglobin (HB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and thrombocytes (PLT).

### 3.2 Material & Methods

In total, 793 differential hemograms of harbor seals were measured between 1997 and 2004. 517 of them were repeat samples of rehabilitated pups. In order to keep the results independent and get more representative results only the pre-release values of the pups were tested for reference (n = 127).

In 2003/2004 the differential hemogram was also performed by flow cytometry (FACS Calibur<sup>®</sup>, Becton Dickenson) in order to compare results with manually counted blood smears. 100µl blood was treated with 2ml of BD Lysis. After a short time (10 min) standing in the dark, it was centrifuged for 10 min at a temperature of 15°C and 1500rpm. It was washed twice with PBS and directly measured by the flow cytometer. The time elapsing between blood withdrawal and the measurement with the flow cytometer varied between 2 and 10 hours.

By detecting the different cell populations according to size (forward scatter = FSC) and granularity (sidescatter = SSC), they can be made visible even without using antibodies. In the case of a differential hemogram the eosinophils are the largest and most granular cells whereas the lymphocytes are the smallest and have the smoothest cell body (Fig. 1).

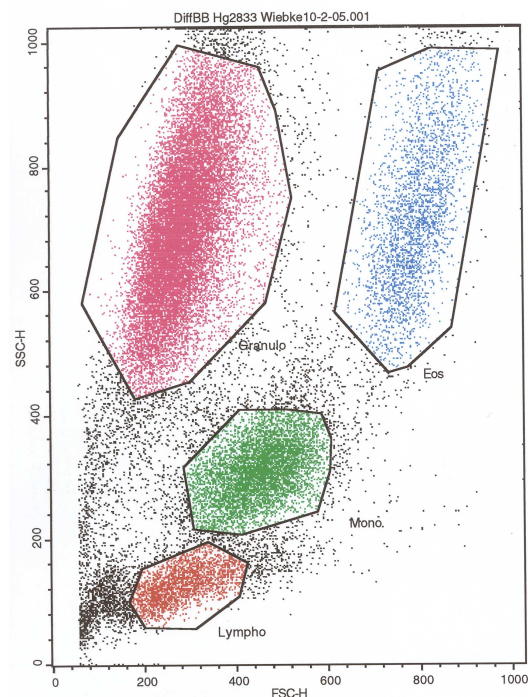


Fig. 1: Dot Plot of granulocytes (pink), eosinophils (blue), monocytes (green), and lymphocytes (red) of harbor seal blood as shown by a FACS Calibur<sup>®</sup> (Becton Dickenson).

### 3.2.1 Statistical considerations

For baseline values the median and inter-percentile range (5-95%) were chosen as the values were not normally distributed. In case of sample sizes <10, the minimum and maximum were depicted. For each seal group, differences among seasons (separately for different age-classes) and ages (separately for different seasons) were assessed using Mann-Whitney U-tests or Kruskal-Wallis H-tests, respectively. Captive seals were tested for differences between individuals with the same tests. In general, non-parametric tests were used because most variables did not match the assumptions of parametric tests. Analyses were conducted only for those age classes (pups, juveniles and adult females, respectively) for which we had sufficient sample sizes. Because 16 different blood values were examined, an alpha-level adjustment was required. For this purpose Fisher's Omnibus test was applied, which combines a number of P-values into a single Chi-square distributed variable with degrees of freedom equalling twice the number of P-values (Haccou and Meelis 1994). Exact tests were used when small samples required their use (Siegel and Castellan 1988; Mundry and Fischer 1998). All indicated P-values are two-tailed and 5% was chosen as significance level. Due to mostly non-normally distributed variables, the median and interpercentile ranges (5-95%) were chosen for baseline values.

### 3.3 Results

#### 3.3.1 White hemogram (Leukocytes)

##### 3.3.1.1 Free-ranging harbor seals

Statistical tests of free-ranging harbor seals from the North Sea indicated blood values varied among seasons and age-classes. In detail, testing for differences between seasons revealed significant effects in all three age-classes (Fisher's Omnibus test, pups:  $\chi^2 = 81.46$ ,  $df = 32$ ,  $P < 0.001$ ; yearlings:  $\chi^2 = 67.54$ ,  $df = 32$ ,  $P < 0.001$ ; older:  $\chi^2 = 217.80$ ,  $df = 32$ ,  $P < 0.001$ ).

During winter at least 50% pups had at least 10% monocytes, whereas at least 50% yearlings in spring had none at all, which represent animals just a few months older (Tab. 1). The medians of the neutrophils [%] also varied extremely between yearlings caught in spring (55%) and older animals in summer (36%; Tab. 1).

Similarly, hemograms of the age-classes of free-ranging seals differed significantly in all three seasons (spring:  $\chi^2 = 72.28$ ,  $df = 32$ ,  $P < 0.001$ ; summer:  $\chi^2 = 84.32$ ,  $df = 32$ ,  $P < 0.001$ ; winter:  $\chi^2 = 61.42$ ,  $df = 32$ ,  $P < 0.001$ ). Within the group of older animals, for example, the neutrophil median ranged from 36% (summer) to 52% (spring; Tab. 1).

##### 3.3.1.2 Rehabilitated harbor seal pups

Overall, results of rehabilitated pups were similar to those of free-ranging animals. Although with a median of 70% neutrophils of rehabilitated pups prior to their release (rehab\_pups\_last, Tab. 1), the proportion was greater than that of free-ranging animals, and almost twice as much as that of free-ranging animals of about the same age (pups caught in the summer; w\_pups\_su, Tab. 1). Eosinophils in the rehabilitated pups were smallest, with only a median of 1%.

##### 3.3.1.3 Captive harbor seals

Results of captive animals were more variable than expected. Within this group only hemograms among the three adult females did not differ ( $\chi^2 = 24.10$ ,  $df = 32$ ,  $P = 0.84$ ).

Significant differences were found between the two pups (*Hinnerk*, *Lilli*) and the juvenile (*Mareike*) ( $\chi^2 = 51.48$ ,  $df = 32$ ,  $P = 0.02$ ) and also between the two adult males *Lümmel* and *Hein* ( $\chi^2 = 58.2$ ,  $df = 32$ ,  $P = 0.003$ ). The WBC not only differed considerably within the captive group, but also from the free-ranging animals and rehabilitated pups. The percentages of the neutrophils were greater than those of the other two groups (rehabilitated pups, free-ranging seals), whereas the eosinophils were intermediate. Within the captive group the animal *Mareike* showed the most and the largest maxima.

### 3.3.2 Red hemogram (Erythrocytes and their derivatives)

Red blood cells and related blood values appeared more homogeneous within groups (Tab. 2). Noteworthy differences could merely be found within the group of rehabilitated pups. Hemoglobin of rehabilitated pups was less than that of the other groups. Accordingly, MCH, MCHC, and MCV were lower than values of older animals. The red cell distribution width, however, was the same as in the free-ranging and captive animals. Another protruding value was that of the platelets. With at least 50% of the rehabilitated pups having  $\geq 583$  [g/l] platelets, they had considerably larger values than the free-ranging animals and most of the captive seals.

Table 1: Differential hemogram of **white blood cells** (WBC) and their derivatives of free-ranging harbor seals (w) divided into age groups (pups, yearlings, older) and catching seasons (spr, su, win), the pre-release blood withdrawal of rehabilitated seal pups (rehab\_pups\_last) and captive animals of the Seal Center Friedrichskoog. The first number indicates the median with the count as index, the numbers within the parentheses depict the percentile range (5-95%). For the free-ranging animals and the pups the sample size reflects individuals, whereas for the captive animals it is the number of hemograms investigated. In case of sample sizes <10 min. and max., rather than percentiles, are depicted.

	WBC [g/l]	Lym [#]	Lym [%]	Mono [#]	Mono [%]	Neutro [#]	Neutro [%]	Eos [%]
w_pups_su	8.6 <sub>20</sub> (4.7-12.4)	2.3 <sub>10</sub> (1.3-3.0)	39 <sub>22</sub> (21-50)	0.2 <sub>10</sub> (0.1-0.2)	3 <sub>22</sub> (0-7)	5.6 <sub>10</sub> (3.3-6.8)	37 <sub>10</sub> (25-54)	17 <sub>22</sub> (10-31)
w_pups_win	9.1 <sub>12</sub> (6.8-12.5)	1.0 <sub>10</sub> (0.5-2.5)	20 <sub>12</sub> (6.5-25)	0.3 <sub>10</sub> (0.1-0.5)	10 <sub>12</sub> (0-19)	7.0 <sub>10</sub> (4.0-11.3)	51 <sub>10</sub> (34-60)	18 <sub>12</sub> (13-32)
w_yearlings_spr	10.5 <sub>48</sub> (8.0-15.0)	2.2 <sub>48</sub> (0.9-4.1)	28 <sub>28</sub> (6.5-43)	0.2 <sub>48</sub> (0.1-0.5)	0 <sub>28</sub> (0-4)	7.9 <sub>48</sub> (5.2-11.9)	55 <sub>28</sub> (35-68)	17 <sub>28</sub> (9-29)
w_yearlings_su	9.0 <sub>9</sub> (6.5-11.2)	2.6 <sub>3</sub> (2.6-3.0)	32.0 <sub>9</sub> (23-44)	0.1 <sub>3</sub> (0.1-0.1)	3 <sub>9</sub> (2-11)	5.4 <sub>3</sub> (5.4-5.9)	48 <sub>3</sub> (41-48)	17 <sub>9</sub> (9-19)
w_yearlings_win	9.3 <sub>13</sub> (6.3-13.1)	1.8 <sub>10</sub> (1.0-2.6)	26 <sub>13</sub> (11-44)	0.3 <sub>10</sub> (0.1-0.7)	1 <sub>13</sub> (0-12)	6.5 <sub>10</sub> (3.6-20.4)	52 <sub>10</sub> (32-80)	21 <sub>13</sub> (3-34)
w_older_spr	9.4 <sub>60</sub> (7.3-13.3)	2.7 <sub>60</sub> (1.1-4.7)	32 <sub>27</sub> (17-47)	0.2 <sub>60</sub> (0.1-0.7)	2 <sub>27</sub> (0-6)	6.2 <sub>60</sub> (4.8-10.0)	52 <sub>27</sub> (38-65)	14 <sub>27</sub> (5-25)
w_older_su	9.0 <sub>54</sub> (7.0-13.1)	2.7 <sub>40</sub> (1.6-4.1)	37 <sub>56</sub> (15-60)	0.2 <sub>40</sub> (0.1-0.4)	9 <sub>56</sub> (0-37)	5.8 <sub>40</sub> (3.8-6.9)	36 <sub>40</sub> (20-60)	18 <sub>56</sub> (7-30)
w_older_win	10.0 <sub>28</sub> (7.6-13.8)	1.7 <sub>24</sub> (1.0-2.5)	21 <sub>27</sub> (10-46)	0.4 <sub>24</sub> (0.1-0.9)	9 <sub>28</sub> (0-21)	7.8 <sub>24</sub> (5.2-10.8)	51 <sub>23</sub> (33-69)	17 <sub>27</sub> (9-32)
rehab_pups_last	8.9 <sub>127</sub> (6.1-12.3)	2.5 <sub>95</sub> (0.7-4.2)	26 <sub>71</sub> (8-43)	0.2 <sub>95</sub> (0.1-0.5)	3 <sub>71</sub> (0-11)	6.6 <sub>95</sub> (4.7-8.9)	70 <sub>71</sub> (53-86)	1 <sub>71</sub> (0-6)
Lümmel (male, adult)	11.3 <sub>22</sub> (7.2-17.9)	1.6 <sub>5</sub> (0.6-4.4)	19 <sub>20</sub> (12-26)	0.3 <sub>5</sub> (0.2-0.5)	3 <sub>20</sub> (0-9)	8.0 <sub>15</sub> (5.6-10.7)	75 <sub>20</sub> (63-82)	3 <sub>17</sub> (1-8)
Nei/Deern/Lütte (female, adult)	7.7 <sub>34</sub> (4.9-16.5)	0.6 <sub>7</sub> (0.4-1.9)	16 <sub>56</sub> (10-31)	0.2 <sub>7</sub> (0.1-0.2)	3 <sub>56</sub> (1-10)	6.3 <sub>6</sub> (3.3-7.6)	77 <sub>36</sub> (63-85)	4 <sub>32</sub> (1-10)
Hein (male, pup→adult)	12.1 <sub>20</sub> (8.4-15.7)	2.2 <sub>9</sub> (1.2-3.5)	24 <sub>18</sub> (12-38)	0.3 <sub>9</sub> (0.1-0.4)	3 <sub>18</sub> (1-11)	8.8 <sub>9</sub> (6.8-12.9)	69 <sub>18</sub> (52-82)	4 <sub>15</sub> (0-9)
Mareike (female, juvenile)	8.9 <sub>7</sub> (7.9-12.0)	2.5 <sub>7</sub> (1.0-3.6)	22 <sub>5</sub> (13-34)	0.3 <sub>7</sub> (0.1-0.3)	8 <sub>5</sub> (2-15)	7.0 <sub>7</sub> (5.7-8.4)	62 <sub>7</sub> (47-83)	8 <sub>5</sub> (4-15)
Lalli (female, pup)	8.3 <sub>5</sub> (5.6-9.4)	2.3 <sub>5</sub> (1.2-3.4)	16 <sub>5</sub> (10-18)	0.2 <sub>5</sub> (0.1-0.3)	12 <sub>5</sub> (6-17)	5.7 <sub>5</sub> (3.8-6.9)	70 <sub>5</sub> (68-70)	2 <sub>5</sub> (2-2)
Hinnerk (male, pup)	10.4 <sub>4</sub> (9.0-11.3)	2.3 <sub>4</sub> (1.6-3.2)	28 <sub>4</sub> (24-30)	0.3 <sub>4</sub> (0.2-0.5)	6 <sub>4</sub> (4-12)	7.1 <sub>4</sub> (6.8-8.9)	64 <sub>4</sub> (62-64)	2 <sub>4</sub> (0-4)

Table 2: Differential hemogram of red blood cells (RBC) and their derivatives of free-ranging harbor seals (w) divided into age groups (pups, yearlings, older) and catching seasons (spr, su, win), the pre-release blood withdrawal of rehabilitated seal pups (rehab\_pups\_last) and captive animals of the Seal Center Friedrichskoog. The first number indicates the median with the count as index, the numbers within the parentheses depict the percentile range (5-95%). For the free-ranging animals and the pups the sample size reflects individuals, whereas for the captive animals it is the number of hemograms investigated. In case of sample sizes <10 min. and max., rather than percentiles are depicted.

	RBC [x10 <sup>12</sup> ]	HB [g/dl]	HCT [%]	MCH [pg]	MCHC [g/dl]	MCV [fl/μm <sup>3</sup> ]	RDW [%]	PLT [g/l]
w_pups_su	4.8 <sub>11</sub> (4.0-5.6)	20.0 <sub>11</sub> (17.5-22)	58 <sub>11</sub> (45-62)	41.5 <sub>11</sub> (37.4-45.2)	34.0 <sub>11</sub> (32.0-38.7)	112 <sub>7</sub> (110-118)	16 <sub>7</sub> (15-17)	298 <sub>11</sub> (175-478)
w_pups_win	5.0 <sub>11</sub> (4.6-5.6)	20.5 <sub>11</sub> (18-22.5)	55 <sub>11</sub> (51-64)	40.4 <sub>11</sub> (37.0-44.4)	36.8 <sub>11</sub> (33.0-39.4)	109 <sub>11</sub> (106-115)	16 <sub>8</sub> (15-19)	380 <sub>11</sub> (276-433)
w_yearlings_spr	4.8 <sub>18</sub> (3.8-5.4)	19.0 <sub>18</sub> (14.5-21.5)	54 <sub>18</sub> (39-61)	39.9 <sub>18</sub> (34.4-43.5)	35.6 <sub>18</sub> (33.5-41.1)	110 <sub>18</sub> (101-116)	15 <sub>18</sub> (15-17)	380 <sub>18</sub> (265-495)
w_yearlings_su	4.6 <sub>8</sub> (3.9-5.9)	19.0 <sub>8</sub> (17-24)	53 <sub>8</sub> (46-69)	41.2 <sub>8</sub> (40.4-44.0)	35.8 <sub>8</sub> (34.2-37.9)	116 <sub>7</sub> (112-119)	16 <sub>7</sub> (15-16)	297 <sub>8</sub> (156-365)
w_yearlings_win	4.9 <sub>11</sub> (3.9-5.3)	20.5 <sub>11</sub> (16-22)	54 <sub>11</sub> (43-64)	41.0 <sub>11</sub> (39.0-44.7)	36.8 <sub>11</sub> (32.6-38.6)	112 <sub>11</sub> (107-119)	16 <sub>4</sub> (15-16)	326 <sub>11</sub> (195-535)
w_older_spr	4.7 <sub>11</sub> (4.3-5.3)	20.5 <sub>11</sub> (18-22)	53 <sub>11</sub> (48-59)	44.1 <sub>11</sub> (36.3-46.7)	38.1 <sub>11</sub> (32.4-41.4)	114 <sub>11</sub> (109-119)	15 <sub>11</sub> (14-16)	292 <sub>11</sub> (228-407)
w_older_su	4.3 <sub>11</sub> (3.7-4.7)	18.5 <sub>11</sub> (16.5-19.5)	50 <sub>11</sub> (43-55)	43.6 <sub>11</sub> (36.6-47.4)	38.0 <sub>11</sub> (33.8-39.2)	116 <sub>11</sub> (112-124)	16 <sub>11</sub> (14-18)	341 <sub>11</sub> (248-407)
w_older_win	4.7 <sub>19</sub> (4.3-5.4)	21.0 <sub>19</sub> (18-23)	56 <sub>19</sub> (47-65)	42.8 <sub>19</sub> (41.0-46.5)	37.3 <sub>19</sub> (32.4-38.9)	114 <sub>19</sub> (112-121)	15 <sub>17</sub> (15-16)	314 <sub>19</sub> (230-373)
rehab_pups_last	4.8 <sub>117</sub> (3.8-5.7)	16.5 <sub>117</sub> (13.5-21)	46 <sub>117</sub> (36-59)	36.0 <sub>117</sub> (28.4-39.8)	36.4 <sub>111</sub> (28.0-39.0)	98 <sub>111</sub> (82-112)	16 <sub>94</sub> (15-19)	583 <sub>114</sub> (316-743)
Lümmel (male, adult)	4.8 <sub>11</sub> (3.8-5.7)	20.0 <sub>11</sub> (17-22)	56 <sub>11</sub> (46-69)	40.5 <sub>11</sub> (38.0-48.6)	35.0 <sub>19</sub> (31.9-40.7)	118 <sub>18</sub> (107-123)	14 <sub>7</sub> (13-15)	329 <sub>11</sub> (155-499)
Nelly/Deerm/Lütke (female, adult)	5.1 <sub>14</sub> (3.7-5.9)	21.0 <sub>14</sub> (15.5-24.5)	59 <sub>14</sub> (45-68)	41.0 <sub>14</sub> (38.7-45.8)	35.0 <sub>11</sub> (32.0-39.7)	117 <sub>14</sub> (106-125)	14 <sub>7</sub> (13-15)	246 <sub>11</sub> (115-458)
Hein (male, pup→adult)	4.8 <sub>10</sub> (4.2-6.0)	19.0 <sub>10</sub> (16-21)	51 <sub>10</sub> (46-61)	39.0 <sub>10</sub> (30.0-48.6)	35.7 <sub>10</sub> (32.8-41.9)	108 <sub>10</sub> (93-117)	14 <sub>9</sub> (13-17)	370 <sub>10</sub> (148-582)
Mareike (female, juvenile)	5.2 <sub>7</sub> (4.0-5.6)	21.4 <sub>7</sub> (20.0-23.8)	59 <sub>7</sub> (47-60)	45.8 <sub>7</sub> (13.6-51.8)	39.1 <sub>7</sub> (36.5-44.4)	117 <sub>7</sub> (102-119)	15 <sub>7</sub> (14-22)	294 <sub>7</sub> (265-348)
Lilli (female, pup)	5.0 <sub>7</sub> (4.4-5.4)	21.0 <sub>7</sub> (19.5-24)	53 <sub>7</sub> (48-62)	46.6 <sub>7</sub> (43.9-49.1)	40.8 <sub>7</sub> (38.2-45.9)	114 <sub>7</sub> (102-117)	16 <sub>7</sub> (13-17)	387 <sub>7</sub> (173-481)
Hinnerk (male, pup)	4.7 <sub>7</sub> (4.0-5.1)	19.0 <sub>7</sub> (17.5-21)	48 <sub>7</sub> (42-53)	40.2 <sub>7</sub> (39.8-44.1)	39.8 <sub>7</sub> (39.4-42.4)	102 <sub>7</sub> (100-104)	15 <sub>7</sub> (14-21)	392 <sub>7</sub> (363-466)



## 3.4 Discussion

### 3.4.1 Statistics

In order to take the mean and standard deviation, as done in a lot of papers (e.g. Wickham 1989, Roletto 1993, Bishop *et al.* 1995, Morgan *et al.* 1998, Lander *et al.* 2003), the blood values must be normally distributed. However, experiences of the present study were, that they were absolutely random. Especially if the sample size is rather small, the SD is very sensitive to any changes. If the mean is accurate, which would mean the data are normally distributed, the median would be the same. In case of the hemograms the median is therefore superior to the mean and was preferred in this study. Especially the monocyte percentage values demonstrate where the danger lies if taking the mean and standard deviation. In some animals or groups the standard deviation would have been even higher than the mean – which would have given a negative value of the minimum of the range – or it lay just below. There were other values, which showed a similar phenomenon. Bossart *et al.* (2001) showed hematological values of Pacific harbor seals (*Phoca vitulina richardsi*) of rehabilitated weanlings of the Marine Mammal Center at Fort Cronkhite, Sausalito, California, and captive adults from Sea World, San Diego, California. The ranges were calculated either by taking the  $\pm 2$  standard deviation around the mean (MMC) or they derived by calculation of the 25 and 75 quartiles around the individual median (SeaWorld). The former represented 42 rehabilitated weaned pups. For the 25 and 75 quartiles the authors used 33 adult individuals of which 184 blood samples were taken. It is difficult to compare these data of two different statistical valuations. As shown in the present study the hemograms of the 8 captive individuals examined in the Rehabilitation Center Friedrichskoog could hardly be pooled. If the medians of the blood samples were put together, the statistics would have shown a completely different and probably deceptive picture.

### 3.4.2 Blood Values

Overall, differences among hemograms in all three investigated harbor seal groups were seen. For the first time, this thesis compares results of three different groups (free-ranging animals, rehabilitated pups and captive animals) with the same analytical methods. Statistical tests proved that it is of vital importance to distinguish between different groups, age and also seasons in order to develop proper baseline hemogram values. This observation was in

compliance with other studies of captive and free-ranging groups (McConnell & Vaughan 1983, Bossart *et al.* 2001, Lander *et al.* 2003, Trumble *et al.* 2006). Usually those hemogram values were depicted as means with the respective SD. In order to have baseline values, however, it seemed more appropriate to provide the median and interpercentile range, as is done for ranges of human blood values. Even after dividing the animals into different subgroups, the sample sizes were still large enough to provide informative values.

As shown in 3.3 (Results), the erythrocyte hemogram (Tab. 2) was more homogeneous than the leukocyte hemogram (Tab. 1). The latter is influenced more by physiological changes of the animals. Especially the life history of the free-ranging animals was unknown. Even though animals were immediately released if they appeared sick or too stressed, seals with minor injuries or infections could have been captured. The large number of animals, however, should alleviate this disadvantage.

### 3.4.3 White hemogram (Tab. 1)

#### *3.4.3.1 Free-ranging harbor seals*

Young free-ranging animals (Tab. 1; w\_pups\_su; about 3 months of age) had the highest proportion of lymphocytes (38%). After 3-4 months the immune system is fully developed. The antibodies imbibed with milk, might have been exhausted and the abundance of pathogens the pups were confronted with, stimulated the immune reaction. An increase in lymphocytes observed in dogs (2 months of age) probably results from an immune response due to numerous new antigens after leaving the litter box (*pers. comm.* R. Mischke, Veterinary School, Hannover, Germany). Interestingly, lymphocytes of all three age groups of seals peaked during the summer. These results corroborated a study by Fonfara *et al.* (*pers. comm.*), who found that cytokines of the specific immune response in the same study group of seals was also elevated during the summer (Lehnert *et al.* submitted, Siebert *et al.* submitted).

Medians of monocytes did not seem to vary much within or among groups. However, differences in the inter-percentile ranges were variable, especially for free-ranging adult animals caught during summer (w\_older\_su; 0-36%). Considerable variations, especially of monocytes, seem to be common in manually prepared blood smears (Stiene-Martin 1980; Bishop and Morado 1995). The high value might also result from different stress answers of the animals or of indiscernible infections.

Free-ranging animals of about 1 ½ years (Tab. 1; w\_yearlings\_win) had the largest proportion of eosinophils. These cells are mostly responsible for parasitic defense. It seemed most likely that the animals of this age group had the highest infestation with parasites. This was confirmed by pathological findings during dissections performed at the Research and Technology Center Westcoast, Germany (Lehnert *et al.* submitted, Siebert *et al.* submitted).

#### 3.4.3.2 Rehabilitated harbor seal pups

Unlike other studies (e.g. McConnell and Vaughan 1983), the WBC counts of rehabilitated pups (9.0g/l) were less than or similar to those of the young free-ranging animals (9.5g/l). This discrepancy may have resulted from McConnell and Vaughan (1983) averaging all blood samples collected during the rehabilitation. In this study only pre-release values were taken into consideration for comparison. When pups were first admitted, their immune system was impaired as indicated by little or no IgG (Hasselmeier *et al.* in prep.) and a lower WBC count (7.6g/l). The results possibly support the conclusion of McConnell and Vaughan (1983) that rehabilitated pups are more susceptible to infections than free-ranging pups. *In vitro* stimulated immune cells of blood samples from admitted pups showed, however, that they are capable of starting a defense mechanism (Kakuschke *et al.* 2005). An explanation for differences in the recovering development during rehabilitation might be found in Beckmann *et al.* (2003), who proved that northern fur seal pups (*Callorhinus ursinus*) of older dams had a more capable immune response than those of young dams. Due to lack of knowledge about the life history of the mothers this problem currently cannot be solved.

Even though the percentage of neutrophils seemed high, the absolute figures showed that the pre-release blood values represented healthy animals.

High eosinophil values were not expected for rehabilitated pups because they were fed inspected and frozen fish, and therefore should not have had any contact with parasites. Occasional necropsies of pups that died of various infections during rehabilitation indicated no infection of parasites. Additionally, water from Friedrichskoog Harbor was cleaned mechanically (different sizes of pebbles) and chemically (ozone) before it was released into the pools.

### 3.4.3.3 Captive harbor seals

Unexpectedly, the variability within the group of captive seals was very high. Only the blood values of the three adult females could be pooled. The adult males *Lümmel* and *Hein* could neither be pooled with the adult females, nor with each other. During the years both males showed infectious signs of high WBC once in a while, which might have biased the median value and therefore made them not suitable for pooling. Though of same age, the two pups born in the center (*Hinnerk* and *Lilli*) couldn't be pooled either. Their sample size is yet quite small, however. For profound baseline values more samples have to be taken from the young animals.

Like the rehabilitated pups, all captive animals had a very low proportion of eosinophils. As they were fed the same inspected and frozen fish and the water in the pool was prepared the same way as it was for the rehabilitation pools, they also shouldn't have had contact with parasites. The relatively high percentage of eosinophils in *Mareike* cannot easily be explained; she came into the Seal Center as a weaned pup of a few months of age, and therefore might have had contact with parasites. As she was treated with anthelmintics and fed the same inspected fish as the other animals, the presence of eosinophilie cannot be explained by parasites. The lifespan of an eosinophilic granulocyte is appr. 10 days, but the retention period in the peripheral blood is only 10 hours (Messow and Hermanns 1990, Begemann 1998, Mahlberg *et al.* 2004). MELISA-tests have shown a significant allergization to molybdenum, however (*pers. comm.* Kakuschke). As the rest of the white hemogram did not show any signs of infection, this might be the first indication of an allergic reaction in harbor seals. More samples should be collected to further investigate this phenomenon.

#### 3.4.4 Red hemogram (Tab. 2)

There was only little variability in the red hemogram. This applied for the blood values within as well as among the three different groups.

##### *3.4.4.1 Free-ranging harbor seals*

McConnell & Vaughan (1983) and Lander *et al.* (2003) found the greatest differences between free-ranging and captive seals in the RBC and HB values. This could not be entirely corroborated with this study.

##### *3.4.4.2 Rehabilitated Pups*

The HB and MCV of the rehabilitated pups were considerably lower than in the other groups. The mean corpuscular volume of harbor seals is greater than in terrestrial animals or man (Knickel *et al.* 2002; Braun and Dormann 2003), but less than compared to other phocids, such as elephant seals (*Mirounga angustirostris*, *M. leonina*) (Wickham *et al.* 1989, Dierauf and Gulland 2001, Boily *et al.* 2006). The larger size of the RBCs in free-ranging and captive animals is most likely an adaptation to diving and more exercise (Wickham *et al.* 1989, Morgan *et al.* 1998, Dierauf and Gulland 2001, Lander *et al.* 2003).

When admitted, the median levels of the RBC (6.1 T/l) and HB (21.1 g/dl) were considerably higher than prior to their release. This phenomenon was also described in other studies (Bossart *et al.* 2001, Lander *et al.* 2003). The pups' lower levels of HB and MCV were most likely due to lack of activity during their rehabilitation.

The exceedingly high median and range of the thrombocytes can not easily be explained without looking at chemical factors of the coagulation factors. The phenomenon was also observed in other rehabilitated young pinnipeds (Bossart *et al.* 2001).

##### *3.4.4.3 Captive Harbor Seals*

Interestingly, the captive animals at the Seal Center had about the same hemoglobin levels as the free-ranging animals. The comparable levels of these two groups were very impressive and possibly indicate that the animals held in captivity at the Seal Center

Friedrichskoog had ample exercise to create a high MCV and HB. As the erythrocytes and hemoglobin are also dependent on exercise and diet, the supplementation with vitamins seems sufficient at the Seal Center Friedrichskoog (Geraci 1994; Trumble and Castellini 2002, Trumble *et al.* 2006).

#### 3.4.4.4 Comparison of rehabilitated and free-ranging pups (Tab. 3, Fig. 4)

The comparison of the median values of the rehabilitated pups with the free-ranging pups showed striking equalities (Tab. 3, Fig. 4). In some cases they were even identical (RBC, RDW, Monos). Both groups were about the same age (~ 3 months). Only the eosinophils, neutrophils and the hematocrit showed significant differences (quasi-Poisson;  $p < 0.05$ ). Other studies showed higher RBC in free-ranging animals (Dierauf and Gulland 2001, Lander *et al.* 2003). The analogy of the blood values spoke for the fitness of the rehabilitated pups, which again falls back to the quality of the rehabilitation procedures at the Seal Center Friedrichskoog. The insignificantly higher values of HB, MCH and MCHC only emphasize this. The elevated HCT in the free-ranging seal pups can be explained by the stress they were exposed to during the catch (Castellini *et al.* 1996). The much higher proportion of the eosinophils was also unsurprising as the rehabilitated seals were not exposed to parasites. The significance of the higher MCV in the wild animals is not as powerful as would have been expected. Especially in the last weeks the rehabilitated pups enjoyed a deeper and bigger pool to get enough exercise in preparation of their release.

Table 3: Erythrocyte and leukocyte comparison between rehabilitated (rehab\_pups\_last) and free-ranging (w\_pup\_su) pups of about 3 months of age. The first number indicates the median with the count as index; the numbers within the parentheses depict the percentile range (5-95%).

	<b>WBC [g/l]</b>	<b>Lym [#]</b>	<b>Lym [%]</b>	<b>Eos [%]</b>
w_pups_su	8.6 <sub>20</sub> (6.5-13.5)	2.3 <sub>5</sub> (1.4-3.0)	39 <sub>17</sub> (21-50)	17 <sub>22</sub> (10-31)
rehab_pups_last	8.9 <sub>127</sub> (6.1-12.3)	2.5 <sub>95</sub> (0.7-4.2)	26 <sub>71</sub> (8-43)	1 <sub>71</sub> (0-6)
	<b>Mono [#]</b>	<b>Mono [%]</b>	<b>Neutro [#]</b>	<b>Neutro [%]</b>
w_pups_su	0.2 <sub>5</sub> (0.1-0.2)	3 <sub>17</sub> (0-7)	5.6 <sub>5</sub> (3.7-6.6)	37 <sub>10</sub> (25-52)
rehab_pups_last	0.2 <sub>95</sub> (0.1-0.5)	3 <sub>71</sub> (0-11)	6.6 <sub>95</sub> (4.7-8.9)	70 <sub>71</sub> (53-86)

	<b>RBC [t/l]</b>	<b>HB [g/dl]</b>	<b>HCT [%]</b>	<b>PLT [g/l]</b>
w_pups_su	4.8 <sub>15</sub> (4.0-5.6)	20.0 <sub>15</sub> (17.5-22)	58 <sub>15</sub> (45-62)	298 <sub>15</sub> (175-478)
rehab_pups_last	4.8 <sub>127</sub> (3.8-5.7)	16.5 <sub>127</sub> (13.5-21)	46 <sub>127</sub> (36-59)	583 <sub>124</sub> (316-743)
	<b>MCH [pg]</b>	<b>MCHC [g/dl]</b>	<b>MCV [fl/μm<sup>3</sup>]</b>	<b>RDW [%]</b>
w_pups_su	41.5 <sub>15</sub> (37.4-45.2)	34.0 <sub>15</sub> (32.0-38.7)	11 <sub>27</sub> (110-118)	16 <sub>5</sub> (15-17)
rehab_pups_last	36.0 <sub>127</sub> (28.4-39.8)	36.4 <sub>126</sub> (28.0-39.0)	99 <sub>116</sub> (82-112)	16 <sub>94</sub> (15-19)

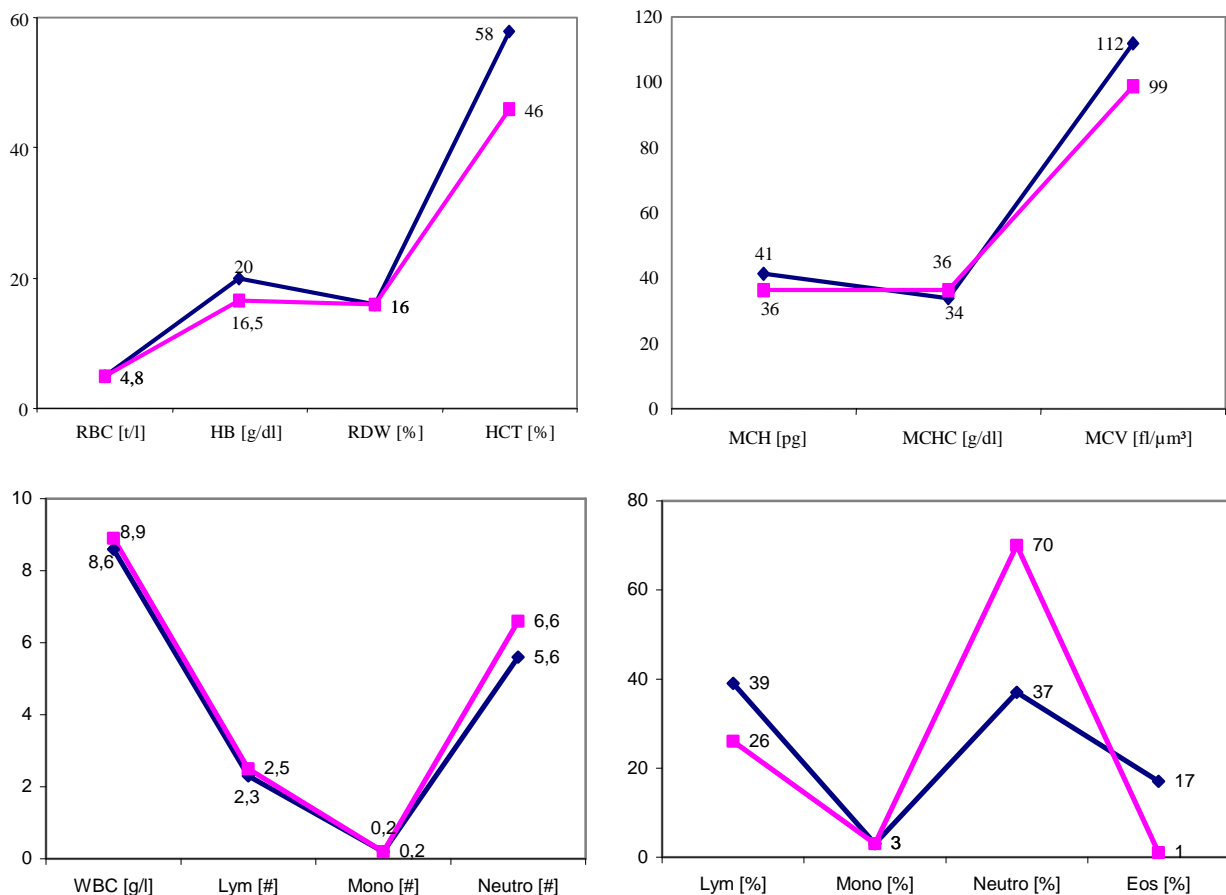


Fig. 4: Display of the median of the white and red hemogram of free-ranging (—) and rehabilitated (—) pups of about 3 months of age.

### 3.4.5 A different approach – Flow cytometer (Tab. 4)

In 2003/2004, some of the differential hemograms (i.e. the sub-groups of leukocytes) were analyzed with a FACS Calibur<sup>®</sup> flow cytometer. The advantage of the flow cytometer to the manually read differential hemogram from a blood smear is the number of cells it can count in a very short time. In a manually read differential hemogram usually 100 cells are counted, which takes about – according to experience – 3-5 minutes. The flow cytometer was set to read 10,000 lymphocytes plus all other leukocytes (granulocytes, eosinophils and monocytes), which usually took less than a minute. The high number of cells also made the results a lot more accurate. According to the time it took the flow cytometer to read the pre-set number of lymphocyte it was also possible to draw conclusions to the total number of WBC the animal had. Another bias of the manual counts lies in different people/labs reading the blood smear (experience, microscope, cell dye).

The disadvantage, however, was that if the dot plots could not be differentiated accurately, it was hard to set the gates to get accurate results. Therefore those readings had to be treated separately. The dot plots showed partly immense discrepancies between the blood smear and the flow cytometer (Tab. 4). The number of animals tested with the flow cytometer is still very small to really compare the values with the blood smears. So far, only adult free-ranging animals caught in summer and winter could be depicted. First results encourage further investigations.

Table 4: Proportion of lymphocytes, monocytes, neutrophil and eosinophil granulocytes counted by blood smear and flow cytometer of adult free-ranging harbor seals (w\_older\_x) divided into two catching seasons (winter = win, summer = su). The first number indicates the median with the count as index; the numbers within the parentheses depict the percentile range (5-95%). In case of sample sizes <10 min. and max., rather than percentiles, are depicted.

	<b>Lym [%]</b>	<b>Mono [%]</b>	<b>Neutro [%]</b>	<b>Eos [%]</b>
w_older_su (smear)	35.5 <sub>56</sub> (15-60)	8.0 <sub>56</sub> (0-37)	33 <sub>40</sub> (20-60)	16 <sub>56</sub> (7-30)
w_older_su (Flowcyt.)	45 <sub>24</sub> (29-54)	12 <sub>24</sub> (10-17)	25 <sub>24</sub> (20-40)	17 <sub>24</sub> (10- 29)
w_older_win (smear)	19 <sub>27</sub> (10-46)	1 <sub>28</sub> (0-21)	46 <sub>23</sub> (33-69)	18 <sub>27</sub> (9-32)
w_older_win (Flowcyt.)	42 <sub>8</sub> (19-56)	12 <sub>8</sub> (7-23)	29 <sub>8</sub> (24-42)	8 <sub>8</sub> (7-9)



### 3.4.6 Yet another approach

The manual blood count of the leukocyte derivatives of the seal catches in April 2005 were dyed and counted at the *Tierärztliche Hochschule* in Hanover, Germany. The samples were stained with *Pappenheim* and counted at a magnification of 100x. *Pappenheim* is a combination of May-Grünwald and Giemsa staining. May-Grünwald consists of eosin, methylene blue, mythelene azur, and mythelene violet. With the *Pappenheim* staining, the cells were better distinguishable and basophils that were hardly recognizable at all in the *Diff Quick* could be seen clearly; another argument for the importance of the verification of the comparability of the results. The more accurate results with the *Pappenheim* were the decisive factor for the Research and Technology Center to switch to that technique in the future.

It was the first time that this many hemogram values have been put together and analyzed. Due to sample size, subdivisions were possible that have never been tested before. The study has shown that it is of vital importance that the animals were separated into different (sub)groups. It is substantial to verify if captive animals could be pooled as done in many analyses (e.g. McConnell and Vaughan 1983; Dierauf and Gulland 2001). Even though the captive animals tested are held in controlled conditions, individual differences made pooling only possible for three adult females.

Blood analyses with different methods (staining, flow cytometry) have shown significant differences and should be taken into consideration if comparing reference values.

This study is an important contribution in understanding and assessing the health status of harbor seals. The three different groups give a very good overview of hemograms of the population of harbor seals in the German North Sea but should be consulted for other populations as well. Further investigations and comparisons should be undertaken, as several of the subgroups are still under-represented. Continuous efforts are also necessary to maintain a stable monitoring and management program.

## Chapter 4

### **Phagocytosis of Peripheral Blood Granulocytes and Monocytes of Harbor Seals (*Phoca vitulina vitulina*) using Flow Cytometry**

#### **4.1 Introduction**

Phagocytosis is the most important and initial defense against many pathogens in mammals. In general, it is the ability of cells to ingest extracellular macromolecules. This can happen in different ways. In order to verify the efficiency of the immune system it is possible to examine the functionality of the phagocytes. The phagocytosis test enables a quantitative analysis of phagocytic function of neutrophils and monocytes in a blood sample using either immunofluorescence microscopy or flow cytometry. Either assay can measure the overall percentage of monocytes and neutrophils showing phagocytosis (ingestion of one or more bacteria per cell) and the individual cellular phagocytic activity (relative number of bacteria per cell). Acquired immunodeficiencies can result in increased susceptibility to infection, some being due to defective neutrophil phagocytosis.

Several studies have been published about the phagocytosis assay itself and its application in assessing human and domestic animal immune systems (Higgins 1992, Brosche and Platt 1995, Esparza *et al.* 1996, de Guise *et al.* 2000, Hoffmann-Jagielska *et al.* 2003, Kampen *et al.* 2004). Some work has also proposed the potential utility of phagocytosis in investigating marine mammal health (de Guise 1995, de Swart *et al.* 1996, Fournier 2000). In this study the Phagotest<sup>®</sup> (Orpegen Pharma, Heidelberg, Germany) has been applied for the first time on harbor seals. It was accomplished in order to test the applicability and the utility, both in a routine evaluation of the health status of harbor seals, and temporal evaluation of animal health during a diseased state.

An overview of the mechanism of the phagocytosis can be found in Appendix B of this thesis.

## 4.2 Material & Methods

A Phagotest® test kit produced by Orpegen Pharma (Czernyring 22, 69115 Heidelberg, Germany) for human medicine application was applied. The test kit contained stabilized and opsonized FITC-labeled *Escherichia coli* (*E. coli*) suspension, Quenching Solution, DNA Staining Solution, Lysing Solution, and Washing Solution. An opsonin is any molecule that acts as a binding enhancer for the process of phagocytosis. The analysis was performed based on statistical results provided by the supplied software CellQuestPro® (Becton Dickinson).

100µl of heparinized whole blood (Sarstedt Monovette, Sarstedt Aktiengesellschaft & Co., 51588 Nümbrecht, Germany) was necessary per sample tube (Falcon Tube, VWR International, Billwerder Ring 19, 21035 Hamburg). As a negative control was always utilized, two tubes (200 µl blood) per animal were required. In order to cool the blood down to 0°C, the tubes were stored on ice for 10 minutes. Afterwards, 20 µl of the pre-cooled bacteria (*E. coli*) were added to the phagocytic test tubes. After each adding of a reagent the tubes were mixed on a vortex mixer. Next, the control sample remained on ice, while the test tubes with the bacteria were incubated in a closed water bath at 37°C for 10 minutes. After the incubation the test tubes joined the controls in the ice bath for another 10 minutes in order to stop the phagocytosis. Now 100 µl of the pre-cooled Quenching Solution (Orpegen Pharma) was added to each tube. This would suppress the fluorescence of bacteria attached to the outside of the phagocytic cells. After mixing, the samples were immediately washed twice with 3 ml Washing Solution (Orpegen Pharma). In between the two washing processes, the cells were spun down in a centrifuge at 250 x g, 4°C, for 5 minutes, the supernatant was discarded. Subsequently, the whole blood was lysed and fixed with 2 ml warm (room temperature) Lysing Solution (Becton Dickinson). The samples were then stored in the dark at room temperature for 10 minutes. Afterwards, the cells were again spun down and the supernatant was discarded. A third washing process and afterwards centrifugation followed. Finally, 200 µl of DNA Staining Solution (Orpegen Pharma) was added to the cells. The Staining Solution facilitates discrimination of leukocytes during flow cytometry analysis. The cells were then incubated in a light-protected ice bath, and measured with the flow cytometer within 30 minutes.

CellQuestPro®:

The plots received by leukocytes and monocytes to be analyzed were identified by their incorporation of propidium iodide using CellQuest™ Pro software as follows:

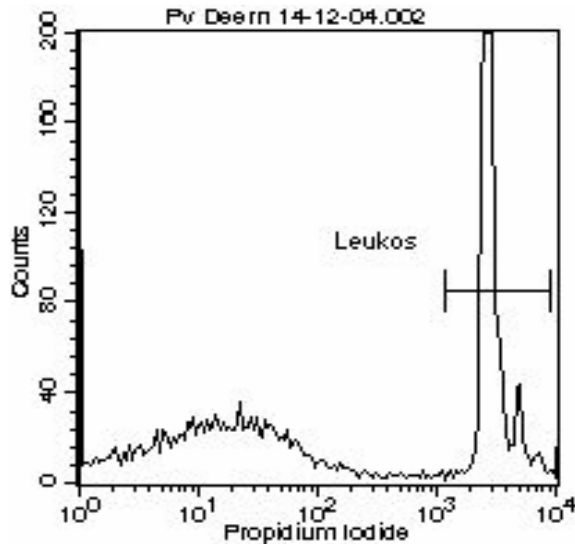


Figure 1: Example of a histogram of all cells detected by the laser. Leukocytes marked by bar.

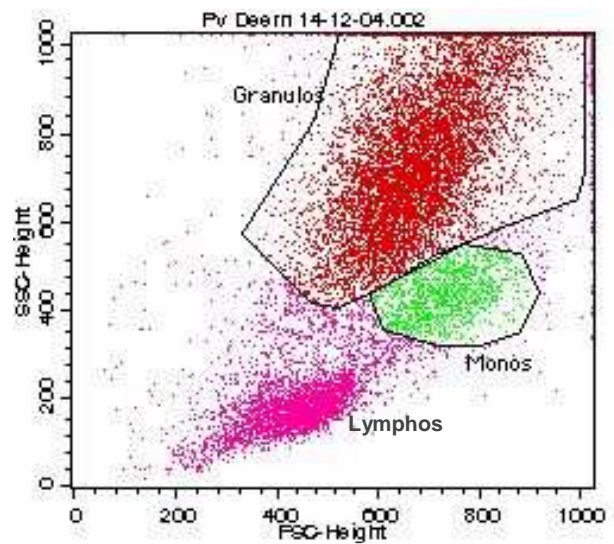


Figure 2: Example of a Dot Plot of all leukocytes.

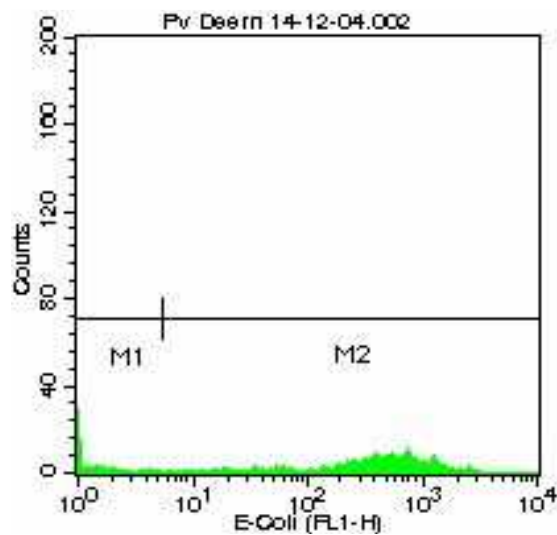


Figure 3: Example of fluorescent histogram of monocytes; M1 represents cells without phagocytosis; M2 represents cells which have phagocytized bacteria.

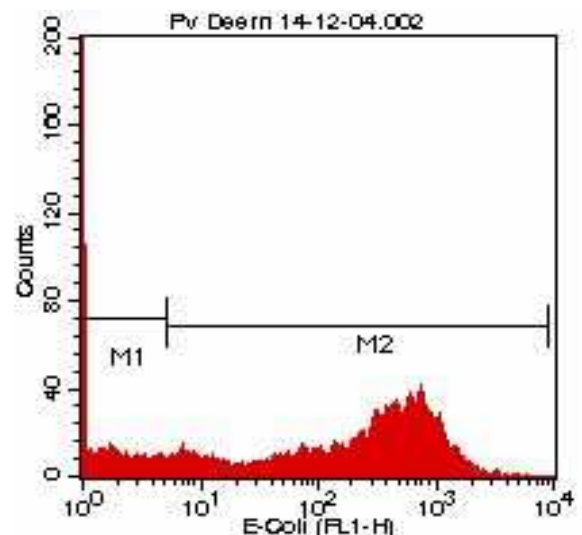


Figure 4: Example of fluorescent histogram of granulocytes; M1 represents cells without phagocytosis; M2 represents cells which have phagocytized bacteria.

Table 1: Example of statistics of Figures 3 and 4.

Sample ID: Pv Deern Tube: E-Coli Panel: Pv Phago Acquisition Date: 14-12-04 Gate: Monos					Sample ID: Pv Deern Tube: E-Coli Panel: Pv Phago Acquisition Date: 14-12-04 Gate: Granulos				
Marker	Events	%Gated	X-Mean	SD	Marker	Events	%Gated	X-Mean	SD
All	751	100	428.42	441.29	All	10793	100	541.29	707.01
M1	116	15.45	1.76	0,7	M1	1615	14.96	2.01	1.12
M2	635	84.55	506.36	548,8	M2	9179	85.05	631.80	700.21

The first (blank) histogram (Fig. 1) depicted all cells detected by the laser. The dot plot displayed all leukocytes (granulocytes, monocytes, and lymphocytes) according to size (FSC) and complexity (SSC) with granulocytes and monocytes gated (Fig. 2). The bar marked the leukocytes, which can be found in the other two histograms separated into granulocytes (red) and monocytes (green) (Fig. 3, 4). The bar M1 in the monocyte/granulocyte histograms represented no phagocytic activity. Bar M2 marked all phagocytizing granulocytes and monocytes. According to the marks set by the bars and gates, respective results are shown in Table 1 and could be analyzed. *Events* in this case represented single cells detected by the laser. The *Mean* described the average amount of fluorescence of the cells. *SD* in this case calculated the standard deviation of the mean fluorescence. The lower the *SD* the more uniform the cells behaved. A high *SD* indicated the cells phagocytized in a much more heterogenous fashion by demonstrating a broad range of fluorescence.

### 4.3 Results

The animals were divided into three groups: free-ranging (WF), captive, and pups subdivided into pups at their admission for rehabilitation (pup\_adm), and pups at pre-release (pup\_rel). It was also marked if animals showed any conspicuity in their differential hemograms (i.e. high numbers of WBC). WBC < 11g/l were considered normal (0), WBC > 12g/l were identified as abnormal (1).

#### 4.3.1 Free-ranging harbor seals (Tab. 2, 3; Fig. 5-7)

The tested free-ranging animals were all older than 2 years according to weight and length. The animals with normal hemograms differ considerably from seals with e.g. elevated leukocytes. The median of phagocytizing neutrophils in animals with regular differential hemograms was 81%, and the median of the mean fluorescence intensity had a maximum of 821, whereas in seals with elevated WBC it was only 69% and 370, respectively. Divided into different months, it could be seen that in the free-ranging animals the median SD was lower in August (SD = 295) and rising towards December (Sept.: 360; Dec.: 480). This conformed to the tested captive seals.

Results of monocyte phagocytosis were slightly different: there was no trend observed. In December, however, monocytes were more active than in other months, although the SD was also higher. Overall the granulocytes were more active than monocytes with the variation of latter cells increased.

#### 4.3.2 Rehabilitated harbor seal pups (Tab. 4, 5; Fig. 7, 8)

It is obvious that cells capable of phagocytosis decreased during rehabilitation. The median of the phagocytizing neutrophils of pups at their admittance to the seal center was 87%, prior to their release it was only 58%. Neither the mean fluorescence intensity (402 => 430) nor its standard deviation (566 => 537) of the neutrophils changed much during the three months of rehabilitation.

There is a striking concurrence with the activated monocytes. The median proportion of phagocytizing monocytes was 86% at admittance and 58% prior to their release. The median

of mean fluorescence intensity and standard deviation were also at a similar level as of the granulocytes (X\_Mean: 405 => 434; SD: 539 => 563).

#### 4.3.3 Captive Harbor Seals (Tab. 7, 8; Fig. 7-9)

It was quite obvious that the percentage of phagocytizing neutrophils of *Hinnerk* decreased considerably over a period of 7 months (15 Jan. 2004 - 13 Aug. 2004). In January 2004 the animal was, according to the differential hemogram, in good apparent health. In March and August, however, his hemogram had changed. The proportion of his phagocytizing granulocytes decreased from 79% to 24%. At the same time the percentage of monocytes phagocytizing E-coli bacteria increased slightly from 76% to 83%. There were fluctuations in the mean fluorescence intensity and its standard deviations but in general the monocytes showed a higher obliquity.

*Mareike*, *Lilli*, and *Hein* showed an opposite phenomenon compared to *Hinnerk*. In January the percentage of phagocytizing neutrophils was rather low but increased over the year (min. 51% => max. 95%). This was in accordance with their monocytes (min. 64% => max. 96%). Again, the X\_Mean and SD depicted high variability with no apparent trend. Monocytes were once again more variable.

*Lümmel* and *Lona*, which were only tested in August and December, had higher percentages of phagocytizing neutrophils and monocytes in December. X\_Mean and SD kept to their irregularity.

*Deern*, also tested only in August and December, had less phagocytizing neutrophils but more monocytes in December. Again, the mean fluorescence intensity and standard deviation displayed no trend.

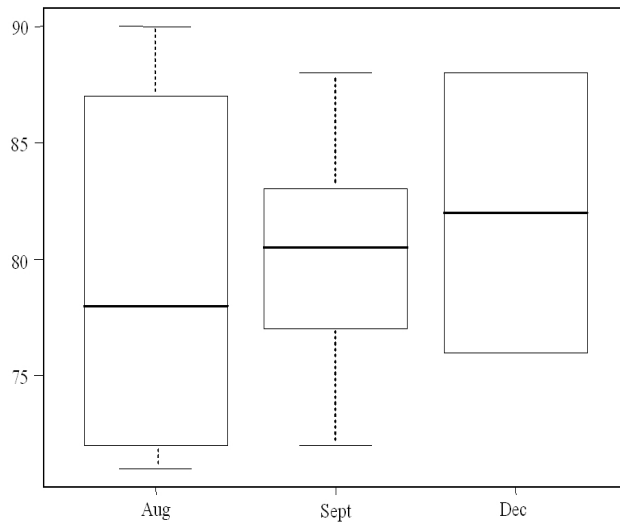


Figure 5: Comparison of percentage of activated neutrophils of free-ranging harbor seals in different months with median (—), 5-95% percentile (□), and range (I); **without** PDV infested animals.

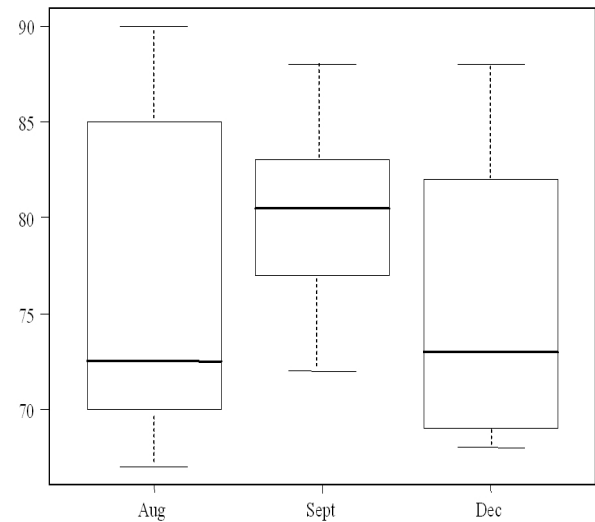


Figure 6: Comparison of percentage of activated neutrophils of free-ranging harbor seals in different months with median (—), 5-95% percentile (□), and range (I); **including** PDV infested animals.

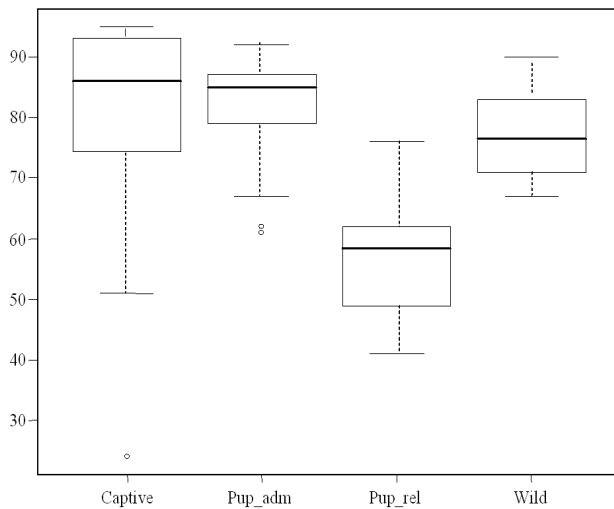


Figure 7: Comparison of percentage of activated neutrophils of all harbor seal groups, with median (—), 5-95% percentile (□), and range (I).

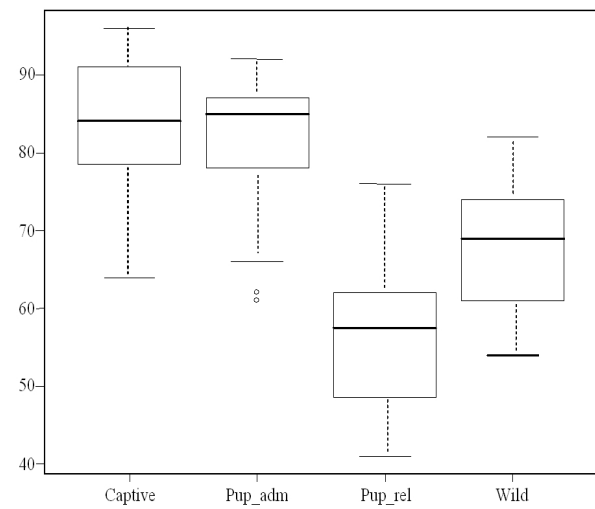


Figure 8: Comparison of percentage of activated monocytes of all harbor seal groups, with median (—), 5-95% percentile (□), and range (I).

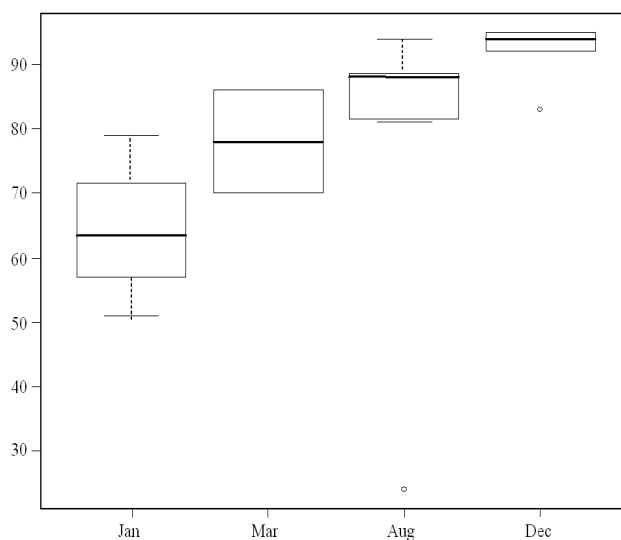


Figure 9: Comparison of percentage of activated neutrophils of captive animals on different blood withdrawals, with median (—), 5-95% percentile (□), and range (I).



Table 2-5: Data of Phagotest<sup>®</sup> of free-ranging seals, pups at admission, and pups prior to release. M2\_%Gated = percentage of phagocytizing **granulocytes** or **monocytes**; M2\_XMean = mean fluorescence intensity of activated cells; M2\_SD = standard deviation of the mean fluorescence of active cells; problem 0 = animal with normal hemogram; 1 = animal with altered differential hemogram (WBC > 12g/l); The first number indicates the median with the count as index; the numbers within the parentheses depict the percentile range (5-95 %). In case of sample sizes <5 the minimum and maximum, rather than percentiles, are depicted.

Table 2: Free-ranging harbor seal values differentiated by months (neutrophils)

group	date	M2_%Gated	M2_XMean	M2_SD	problem
WF	Aug/Dec	69 <sub>4</sub> (67-70)	307 <sub>4</sub> (279-370)	350 <sub>4</sub> (300-427)	1
WF	Aug	78 <sub>6</sub> (71-89)	281 <sub>6</sub> (244-323)	295 <sub>6</sub> (260-313)	0
WF	Sept	80 <sub>6</sub> (73-87)	362 <sub>6</sub> (241-440)	337 <sub>6</sub> (275-429)	0
WF	Dec	82 <sub>2</sub> (76-88)	669 <sub>2</sub> (518-821)	569 <sub>2</sub> (488-650)	0

Table 3: Free-ranging harbor seal values differentiated by months (monocytes)

group	date	M2_%Gated	M2_XMean	M2_SD	problem
WF	Aug/Dec	67 <sub>2</sub> (54-79)	452 <sub>2</sub> (344-498)	480 <sub>2</sub> (402-526)	1
WF	Aug	70 <sub>6</sub> (62-79)	560 <sub>6</sub> (406-663)	538 <sub>6</sub> (426-655)	0
WF	Sept	65 <sub>6</sub> (58-73)	462 <sub>6</sub> (293-703)	525 <sub>6</sub> (342-827)	0
WF	Dec	76 <sub>2</sub> (71-86)	740 <sub>2</sub> (588-892)	648 <sub>2</sub> (535-761)	0

Table 4: Pups at admission and prior to release (neutrophils)

group	M2_%Gated	M2_XMean	M2_SD	problem
pup_adm	65 <sub>4</sub> (61-70)	433 <sub>4</sub> (367-477)	643 <sub>4</sub> (537-986)	1
pup_adm	87 <sub>14</sub> (82-92)	402 <sub>14</sub> (254-624)	524 <sub>14</sub> (387-736)	0
pup_rel	58 <sub>12</sub> (45-72)	430 <sub>12</sub> (308-604)	537 <sub>12</sub> (373-660)	0

Table 5: Pups at admission and prior to release (monocytes)

group	M2_%Gated	M2_XMean	M2_SD	problem
pup_adm	64 <sub>4</sub> (61-70)	437 <sub>4</sub> (372-714)	678 <sub>4</sub> (564-1012)	1
pup_adm	85 <sub>14</sub> (82-92)	405 <sub>14</sub> (254-628)	590 <sub>14</sub> (287-769)	0
pup_rel	58 <sub>12</sub> (44-72)	434 <sub>12</sub> (309-607)	563 <sub>12</sub> (374-694)	0

Table 6: Data of different Phagotest<sup>®</sup>-results of captive seals. M2\_%Gated = percentage of phagocytizing **neutrophils**; M2\_XMean = mean fluorescence intensity of activated cells; M2\_SD = standard deviation of the mean fluorescence of active cells; problem 0 = animal with normal hemogram; 1 = animal with altered differential hemogram (WBC > 12g/l).

group	animal	date	M2_%Gated	M2_XMean	M2_SD	problem
captive	Hinnerk	15-1-04	79	529	436	0
captive	Hinnerk	23-3-04	71	631	566	1
captive	Hinnerk	13-8-04	24	487	558	1
captive	Mareike	15-1-04	63	461	504	1
captive	Mareike	23-3-04	86	830	716	0
captive	Mareike	20-8-04	94	550	409	0
captive	Mareike	14-12-04	95	767	668	0
captive	Lilli	15-1-04	51	422	423	0
captive	Lilli	20-8-04	89	411	351	0
captive	Lilli	14-12-04	94	758	672	0
captive	Hein	15-1-04	70	512	509	1
captive	Hein	20-8-04	81	357	342	0
captive	Hein	14-12-04	92	696	644	0
captive	Lümmel	20-8-04	82	380	384	0
captive	Lümmel	14-12-04	95	615	556	0
captive	Lona	20-8-04	88	427	360	0
captive	Lona	14-12-04	95	823	665	0
captive	Deern	20-8-04	89	487	369	0
captive	Deern	14-12-04	83	440	424	0

Table 7: Data of different Phagotest<sup>®</sup>-results of captive seals. M2\_%Gated = percentage of phagocytizing **monocytes**; M2\_XMean = mean fluorescence intensity of activated cells; M2\_SD = standard deviation of the mean fluorescence of active cells; problem 0 = animal with normal hemogram; 1 = animal with altered differential hemogram (WBC > 12g/l).

group	animal	date	M2_%Gated	M2_XMean	M2_SD	problem
captive	Hinnerk	15-1-04	76	766	659	0
captive	Hinnerk	23-3-04	81	807	709	1
captive	Hinnerk	13-8-04	83	676	554	1
captive	Mareike	15-1-04	65	533	558	1
captive	Mareike	23-3-04	82	1216	811	0
captive	Mareike	20-8-04	91	691	496	0
captive	Mareike	14-12-04	93	827	562	0
captive	Lilli	15-1-04	65	485	475	0
captive	Lilli	20-8-04	87	496	418	0
captive	Lilli	14-12-04	91	766	590	0
captive	Hein	15-1-04	64	413	397	1
captive	Hein	20-8-04	83	823	711	0
captive	Hein	14-12-04	96	860	563	0
captive	Lümmel	20-8-04	72	522	503	0
captive	Lümmel	14-12-04	96	672	491	0
captive	Lona	20-8-04	84	637	503	0
captive	Lona	14-12-04	95	745	590	0
captive	Deern	20-8-04	85	871	597	0
captive	Deern	14-12-04	89	558	447	0

## 4.4 Discussion

The results of the phagocytosis test demonstrated differences (Fig. 7, 8) between the three harbor seal groups investigated in this study. It was the first time the Phagotest<sup>®</sup> was employed in pinnipedia.

### 4.4.1 Free-Ranging Harbor Seals

The results demonstrated the applicability of the Phagotest<sup>®</sup> to free-ranging harbor seals. There is an indication that animals with elevated WBC showed less phagocytic activity than seals with normal hemograms (quasi-Poisson;  $p = 0.05$ ; Tab. 2, 3). For secure verification the sample size is yet too small. It indicates a reduced response of the innate immune system, which is supported by previous publications (de Swart *et al.* 1996, de Guise *et al.* 2000, Tabuchi *et al.* 2005).

Seasonal changes could also be observed. The phagocytic capacity of leukocytes increased from August to December (Fig. 5) Not only had the proportion of phagocytizing neutrophils increased, the X\_Mean (relative number of bacteria ingested/cell) increased and the SD values also doubled in December (Tab. 2; median of Aug. - Dec.: %Gated = 78% - 82%, X\_Mean = 281 - 669, SD = 295 - 569). Some granulocytes seem to be very active whereas others only slightly take part in the process. The percentage of phagocytizing monocytes increased almost the same as neutrophils, but differences within this cell group were less distinct (Tab. 3; median of Aug. - Dec.: %Gated = 70 - 76, X\_Mean = 560 - 740, SD = 538 - 648). Neutrophils therefore seem to react more dominant to seasonal changes than monocytes. It would be interesting to see the phagocytic activity of free-ranging animals in spring. Unfortunately, no tests were available for the seal catches in March and April.

De Swart *et al.* (1996) also found seasonal changes characterized by lower NK-cell activity during winter. Animals exposed to seasonal variations may also express differences in their capability of defense mechanisms without obvious signs (e.g. elevated WBC). Seasonal changes in diet may also have an effect on the phagocytic activity (de Swart *et al.* 1996, de Guise *et al.* 1998, Levin *et al.* 2005).

#### 4.4.2 Rehabilitated harbor seal pups

Results of rehabilitated harbor seal pups were unexpected. At admission it was evident that neutrophils of pups with elevated hemogram values (WBC > 12 g/l) had less phagocytic activity than those of pups with normal hemograms (quasi-Poisson,  $p = 0.02$ ). The innate immune response was obviously very active at admission. This was also observed by Kakuschke (2006) and Ross *et al.* (1994). In the same animals Kakuschke (2006) found that, even without stimulation, the lymphocyte proliferation was higher than expected. The stress the pups were exposed to coming into the rehabilitation center was doubtlessly one elicitor of the high phagocytosis reaction. In addition, some of the animals had infected umbilicus, were dehydrated and/or had diarrhea without showing any increase of WBC. The results of this study illustrated an apparently efficient phagocytic capability of neutrophils and monocytes of new-born pups shortly after birth and the capability of answering challenges by minor bacterial infections. Lalancette *et al.* (2003) studied lymphocyte transformation and phagocytosis in developing gray seal pups. They found an increase of the immune response and concluded the immune system was not completely developed in the first few weeks of life. The animals studied were in a post-weaning stage, however, i.e. had the full advantage of their mother's milk. Unfortunately, no free-ranging pups could be tested for this study.

Prior to release the 2 ½ - 3 months old rehabilitated pups showed a considerable decrease of neutrophil phagocytic activity (Fig. 7; Tab. 4: median = 58%  $\pm$ 9.8). None of the animals had any visible infections and had a good body condition. None of the rehabilitated pups had increased WBC. Nevertheless, the phagocytic response to E-coli was significantly reduced (Fig. 7). During rehabilitation pups are held in small, relatively shallow pools with up to 6 animals. They are fed surrogate milk, then mashed herring, pieces of herring and finally whole fish. After they have attained a certain weight ( $\geq 12$ kg) and went through another hemogram check, they are maintained in a bigger, deeper pool with a counter current flow and, to a certain extent, imitation of tides. In this pre-release-pool pups are intermittently fed live fish. These changes in environment and feeding regime might influence phagocytosis on an individual basis. However, it is unlikely that all animals would show an equal decrease as found in this study. Kakuschke (2006) identified a significantly higher haptoglobin (Hp) value in pups prior to release than when admitted. One of the functions of this acute phase protein is the binding of free hemoglobin (Hb). In human medicine a high level of Hb a few days after birth is known, which decreases in the following months (Speer 2000). When handling the serum samples of the admitted pups mostly a yellow coloration was evident. This again

suggested a high bilirubin value, a degradation product of Hb. Compared to adults, newborn have higher Hb concentrations and RBC with shorter life-spans of the erythrocytes. Latter causes the increase of Hb concentration and at the same time a need for Hp (Bergmann 1998, Andersson and Sevelius 2001, Mahlberg *et al.* 2004). Hp, however, is known to inhibit phagocytosis and dampen the acute inflammatory response (Rossbacher *et al.* 1999). The higher Hp value of pups in their pre-release blood samples found by Kakuschke could be one reason why the phagocytic activity was reduced. It might also denote a development of the immunocompetence.

Monocytes were in full conformity with the granulocytes. Phagocytosis of the newly admitted pups was high, whereas the monocyte activity decreased during the rehabilitation period (Fig. 8; Tab. 5).

#### 4.4.3 Captive harbor seals

In Table 6 the decrease of phagocytizing granulocytes in *Hinnerk* is very conspicuous. *Hinnerk* was born in the Rehabilitation Center of Friedrichskoog early June 2003. In August 2004 an infestation of *Clostridium perfringens* was diagnosed in all animals by anal swabs. On 20 March *Hinnerk* showed first signs of elevated WBC, but not beyond normal range (< 12g/l). The differential hemogram displayed an increase in neutrophil granulocytes and monocytes, which had subsided again in June. On 13 August 2004, one day before his death, the differential hemogram depicted very low values for leukocytes (WBC = 2.3g/l, lymphocytes = 0.7g/l, monocytes = 0.1g/l, neutrophils = 1.6g/l).

Table 7 demonstrates the contrasting reaction of the monocytes. The phagocytic reaction increased from January to August. The cells themselves, however, responded in the same way as neutrophils. At the beginning of the infection some of the monocytes exhibited high phagocytic activity (X\_Mean = 807), whereas others were rather inconspicuous (SD = 709). In the same blood sample Kakuschke (2006) found a high level of non-stimulated lymphocyte proliferation, which also indicates a cellular immune reaction. One day before his death monocytic activity had again slightly increased, but the phagocytosis of the single cells had decreased. An incompetence of the immune system became evident.

Other than flatulence neither he nor the other animals showed any symptoms of disease. Nevertheless, *Hinnerk* died on 14 August 2004 of triple intestine invagination, one day after the last phagocytosis test. The results of the Phagotests<sup>®</sup> depict very well the course of the disease.

In the animals *Mareike*, *Lilli*, and *Hein* the phagocytic activity coincides with free-ranging harbor seals. In January the captive animals demonstrated a rather low phagocytic activity of neutrophils as well as monocytes (Tab. 6, 7). The proportion of phagocytizing cells enhanced over the course of the year. Even though *Clostridium perfringens* was diagnosed in all captive seals, neither *Mareike* nor *Lilli* or *Hein* showed any effect of the Gram-positive bacterium, except for flatulence.

The results from January make it even more interesting to test free-ranging animals early in the year.

*Luemmel* was born in June 1987, brought to the seal center, but could not be released for medical reasons. *Lona* was born in the seal center in June 2004 as daughter of *Deern*. These two animals were only tested in August and December. Thus comparison with other animals is difficult. However, they also show an increase in phagocytic activity in December, both in neutrophils as well as in monocytes.

*Deern* was born in June 1985, and was also brought to the seal center for rehabilitation. Due to an infection she was not allowed to be released. She was also only tested in August and December. Her phagocytic activity of granulocytes decreased, whereas monocytic reaction increased. Both values were still in normal ranges. Differential hemograms for both dates do not show any abnormalities.

Results of the Phagotest<sup>®</sup> have shown the applicability of the test kit for harbor seals. It is a powerful test to investigate group and individual differences. The test kit allows easy handling with an option to adjustment to given circumstances.

Results have reflected that the three harbor seal groups differ and have to be compared separately. It has also been suggested that seasonal variability can be expected in free-ranging and captive animals. Sample size is yet too small to be conclusive, however. Captive animals and pups at admission for rehabilitation have nearly the same phagocytic activity of

neutrophils and monocytes, whereas both leukocytes of free-ranging seals and pups prior to release present considerably less phagocytosis.

Further investigations should focus on more in-depth studies of why rehabilitated pups show less phagocytosis and if an increased Hp is the only reason. Additional interest should concentrate on seasonal changes within the captive and free-ranging harbor seals as it has never been mentioned before.

De Guise *et al.* (1995) observed a sex dimorphism in phagocytosis activity. In their study higher phagocytosis in females was speculated as an adaptation to compensate for lower respiratory burst. This observation could not be supported by this study. Even though sample size was small, in all groups females had lower phagocytosis and higher respiratory burst results than males.

The Phagotest<sup>®</sup> was described in order to show its applicability for harbor seals. It has been developed for human application but has been successfully adapted to terrestrial mammals as well (Menge *et al.* 1998, Werling *et al.* 1998, Fachinger *et al.* 2000).

Phagocytosis has already been shown to be affected by numerous xenobiotics in other species, e.g., exposure to dieldrin, mercury, cadmium and lead (Krzystyniak *et al.* 1987, Guillard and Lauwerys 1989). Fournier (2000) also suggested the phagocytosis as a good tool for monitoring exposure to heavy metals in wildlife species.



## Chapter 5

### **Oxidative Burst Activity of Peripheral Blood Granulocytes of Harbor Seals (*Phoca vitulina vitulina*) using Flow Cytometry**

#### **5.1 Introduction**

The phagocytic process can be separated into several major stages: chemotaxis (migration of phagocytes to inflammatory sites), attachment of particles to the cell surface of phagocytes, ingestion (phagocytosis) and intracellular killing by oxygen-dependent (oxidative burst) and oxygen-independent mechanisms (Roitt *et al.* 1996, Donadebian 1989). After ingestion of bacteria, neutrophils display a special metabolic change known as the respiratory or oxidative burst, in which large amounts of superoxide anion are produced by the enzyme complex NADPH oxidase.

The Phagoburst<sup>®</sup> test takes a step beyond the Phagotest<sup>®</sup>. Instead of looking at the number of particles the neutrophils have taken in, it investigates post-phagocytic events more closely linked to killing of ingested pathogens. If the oxidative burst malfunctions the whole immune response can be involved or paralyzed respectively.

This study was conducted in order to investigate the applicability and the utility of the Phagoburst<sup>®</sup> test in the routine evaluation of the health status of harbor seals. It has never been applied to marine mammals before.

An overview of the mechanism of the respiratory burst can be found in Appendix C of this thesis.

## 5.2 Material & Methods

A test kit for Phagoburst<sup>®</sup> Test, produced by Orpegen Pharma for human medicine, application was used. The test kit contained opsonized *E. coli* suspension; protein kinase C ligand, phorbol 12-myristate 13-acetate (PMA), was induced as a potent stimulus, and the chemotactic peptide, N-formyl-methionyl-leucyl-phenylalanine (fLMP), as a relatively mild physiological stimulus. The induced “burst” was measured using a fluorogenic substrate, dihydrorhodamine (DHR) 123. During the oxidative burst the non-fluorescent DHR was reduced to fluorescent rhodamine 123. The percentage of fluorescent phagocytic cells which produced reactive oxidants (ROS) was determined by flow cytometry. The analysis was performed utilizing CellQuest<sup>™</sup> Pro, BD.

Four test tubes of blood (100 µl blood/tube) per animal were required (one control and three stimulated). Blood tubes were chilled to 0°C by incubation on ice for 10 minutes. Afterwards, 20µl PBS was added to the control tube, 20 µl of the pre-cooled bacteria (*E. coli*), fMLP and PMA were added to the corresponding tubes. Tubes were immediately mixed on a vortex mixer and incubated in a closed water bath at 37°C for 10 minutes. After the incubation they were put on ice for another 10 minutes in order to stop the oxidative burst reaction. Following incubation the samples were washed with 3ml Washing Solution. The cells were spun down in a centrifuge at 250 x g, 4°C, for 5 minutes and the supernatant discarded. Subsequently, the blood was lysed and fixed with 2ml (room temperature) Lysing Solution. The samples were then stored in the dark at room temperature for 10 minutes. Cells were spun down as before and the supernatant was discarded followed by a second washing process, centrifugation, and addition of 200 µl of DNA Staining Solution. The Staining Solution was used to facilitate discrimination of bacteria during leukocyte analysis. The cells were then held in a light-protected ice bath and measured with the flow cytometer within 30 minutes.

CellQuest™ Pro

Leukocytes to be analyzed were identified by their incorporation of propidium iodide using CellQuest™ Pro software as follows:

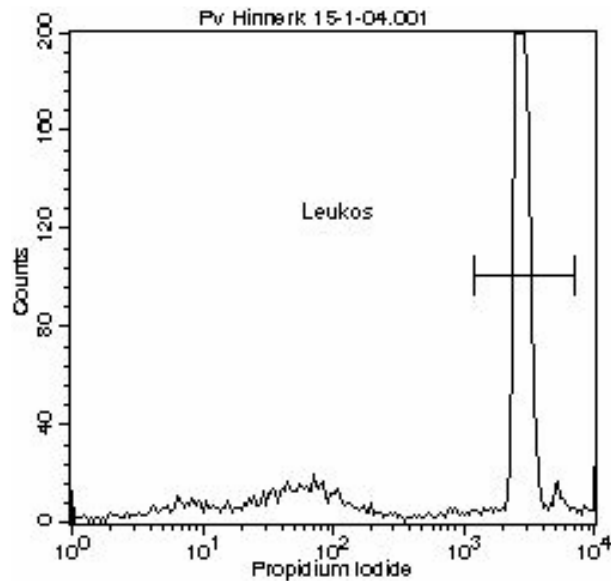


Figure 1: Histogram of all cells detected by the laser. Leukocytes marked by bar.

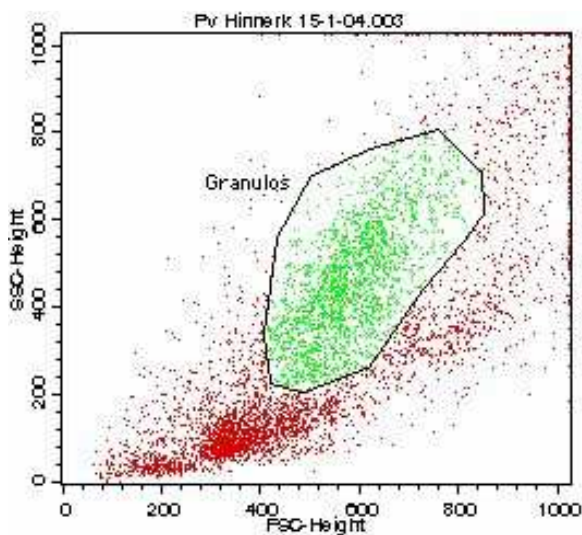


Figure 2: Dot Plot of all leukocytes stimulated by E-coli. The granulocyte gate is illustrated in green.

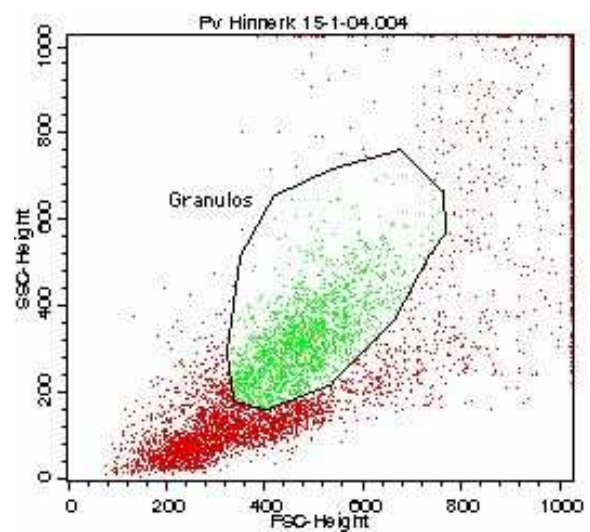


Figure 3: Dot Plot of all leukocytes stimulated by PMA. The granulocyte gate is illustrated in green.

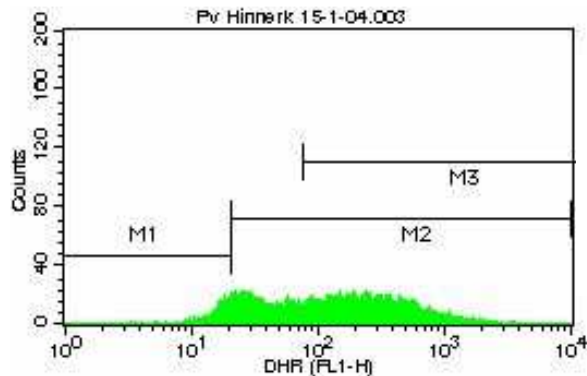


Figure 4: Fluorescent histogram of granulocytes; M1 represents non-stimulated cells; M2 and M3 represent cells which have been activated by *E. coli*.

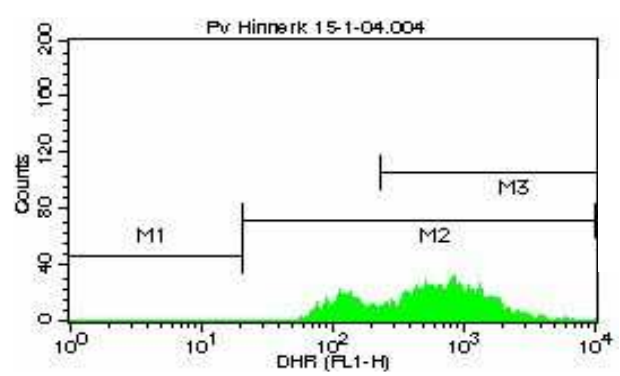


Figure 5: Fluorescent histogram of granulocytes; M1 represents non-stimulated cells; M2 and M3 represent cells which have been activated by PMA.

Table 1: Statistical analysis by CellQuest™Pro of Figures 4 and 5.

Sample ID: Pv_Hinnerk Tube: E-Coli Panel: Burst Acquisition Date: 15-1-04 Gate: Granulos					Sample ID: Pv_Hinnerk Tube: PMA Panel: Burst Acquisition Date: 15-1-04 Gate: Granulos				
Marker	Events	% Gated	X_Mean	SD	Marker	Events	% Gated	X_Mean	SD
All	5383	100	217.51	301.64	All	4175	100	745.09	96.74
M1	388	7.21	16.37	3.03	M1	0	0	0	0
M2	4995	92.79	233.13	307.68	M2	4175	100	745.09	96.74
M3	3325	61.77	330.36	337.36	M3	3913	97	748.01	96.39

The histogram on top (Fig. 1) depicts all leukocytes (which have incorporated propidium iodide into their DNA) detected by the laser. The bar marks all leukocytes. In Figure 2 and 3 the dot plots again show all leukocytes with the granulocyte population gated (green). The colored histograms (Fig. 4, 5) display burst profile of neutrophils. The bar M1 represents no phagocytic burst. Bar M2 and M3 mark all activated granulocytes. The second marker within the activated cells' histogram was used such that the 2<sup>nd</sup> distinct peaks could be analyzed independently. Gated neutrophils were analyzed as either being non-stimulated, stimulated or highly stimulated (Tab. 1). The *Mean* in the tables described the average fluorescence of the cells. *SD* in this case calculated the standard deviation of the mean

fluorescence. The lower the SD the more uniform the cells behaved. A high SD indicated the cells acted independently by demonstrating a broad range of fluorescence.

After stimulation with E-Coli and PMA it was impossible to identify a clear monocyte population in the FSC/SSC dot plot. The monocytes were difficult to isolate by light scatter characteristics in the control tube and, therefore, only neutrophils were analyzed.

Stimulation with fMLP had no demonstrable effect on monocytes nor neutrophils from any animal. After an adequate number of tests (n = 19) fMLP was dropped from the protocol.

## 5.3 Results

The animals were again divided into three groups, with rehabilitated pups redivided: captive, free-ranging (WF), pups at their admittance for rehabilitation (pup\_adm), and pups at their pre-release blood withdrawal (pup\_rel). It was also distinguished if the animals showed any conspicuities in their differential hemograms (e.g. high numbers of WBC).

### 5.3.1 Free-ranging harbor seals (Tab. 2, 3)

Table 2 and 3 contain results of stimulated neutrophils of free-ranging harbor seals. In most cases only neutrophils triggered by *E. coli* could be clearly identified for analysis. There was no recognizable trend in animals with normal or altered hemogram. The same applies for different months. Most granulocytes stimulated by PMA could not be gated (i.e. see Fig. 3) and therefore statistical analysis could not be conducted. If cells could be distinguished by light scatter (FSC/SSC) the stimulation was usually 100% with a high mean fluorescence intensity and standard deviation of the mean fluorescence.

### 5.3.2 Rehabilitated Harbor Seal Pups (Tab. 4-7)

Only four animals could be tested both at admission and prior to release (marked) as the coordination of the blood withdrawal with the veterinarian, the seal center and the laboratory was often difficult. In most cases both *E. coli* and PMA stimulated granulocytes could be gated and statistically analyzed.

*E. coli*-induced reactive oxygen species (ROS) were evident in varying percentages of neutrophils in harbor seal pups at admission to the seal center (Table 4). Animals tested on 17 June 2003 (4 with normal hemograms and one with a high WBC), had relatively low burst activity; no trends associated with abnormal hemogram were recognizable.

Seal pups prior to release showed a relative reduction in *E. coli*-induced respiratory burst compared to results at admission (adm. median: 84%; pre-rel. median: 77%).

PMA induced ROS were also evident in a great majority of neutrophils of most pups at admission (Table 5) with the exception of two animals (Pv\_2284 & Pv\_2285) that had 53 and

60% reactive cells. There was no significant difference (quasi-Poisson:  $p = 0.07$ ) between animals with altered and normal hemograms.

Even though the median fluorescent intensity of PMA stimulated neutrophils in the M2 gate is very similar between pups at admission, and prior to release (adm. median: 99%; pre-rel. median: 97%), the animals differ significantly. The most significant difference is the individual animal variation in M2-values (adm.:  $100 - 95.6 \Rightarrow 4.4$ ; pre-rel.:  $99 - 76.8 \Rightarrow 22.2$ ; Mann-Whitney-U-Test:  $U = 65$ ,  $N \text{ Pups}_{\text{adm}} = 17$ ,  $N \text{ Pups}_{\text{rel}} = 15$ ,  $p = 0.017$ ).

### 5.3.3 Captive harbor seals (Tab. 8, 9)

While *Hinnerk* was only tested twice a dramatic decrease in both *E. coli*- and PMA-induced ROS was evident from January to August 2004 (Tab. 8, 9). 100% of neutrophils produced ROS in January, whereas in August none could be stimulated.

*Lilli* exhibited the opposite profile, but it was only evident in burst activity stimulated by *E. coli*. While her hemogram showed no abnormalities in January, the percentage of *E. coli* generated ROS was only 55% in M2 and 21% in M3 (Table 8). Results improved, however, over the course of the year and in December 100% of *Lilli's* neutrophils were activated by *E. coli*. Reaction to PMA was 100% in all three tests in M2 (Table 9).

All other captive animals depicted no differences in their production of ROS over the course of 2004. Percentage of gated cells within M2 and M3 stimulated by *E. coli* and PMA were similar.

The mean fluorescence intensity, especially in PMA-triggered neutrophils, demonstrated extensive cell-to-cell variation, i.e. standard deviation was very high (Table 9). This refers to both M2 and M3 gated cells.

Table 2: Data of Phagoburst® of free-ranging (WF) seals. M2\_/M3\_%Gated = percentage of activated neutrophils with *E. coli* as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l); problem 0-1 = borderline hemogram values (WBC = 11-12g/l).

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
WF	Pv_2689	26-8-04	53	246	316	24	493	336	1
WF	Pv_2691	26-8-04	57	164	239	18	423	288	0-1
WF	Pv_2443	18-9-03	74	225	211	48	316	213	0-1
WF	Pv_2825	2-12-04	90	174	287	30	430	378	0-1
WF	Pv_2828	2-12-04	93	309	389	53	505	417	0-1
WF	Pv_2687	26-8-04	72	287	283	45	426	274	0
WF	Pv_2688	26-8-04	84	159	187	34	317	208	0
WF	Pv_2690	26-8-04	54	191	243	22	404	261	0
WF	Pv_2692	26-8-04	70	319	324	45	462	321	0
WF	Pv_2693	26-8-04	57	193	233	25	374	251	0
WF	Pv_2694	26-8-04	62	246	314	30	455	341	0
WF	Pv_2445	18-9-03	93	303	267	74	364	266	0
WF	Pv_2702	18-9-04	63	204	262	30	370	300	0
WF	Pv_2704	18-9-04	67	216	259	32	392	277	0
WF	Pv_2822	2-12-04	8	121	274	1	740	455	0
WF	Pv_2830	2-12-04	95	219	253	50	374	263	0



Table 3: Data of Phagoburst® free-ranging (WF) seals. M2\_/M3\_%Gated = percentage of activated neutrophils with PMA as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l); problem 0-1 = borderline hemogram values (WBC = 11-12g/l); n.p. = not possible to distinguish granulocytes from lymphocytes.

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
WF	Pv_2689	26-8-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	1
WF	Pv_2691	26-8-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0-1
WF	Pv_2443	18-9-03	77	217	208	48	312	213	0-1
WF	Pv_2825	2-12-04	100	1036	692	91	1132	654	0-1
WF	Pv_2828	2-12-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0-1
WF	Pv_2687	26-8-04	100	539	316	89	598	283	0
WF	Pv_2688	26-8-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0
WF	Pv_2690	26-8-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0
WF	Pv_2694	26-8-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0
WF	Pv_2445	18-9-03	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0
WF	Pv_2702	18-9-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0
WF	Pv_2704	18-9-04	n.p.	n.p.	n.p.	n.p.	n.p.	n.p.	0
WF	Pv_2822	2-12-04	100	841	537	82	1015	434	0
WF	Pv_2830	2-12-04	100	571	571	93	869	553	0

Table 4: Data of Phagoburst® of seal pups at admission. M2/M3\_%Gated = percentage of activated neutrophils with *E. coli*, as stimulus; M2/M3\_XMean = amount of average fluorescence; M2/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l); problem 0-1 = borderline hemogram values (WBC = 11-12g/l); marked = animals tested at admission and prior to release.

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
Pup_adm	Pv_2540	7-6-04	65	337	408	27	692	440	1
Pup_adm	Pv_2287	17-6-03	21	212	195	8	413	185	1
Pup_adm	Pv_2532	2-6-04	99	284	366	38	637	381	0
Pup_adm	Pv_2536	6-6-04	71	247	271	26	493	319	0
Pup_adm	Pv_2537	7-6-04	80	143	211	19	427	266	0
Pup_adm	Pv_2538	7-6-04	81	342	403	36	645	445	0
Pup_adm	Pv_2539	7-6-04	63	322	387	26	641	429	0
Pup_adm	Pv_2541	7-6-04	86	312	338	39	572	359	0
Pup_adm	Pv_2542	8-6-04	100	197	291	10	911	448	0
Pup_adm	Pv_2543	9-6-04	100	212	325	16	845	429	0
Pup_adm	Pv_2544	9-6-04	100	200	268	20	632	335	0
Pup_adm	Pv_2545	9-6-04	100	242	324	17	852	366	0
Pup_adm	Pv_2546	9-6-04	97	110	161	19	382	191	0
Pup_adm	Pv_2547	9-6-04	67	178	301	11	710	457	0
Pup_adm	Pv_2548	9-6-04	97	214	317	30	591	359	0
Pup_adm	Pv_2549	9-6-04	99	166	251	15	651	325	0
Pup_adm	Pv_2289	17-6-03	52	261	241	24	450	240	0
Pup_adm	Pv_2290	17-6-03	19	259	252	8	475	249	0
Pup_adm	Pv_2291	17-6-03	54	302	320	24	572	318	0
Pup_adm	Pv_2292	17-6-03	27	252	241	12	458	242	0

Table 5: Data of Phagoburst<sup>®</sup> of seal pups at admission. M2\_/M3\_%Gated = percentage of activated neutrophils with PMA as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l); problem 0-1 = borderline hemogram values (WBC = 11-12g/l); marked = animals tested at admission and prior to release.

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
Pup_adm	Pv_2540	7-6-04	91	413	279	75	487	270	1
Pup_adm	Pv_2284	16-6-03	60	131	96	10	301	106	0-1
Pup_adm	Pv_2285	16-6-03	53	133	102	11	305	125	0-1
Pup_adm	Pv_2287	17-6-03	89	333	305	50	507	307	1
Pup_adm	Pv_2294	22-6-03	100	263	225	72	346	214	0-1
Pup_adm	Pv_2536	6-6-04	95	467	312	82	525	302	0
Pup_adm	Pv_2537	7-6-04	100	714	497	87	771	480	0
Pup_adm	Pv_2538	7-6-04	96	479	379	80	558	377	0
Pup_adm	Pv_2539	7-6-04	88	305	216	56	409	210	0
Pup_adm	Pv_2541	7-6-04	97	784	483	93	824	469	0
Pup_adm	Pv_2542	8-6-04	100	167	168	19	583	199	0
Pup_adm	Pv_2543	9-6-04	100	189	182	27	577	210	0
Pup_adm	Pv_2544	9-6-04	100	460	312	84	520	295	0
Pup_adm	Pv_2545	9-6-04	100	459	301	84	520	291	0
Pup_adm	Pv_2546	9-6-04	100	394	276	78	480	257	0
Pup_adm	Pv_2547	9-6-04	100	407	319	81	492	306	0
Pup_adm	Pv_2548	9-6-04	100	345	315	60	449	313	0
Pup_adm	Pv_2549	9-6-04	99	166	156	23	537	225	0
Pup_adm	Pv_2289	17-6-03	98	798	671	89	873	665	0
Pup_adm	Pv_2290	17-6-03	96	565	418	79	664	399	0
Pup_adm	Pv_2291	17-6-03	97	606	536	83	694	539	0
Pup_adm	Pv_2292	17-6-03	97	478	372	78	570	367	0

Table 6: Data of Phagoburst® of seal pups prior to release. M2\_/M3\_%Gated = percentage of activated neutrophils with *E. coli* as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l); problem 0-1 = borderline hemogram values (WBC = 11-12g/l); marked = animals tested at admission and prior to release.

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
Pup_rel	Pv_2270	19-8-04	87	226	300	27	496	424	0
Pup_rel	Pv_2540	19-8-04	83	190	230	20	452	348	0
Pup_rel	Pv_2542	19-8-04	77	224	313	24	486	453	0
Pup_rel	Pv_2543	19-8-04	82	191	239	20	480	340	0
Pup_rel	Pv_2548	19-8-04	60	209	205	21	417	233	0
Pup_rel	Pv_2287	29-8-03	86	210	213	30	436	226	0
Pup_rel	Pv_2315	29-8-04	96	230	270	34	479	330	0
Pup_rel	Pv_2284	24-9-03	56	245	265	23	454	301	0
Pup_rel	Pv_2290	24-9-03	69	255	282	30	456	333	0
Pup_rel	Pv_2293	24-9-03	53	209	207	19	404	236	0
Pup_rel	Pv_2294	24-9-03	83	322	323	43	522	339	0
Pup_rel	Pv_2313	24-9-03	70	317	296	37	517	287	0
Pup_rel	Pv_2314	24-9-03	60	225	311	21	461	430	0
Pup_rel	Pv_2324	24-9-04	78	270	339	36	465	418	0

Table 7: Data of Phagoburst® of seal pups prior to release. M2\_/M3\_%Gated = percentage of activated neutrophils with PMA as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l); problem 0-1 = borderline hemogram values (WBC = 11-12g/l); marked = animals tested at admission and prior to release.

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
Pup_rel	Pv_2270	19-8-04	99	319	211	73	391	202	0
Pup_rel	Pv_2540	19-8-04	76	196	137	29	323	136	0
Pup_rel	Pv_2542	19-8-04	99	318	331	60	446	369	0
Pup_rel	Pv_2543	19-8-04	97	233	150	49	331	152	0
Pup_rel	Pv_2548	19-8-04	99	433	534	66	599	587	0
Pup_rel	Pv_2287	29-8-03	97	354	283	71	442	283	0
Pup_rel	Pv_2284	29-8-04	74	329	301	47	453	314	0
Pup_rel	Pv_2290	24-9-03	92	458	411	73	548	415	0
Pup_rel	Pv_2293	24-9-03	78	352	247	54	449	375	0
Pup_rel	Pv_2294	24-9-03	99	596	474	76	755	435	0
Pup_rel	Pv_2313	24-9-03	98	534	458	71	700	429	0
Pup_rel	Pv_2314	24-9-03	89	428	369	66	543	362	0
Pup_rel	Pv_2315	24-9-03	100	511	399	74	650	377	0
Pup_rel	Pv_2324	24-9-04	90	450	390	70	551	384	0

Table 8: Data of Phagoburst® of captive seals. M2\_/M3\_%Gated = percentage of activated neutrophils with *E. coli* as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l).

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
Captive	Hinnerk	15-1-04	93	233	308	62	330	337	0
Captive	Hinnerk	13-8-04	29	75	104	6	199	173	1
Captive	Mareike	15-1-04	99	364	124	58	388	82	1
Captive	Mareike	20-8-04	99	300	333	77	373	344	0
Captive	Mareike	15-12-04	100	333	363	74	433	375	0
Captive	Lilli	15-1-04	55	104	122	21	209	146	0
Captive	Lilli	20-8-04	99	259	314	66	367	335	0
Captive	Lilli	15-12-04	100	306	393	76	387	420	0
Captive	Hein	15-1-04	98	253	247	70	337	246	1
Captive	Hein	20-8-04	100	446	511	76	569	530	0
Captive	Hein	15-12-04	100	429	502	75	551	525	0
Captive	Lümmel	20-8-04	100	363	408	64	539	416	0
Captive	Lümmel	15-12-04	100	459	496	84	533	506	0
Captive	Lona	20-8-04	100	233	322	65	333	362	0
Captive	Lona	15-12-04	100	294	291	80	354	296	0
Captive	Deern	20-8-04	100	380	389	82	452	393	0
Captive	Deern	15-12-04	100	271	339	57	443	366	0

Table 9: Data of Phagoburst® of captive seals. M2\_/M3\_%Gated = percentage of activated neutrophils with PMA as stimulus; M2\_/M3\_XMean = amount of average fluorescence; M2\_/M3\_SD = standard deviation of the mean fluorescence; problem 0 = animal had normal hemogram; problem 1 = animal had altered differential hemogram (WBC > 12g/l).

Group	Animal	Date	M2_%Gated	M2_XMean	M2_SD	M3_%Gated	M3_XMean	M3_SD	Problem
Captive	Hinnerk	15-1-04	100	745	721	100	748	721	0
Captive	Hinnerk	13-8-04	0	0	0	0	0	0	1
Captive	Mareike	15-1-04	100	1008	1005	100	1008	1005	1
Captive	Mareike	20-8-04	100	862	586	100	865	585	0
Captive	Mareike	15-12-04	100	1691	938	100	1691	938	0
Captive	Lilli	15-1-04	100	324	373	85	374	384	0
Captive	Lilli	20-8-04	100	1096	944	100	1100	944	0
Captive	Lilli	15-12-04	100	855	676	99	863	674	0
Captive	Hein	15-1-04	100	615	545	97	630	544	1
Captive	Hein	20-8-04	100	998	838	100	999	838	0
Captive	Hein	15-12-04	100	1607	948	100	1607	948	0
Captive	Lümmel	20-8-04	100	1213	1060	98	1232	1058	0
Captive	Lümmel	15-12-04	100	1900	980	100	1900	980	0
Captive	Lona	20-8-04	100	943	954	99	953	955	0
Captive	Lona	15-12-04	100	1054	676	100	1055	676	0
Captive	Deern	20-8-04	100	739	681	99	746	681	0
Captive	Deern	15-12-04	100	1677	1066	100	1677	1066	0

## 5.4 Discussion

The Phagoburst<sup>®</sup> has been successfully applied to human and terrestrial mammal blood samples (Esparza *et al.* 1996, Gessler *et al.* 1996, Menge *et al.* 1998, Hoffmann-Jagielska *et al.* 2003). This project represents the first reported use of Phagoburst<sup>®</sup> in pinnipedia.

De Guise *et al.* (1995) have described extensive variability in both phagocytosis and respiratory burst in free-ranging beluga whales (*Delphinapterus leucas*); they speculated the variation may have been due to individual response to capture. These authors also observed a sex dimorphism, in which higher phagocytosis was observed in females and speculated this might be due to compensation for lower respiratory burst. Such was not the case in this study on harbor seals. Even though sample size was small in all groups and the sexes were not divided, females had lower phagocytosis and higher respiratory burst as compared to males. Further analysis of a larger number of animals may help to identify potential differences between the sexes.

### 5.4.1 Free-Ranging Harbor Seals

No trends in *E. coli*-induced respiratory burst were recognizable in animals with normal versus those with altered hemograms (Tab. 2). The same applies for respiratory burst measured in different months. Table 3 illustrates the values for stimulation with PMA. Limited analysis of neutrophils stimulated with PMA was possible due to the inability to distinguish between bursted granulocytes and lymphocytes (Fig. 6); PMA stimulated granulocytes apparently were induced to degranulate such that their side scatter characteristics became similar to lymphocytes.

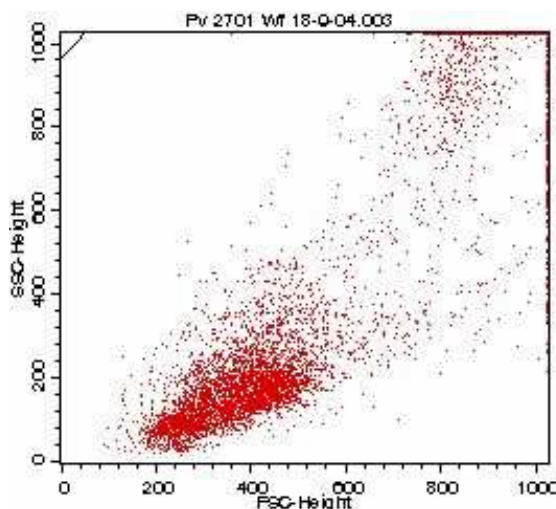


Figure 6: Dot plot of free-ranging harbor seals; PMA stimulated granulocytes cannot be distinguished from lymphocytes anymore.



### 5.4.2 Rehabilitated Harbor Seal Pups

Pups admitted to the seal center for rehabilitation generally demonstrated high neutrophil activity relative to creating ROS with only few exceptions. This was evident with both stimuli, *E. coli* and PMA.

Granulocytes from two animals (#2540 & #2287) with increased WBC demonstrated rather low activity triggered by *E. coli* (Table 4). In addition, pup # 2539 bled on June 7 and all animals bled on June 17 (four animals) demonstrated poor *E. coli*-induced ROS. An impaired immune system can be assumed in all seven cases. The underlying reason for the poor activation of PMNs in all animals tested on 17 June 2003 is unknown. Blood samples were all treated in accordance with the manual.

PMA stimulated granulocytes depicted high activity in all animals, even those with low *E. coli* stimulation (Table 5). We would hypothesize that opsonized *E. coli* represent a “more” physiologically relevant stimuli, and thus these results would better represent immunologic health.

Similar to the immunologic analysis of admitted and pre-release harbor seal pups using the phagocytosis test, blood samples from the pre-release examination showed a decrease in *E. coli*-stimulated neutrophil respiratory burst. If phagocytosis can be inhibited by haptoglobin (Rossbacher *et al.* 1999) as described in Chapter 4, the reduced respiratory burst may have also been inhibited.

Neutrophils activated by PMA of seal pups at the end of rehabilitation showed a slight but not significant decrease in producing ROS ( $p < 0.05$ ). As previously described for the free-ranging animals, PMA stimulus of the neutrophils often rendered the neutrophils indistinguishable from other leukocytes (Fig. 6). Many more animals have been sampled and examined but only those listed in Tables 5 & 7 could be successfully analyzed.

### 5.4.3 Captive harbor seals

Similar to the data generated using the phagocytosis test, *Hinnerk* showed a major decrease of both *E. coli*- and PMA-induced neutrophil burst activity in August as compared to the previous January (Table 8, 9). The variation (SD) in mean fluorescence was not high, which indicated a consistency among the leukocytes. The first test with PMA in January 2004 resulted in very strong burst activity as all gated neutrophils were within the M3 marker of the histogram. One day prior to death no reaction to PMA could be identified. *E. coli*, however, still stimulated 29% of granulocytes with only 6% being within M3. Typically, PMA is a stronger neutrophil stimulus compared to *E. coli*. I would speculate the neutrophils were sufficiently fragile that the PMA stimulus was sufficiently strong that death rather than activation was induced.

Blood neutrophils from *Deern*, *Mareike*, *Lümmel*, *Lona*, and *Hein* produced similar numbers in the M2-marker whether stimulated by either *E. coli* or PMA. The same was true for numbers in the M3-marker of PMA activated cells. Numbers of *E. coli*-stimulated neutrophils that fell within the M3-marker, varied throughout the course of 2004. The results were similar to the results of the Phagotest<sup>®</sup>. Again, the ability to identify temporal changes in immune function, as measured by respiratory burst, were best identified using the physiologic stimulus of opsonized *E. coli*.

*Lilli* was the only other animal in which temporal changes in respiratory burst were observed, similar to the phagocytosis data. This was especially evident when stimulated by *E. coli*. Temporal changes in PMA-induced burst were only evident in the M3-marker.

The consistency in response to *E. coli*, identified between neutrophils from an individual animal, was not evident in PMA-stimulated samples, where the mean fluorescence and standard deviation were high.

Respiratory burst in neutrophils has previously been shown to be influenced by numerous xenobiotics in multiple species, e.g., exposure to dieldrin, mercury, cadmium and lead (Krzystyniak *et al.* 1987, Guillard and Lauwerys 1989). Further investigations in that respect might help understand the inconsistency between individual animals of the same study group (free-ranging, rehabilitated and captive).

While the results obtained using the Phagoburst<sup>®</sup> test kit were not as insightful as those from the Phagotest kit, some interesting data was generated. The changes in respiratory burst that were identified both between animals, and with individual animals across time, would suggest animals have the potential to be categorized relative to immunologic health using this procedure. Future attempts to expand this study should probably focus on the use of *E. coli* as a stimulus with PMA appears to work more like a hammer than a physiologically relevant stimulus of oxidative burst. Additional studies should also be directed at a more in-depth study of the events involved in respiratory burst of pinniped neutrophils as Drath and Karnovsky (1975) previously described considerable species differences in O<sub>2</sub>-release by different leukocytes that might reflect both the production and/or destruction (e.g. by dismutase) of that substance.

## Chapter 6

### Serum Protein Electrophoresis in Harbor Seals

#### (*Phoca vitulina vitulina*)

### 6.1 Introduction

Subsidiary to the previous chapters, the serum electrophoresis takes a closer look at the protein groups, operative in the immune response. Serum electrophoresis has been conducted on several marine mammals including cetaceans and pinnipeds. However, studies on harbor seals (*Phoca vitulina*) have been scarce and basic results from the above mentioned groups were hard to find. So far nothing has been published on serum protein electrophoresis (SPEP) in harbor seals.

The aim of this study was to give reference values for routine examinations of the health status of harbor seals. Furthermore the rehabilitation of pups in the Seal Rehabilitation Center each year, gave an excellent chance to evaluate the immune system of young animals. The serum electrophoresis should be a simple tool to evaluate a possible threat to the health of an animal.

Proteins are made of amino acids and are important constituents of all cells and tissues. There are many different kinds of proteins in the body with many different functions, e.g. enzymes, antibodies, hormones, hemoglobin. Serum proteins are separated into albumin and globulins. Each of the classic 6 sub-units contains single proteins and protein groups (pre-albumin, albumin,  $\alpha_1$ ,  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -proteins). The concentrations of the protein groups, either isolated or in combination with proteins of other units, are regulated by different mechanisms.

About half of the protein in blood serum is albumin. In addition to carrying substances through the bloodstream, albumin proteins are of prime importance in maintaining the oncotic pressure of the blood. A small portion of the total osmotic pressure is due to the presence of large protein molecules (e.g. albumin), which is called the colloidal osmotic pressure or oncotic pressure. It may also help with tissue growth and healing.

Globulins are roughly divided into  $\alpha_1$ ,  $\alpha_2$ ,  $\beta$ - and  $\gamma$ -globulins. These can be separated and quantified in the laboratory by serum protein electrophoresis and densitometry.

- The  $\alpha_1$  fraction includes  $\alpha_1$  antitrypsin- (an acute-phase protein) and thyroxine-binding globulins (triiodothyronine –  $T_3$ , thyroxine –  $T_4$ , resin  $T_3$  uptake –  $RT_3U$ ).
- The  $\alpha_2$  fraction contains haptoglobin (an acute-phase protein, binds with hemoglobin), ceruloplasmin, high density lipoproteins (HDL), and  $\alpha_2$  macroglobin. In general,  $\alpha_1$  and  $\alpha_2$  protein levels increase in the presence of inflammation.
- The  $\beta$ -fraction includes transferrin (carries iron through bloodstream), plasminogen, and low density lipoproteins (LDL). The protein complement C3, also found within this fraction, is needed to destroy bacteria.

With the exception of the immunoglobulins almost the entire serum proteins are synthesized in the hepatocytes. With the help of the serum protein electrophoresis serum proteins can be separated into different fractions.

SPEP as a marker for the immune system has long been used in human medicine. Applied on cellulose acetate or gel it imparts a global insight into variations of the composition of serum proteins. The IgG- and IgA-immunoglobulins migrate to the  $\gamma$ -globulin fraction. Due to less electrophoretic mobility, other immunoglobulins, especially IgD, are primarily found in the  $\beta$ -globulin fraction. IgM migrates to the  $\alpha_2$ - and  $\beta$ -globulin fraction (Burmester *et al.* 1998).

The serum electrophoresis usually is an important base analysis, because it allows the following:

1. Detection of a disease after showing a constellation of a dysproteinemia.
2. Allocation of certain diseases or disease groups to characteristic constellation types. Diseases and reaction state are therefore detectable.
3. Process evaluation of a disease status (Thomas 1994).

Indications for identification of the protein fractions are especially all forms of hyper- or hypoproteinemia, which are caused by numerous diseases, such as liver disease, nephrotic syndrome or nutritional deficiency. One important symptom, however, is chronic inflammation.

### 6.1.1 Characterization of Disproteinemia

In Dierauf and Gulland (2001) the different possible causes for hyper- or hypoproteinemia in marine mammals were described as follows:

#### *Albumin (in whole blood)*

high: dehydration or shock

low: malnutrition, renal disease (protein-losing nephropathy), gastrointestinal disease (e.g. parasites, maldigestion, protein-losing enteropathy), advanced hepatic disease, secondary to hyperglobulinemia hemorrhage, extensive and severe skin lesions with epidermal compromise (e.g. burns).

#### *Globulins (by SPEP)*

high  $\alpha$ -globulin: acute inflammatory disease (can occur before other diagnostic signs occur), severe active hepatitis, acute glomerular disease and the nephrotic syndrome

high  $\beta$ -globulin: acute hepatitis, suppurative dermatopathies, nephrotic syndrome

high  $\gamma$ -globulin: respiratory infections, skin disease

low  $\gamma$ -globulin: in fetal serum, precolostral neonate, acquired immunodeficiency of chronic-disease states (suggesting humoral immunological exhaustion).

## 6.2 Material & Methods

Due to lack of serum samples of younger free-ranging animals, only electrophoresis of free-ranging seals of the age class “older” could be conducted. Total protein values were available of all three age-classes. Samples of captive harbor seals were also limited. No serum of the animal *Deern* was available.

The serum was separated of the blood corpuscles and platelets by centrifugation (3000 rpm, 15°C, 20 min). Blood was taken over several years, and serum was stored at -20°C before examination. Unlike most clinical methods where it is of vital importance that the blood is handled fresh and within 24 - 48 hours, the protein fractions of frozen serum separate better than those of fresh serum (pers. comm. Horn). After slow defrosting the serum was placed on cellulose acetate and exposed to an electric current. The various proteins migrated to form bands that indicated the relative proportion of each protein fraction. A citrate buffer served as separating medium with a pH between 8.0 and 9.0. In this buffer the proteins' charge became negative. According to their charge they migrated differently far from the cathode to the anode. After separation they were fixed and dyed. The dyed electropherogram was assessed visually and by standard electrophoresis software. The levels of protein fractions were measured by taking the total serum protein (TSP) and multiplying the relative percentage of each component protein fraction.

In this study the use of cellulose acetate was preferred to gel, to be able to manually adjust the aperture for best possible results. An *Elphoscan Mini Plus* by Eltest (Bornheimer Str.33, 53111 Bonn, Germany) was used, which was located at the WestKüstenKlinikum (WKK) Heide. After several tests a voltage of 115, 20 mA and a separation time of 18 min proved to be the optimum. For reference values of the protein fractions, TSP was also measured by the laboratory of the WKK Heide.

All sera collected from wild catches, pups and animals held in captivity were more or less lipemic. Due to indistinct results, it was tried to first dilute the sera of wild catches with a buffer, with no resounding effect. It was then washed/shaken with trichlorine-trifluoroethane (C<sub>2</sub>Cl<sub>3</sub>F<sub>3</sub>) to extract the lipoproteins. This method had been successfully used with human serum but showed no effect on the harbor seal serum. It was therefore very difficult to separate the globulin fractions in the densitograms. The first separation was done with one relatively distinct densitogram and copied to all other wild animal results.

### 6.3 Results

As could be seen in the densitograms (Fig. 1-8), the results of the three harbor seal groups differ immensely. Electropherograms of free-ranging animals were mostly very indistinct (Fig. 1, 2).

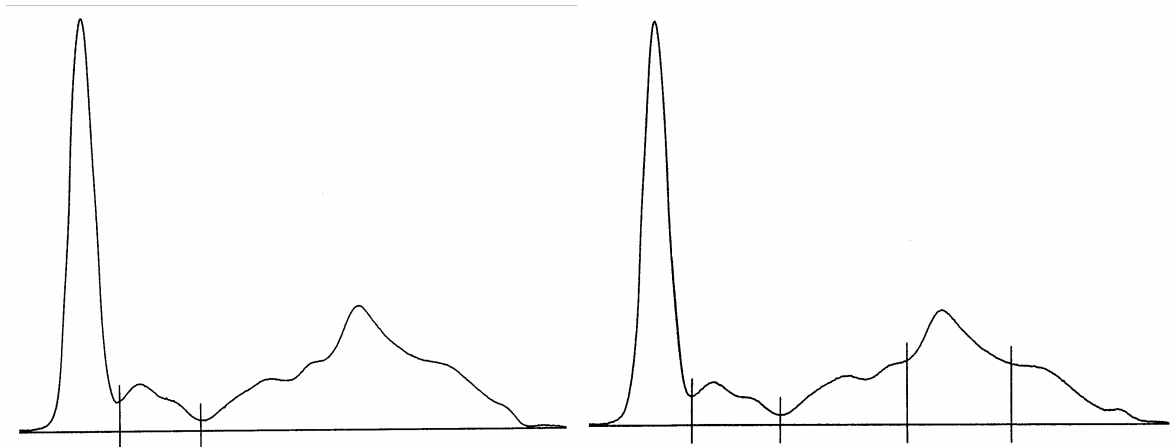


Figure 1: Densitograms of a free-ranging adult harbor seal (Pv\_2474) with and – for a better overview – without marks for serum protein fractions.

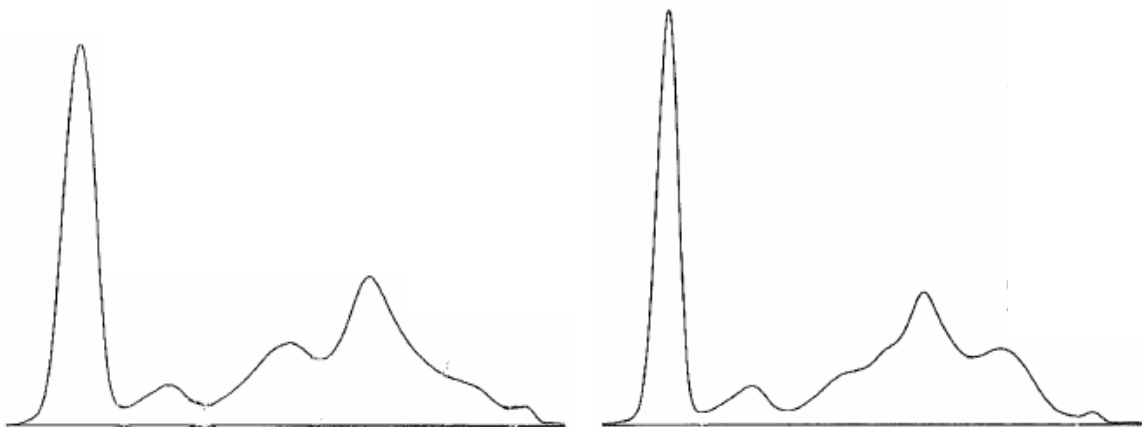


Figure 2: Densitograms of two adult free-ranging harbor seals (Pv\_2216, Pv\_1985).



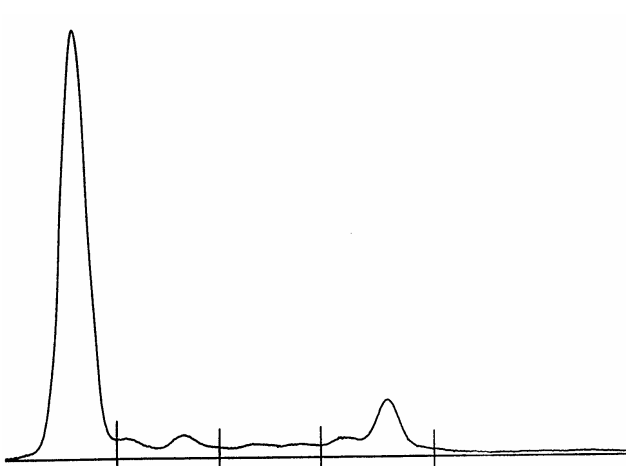


Figure 3: Densitogram of a male harbor seal pup admitted for rehabilitation (Pv\_2275).

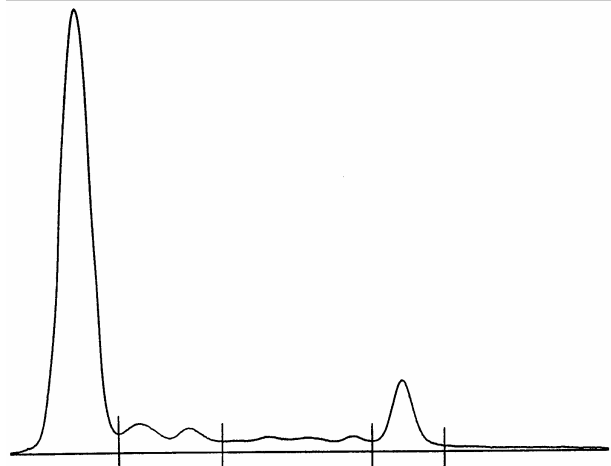


Figure 4: Densitogram of a female harbor seal pup admitted for rehabilitation (Pv\_2314).

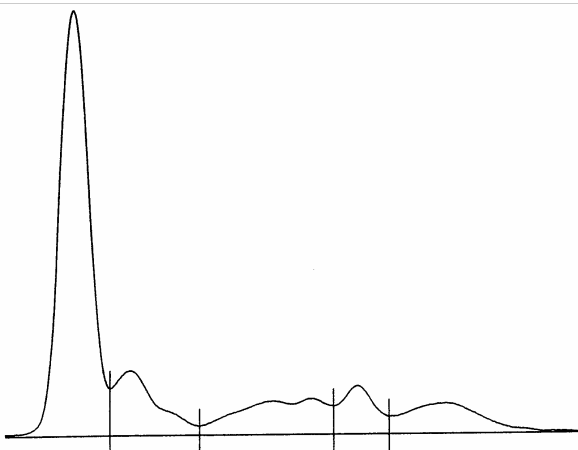


Figure 5: Densitogram of a female juvenile captive harbor seal (19 months; Pv\_Mareike).

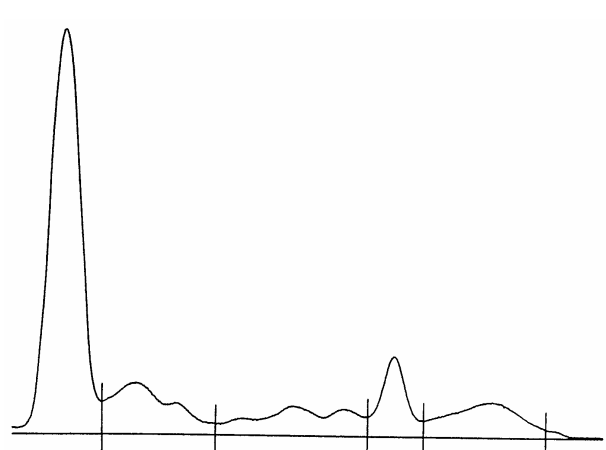


Figure 6: Densitogram of a male harbor seal pup (3 weeks), born in captivity (Pv\_Hinnerk).

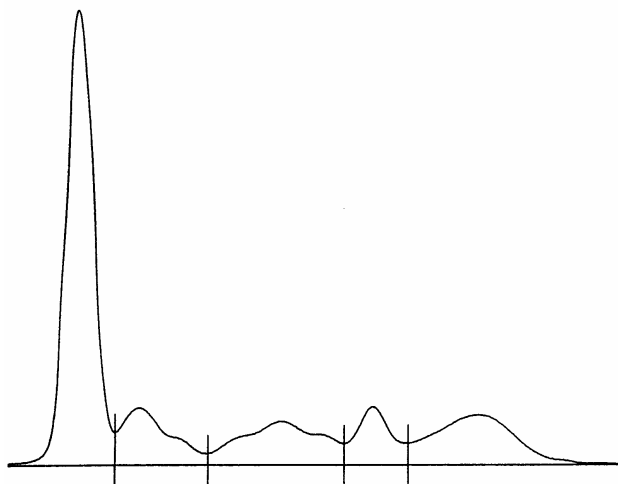


Figure 7: Densitogram of a male adult captive harbor seal (Pv\_Hein).

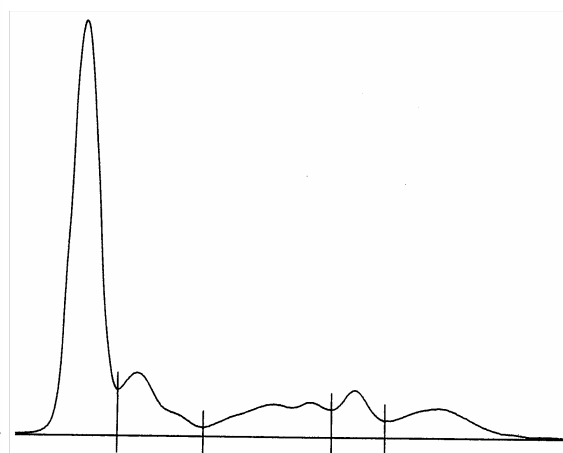


Figure 8: Densitogram of a male adult captive harbor seal (Pv\_Lümmel).

### 6.3.1 Free-Ranging Harbor Seals (Tab. 1-3)

As there were only few pups caught in the field, no significant differences of TSP could be calculated between sexes.

Concerning the yearlings, the interpercentile range of males was slightly larger, but no significant differences of TSP between the sexes could be found (quasi-Poisson;  $p > 0.05$ ). Still, the number of animals was too small to conduct reliable statistics.

Looking at the age class of animals older than 2 years, a conspicuous difference was found in female animals. They were subdivided into animals caught right after the PDV epidemic (12 Nov. 2002) and females caught around the PDV epidemic. The median TSP of infected female seals was significantly ( $p = 0.00005$ ) higher (14g/l) than that of uninfected. It seemed that the virus caused a decrease in albumin and an increase in the  $\beta$ - and  $\gamma$ -fraction of the serum proteins (Tab. 2, 3).

There was no difference found between the three male animals caught on 12 November 2002 and the other males. Therefore the male values of TSP as well as electrophoresis include those three animals. No significant difference was found between uninfected females and males.

Table 1: Total serum protein (TSP) of free-ranging harbor seals differentiated by sex and age-classes. The first number indicates the median with the count as index, the numbers within the parentheses depicted the percentile range (5-95 %). In case of sample sizes  $<10$ , min. and max., rather than percentiles were depicted.

Animal	Sex	Age	TSP [g/l]
wild	f	pup	72 <sub>2</sub> (67-77)
wild	f	yearling	84 <sub>11</sub> (74-95)
wild*	f	older	80 <sub>27</sub> (69-91)
wild**	f	older	94 <sub>16</sub> (79-104)
wild	m	pup	74 <sub>4</sub> (72-79)
wild	m	yearling	80 <sub>10</sub> (74-92)
wild	m	older	80 <sub>72</sub> (70-89)

\* Values without animals caught directly after PVD 2002

\*\* Values of animals caught directly after PDV 2002

Table 2: Proportions of serum protein fractions of free-ranging harbor seals of the age-class “older” (>2yrs) differentiated by sex. The first number indicates the median with the count as index, the numbers within the parentheses depicted the percentile range (5-95 %).

Animal	Sex	Alb (%)	$\alpha_1$ (%)	$\alpha_2$ (%)	$\beta$ (%)	$\gamma$ (%)	x (%)
wild*	f	43 <sub>11</sub> (39-50)	8 <sub>11</sub> (6-10)	16 <sub>11</sub> (11-19)	21 <sub>11</sub> (16-30)	10 <sub>11</sub> (7-13)	2 <sub>11</sub> (1-3)
wild**	f	39 <sub>16</sub> (30-43)	6 <sub>16</sub> (5-8)	17 <sub>16</sub> (16-19)	25 <sub>16</sub> (18-33)	13 <sub>16</sub> (6-19)	2 <sub>16</sub> (1-3)
wild	m	41 <sub>22</sub> (36-45)	7 <sub>22</sub> (4-12)	15 <sub>22</sub> (14-20)	22 <sub>22</sub> (16-26)	11 <sub>22</sub> (8-16)	2 <sub>22</sub> (1-4)

\* Values without animals caught directly after PVD 2002

\*\* Values of animals caught directly after PDV 2002

Table 3: Total serum protein fractions of free-ranging harbor seals of the age-class “older” (>2yrs) differentiated by sex. The first number indicates the median with the count as index, the numbers within the parentheses depicted the percentile range (5-95 %).

Animal	Sex	Alb (g/l)	$\alpha_1$ (g/l)	$\alpha_2$ (g/l)	$\beta$ (g/l)	$\gamma$ (g/l)	x (g/l)
wild*	f	34 <sub>11</sub> (31-38)	6 <sub>11</sub> (4-8)	12 <sub>11</sub> (9-15)	16 <sub>11</sub> (11-27)	8 <sub>11</sub> (5-10)	1 <sub>11</sub> (1-2)
wild**	f	31 <sub>16</sub> (29-36)	6 <sub>16</sub> (4-7)	16 <sub>16</sub> (13-18)	21 <sub>16</sub> (15-33)	11 <sub>16</sub> (6-18)	2 <sub>16</sub> (1-3)
wild	m	32 <sub>22</sub> (27-36)	6 <sub>22</sub> (3-8)	12 <sub>22</sub> (10-15)	16 <sub>22</sub> (11-23)	9 <sub>22</sub> (6-13)	2 <sub>22</sub> (1-3)

\* Values without animals caught directly after PVD 2002

\*\* Values of animals caught directly after PDV 2002

### 6.3.2 Rehabilitated harbor seal pups (Tab. 4-6)

In Table 4 no significant difference was found between TSP in female and male pups at admission nor prior to release of the seal center for rehabilitation (quasi-Poisson;  $p > 0.05$ ). However, values compared between admission and release were significantly different. In males only a trend was recognizable ( $p = 0.08$ ), but in females a highly significant increase was shown ( $p = 0.0009$ ).

The same applies for Tables 5 and 6. Except for the percentage of albumin in pups prior to release ( $p = 0.03$ ), no difference could be found between males and females. By contrast, significant shifts were found in the two sexes compared at admission and pre-release blood withdrawals ( $p < 0.05$ ). Albumin and  $\alpha_1$ -globulin displayed a decrease, whereas the other globulins increased during rehabilitation.

Table 4: Total serum protein (TSP) of rehabilitated pups at admittance (pups\_adm) and release (pups\_rel) divided by sex. The first number indicates the median with the count as index, the numbers within the parentheses depicted the percentile range (5-95 %).

Animal	Sex	Age	TSP [g/l]
pups_adm	f	days	60 <sub>25</sub> (48-70)
pups_adm	m	days	66 <sub>19</sub> (43-68)
pups_rel	f	~ 3 months	68 <sub>12</sub> (63-74)
pups_rel	m	~ 3 months	66 <sub>10</sub> (65-70)

Table 5: Proportions of serum protein fractions of rehabilitated pups at admittance (pups\_adm) and release (pups\_rel) divided by sex. The first number indicates the median with the count as index, the numbers within the parentheses depicted the percentile range (5-95 %).

Animal	Sex	Alb (%)	$\alpha_1$ (%)	$\alpha_2$ (%)	$\beta$ (%)	$\gamma$ (%)
pups_adm	f	65 <sub>25</sub> (60-70)	11 <sub>25</sub> (8-16)	10 <sub>25</sub> (7-16)	9 <sub>25</sub> (6-12)	3 <sub>25</sub> (2-4)
pups_adm	m	65 <sub>19</sub> (59-73)	11 <sub>19</sub> (9-15)	12 <sub>19</sub> (9-15)	8 <sub>19</sub> (7-10)	3 <sub>19</sub> (2-6)
pups_rel	f	53 <sub>12</sub> (46-56)	7 <sub>12</sub> (5-13)	20 <sub>12</sub> (15-24)	13 <sub>12</sub> (11-15)	7 <sub>12</sub> (3-12)
pups_rel	m	55 <sub>10</sub> (50-57)	9 <sub>10</sub> (5-14)	18 <sub>10</sub> (14-22)	12 <sub>10</sub> (10-14)	6 <sub>10</sub> (3-7)

Table 6: Total serum protein fractions of rehabilitated pups at admittance (pups\_adm) and release (pups\_rel) divided by sex. The first number indicates the median with the count as index, the numbers within the parentheses depicted the percentile range (5-95 %).

Animal	Sex	Alb (g/l)	$\alpha_1$ (g/l)	$\alpha_2$ (g/l)	$\beta$ (g/l)	$\gamma$ (g/l)
pups_adm	f	40 <sub>25</sub> (30-46)	7 <sub>25</sub> (4-10)	6 <sub>25</sub> (4-9)	5 <sub>25</sub> (3-7)	2 <sub>25</sub> (1-3)
pups_adm	m	41 <sub>19</sub> (27-45)	7 <sub>19</sub> (3-11)	8 <sub>19</sub> (5-9)	5 <sub>19</sub> (4-7)	2 <sub>19</sub> (1-3)
pups_rel	f	35 <sub>12</sub> (31-37)	4 <sub>12</sub> (3-10)	13 <sub>12</sub> (11-16)	9 <sub>12</sub> (7-10)	4 <sub>12</sub> (2-9)
pups_rel	m	36 <sub>10</sub> (32-39)	6 <sub>10</sub> (3-10)	12 <sub>10</sub> (10-15)	8 <sub>10</sub> (7-9)	4 <sub>10</sub> (2-5)

### 6.3.3 Captive harbor seals (Tab. 7-9)

The densitograms of the harbor seals held in captivity all look very much alike (Fig. 5-8). There is no significant difference in age or sex visible.

The TSP in the young male and female seals (*Hinnerk*: 3 weeks; *Lilli*: 2 weeks) differed significantly from the older captive animals (*Lümmel*: 17 years, *Hein*: 6½ years, *Mareike*: 19

months; Tab. 7;  $p < 0.05$ ). The differences between the older animals as well as variations within one animal are also substantial.

Proportions and total numbers of the serum protein fractions (Tab. 8, 9) only show conformability in the age class of the young animals. The two adult males (*Lümmel* and *Hein*) differ significantly between each other as well as from the 19 months old female *Mareike*. Unfortunately, sample sizes are very small yet.

Table 7: Total serum protein (TSP) of the captive animals of the Seal Center Friedrichskoog. The first number indicates the mean with the count as index, the numbers within the parentheses depicted the min. and max., if more than one sample was taken.

Animal	Sex	Age	TSP [g/l]
Lümmel	m	older	90 <sub>2</sub> (82-99)
Hein	m	older	79 <sub>3</sub> (68-85)
Mareike	f	19 months	85
Hinnerk	m	3 weeks	66
Lilli	f	2 weeks	68

Table 8: Proportion of serum protein fractions of captive animals of the Seal Center Friedrichskoog. The first number indicates the mean with the count as index, the numbers within the parentheses depicted the min. and max., if more than one sample was taken.

Animal	Sex	Alb (%)	$\alpha_1$ (%)	$\alpha_2$ (%)	$\beta$ (%)	$\gamma$ (%)
Lümmel	m	47 <sub>2</sub> (42-50)	4 <sub>2</sub> (3-4)	18 <sub>2</sub> (18-18)	13 <sub>2</sub> (12-14)	17 <sub>2</sub> (12-18)
Hein	m	54 <sub>3</sub> (49-61)	5 <sub>3</sub> (4-8)	17 <sub>3</sub> (15-19)	10 <sub>3</sub> (7-9)	14 <sub>3</sub> (7-18)
Mareike	f	57	6	16	10	11
Hinnerk	m	53	14	12	9	12
Lilli	f	53	14	13	10	10

Table 9: Total serum protein fractions of captive animals of the Seal Center Friedrichskoog. The first number indicates the mean with the count as index, the numbers within the parentheses depicted the min. and max., if more than one sample was taken.

Animal	Sex	Alb (g/l)	$\alpha_1$ (g/l)	$\alpha_2$ (g/l)	$\beta$ (g/l)	$\gamma$ (g/l)
Lümmel	m	41 <sub>2</sub> (40-42)	3 <sub>2</sub> (3-4)	16.5 <sub>2</sub> (15-18)	12 <sub>2</sub> (10-14)	15 <sub>2</sub> (12-18)
Hein	m	40 <sub>3</sub> (37-42)	4 <sub>3</sub> (3-5)	13 <sub>3</sub> (10-14)	6 <sub>3</sub> (5-8)	10 <sub>3</sub> (5-15)
Mareike	f	49	5	13	7	6
Hinnerk	m	35	9	8	6	8
Lilli	f	36	10	9	7	7

## 6.4 Discussion

As neither dilution with buffer nor shaking with  $C_2Cl_3F_3$  had any effect at all, only unbiased densitograms are discussed.

### 6.4.1 Free-Ranging Harbor Seals

Looking at the TSP it is obvious that there was no difference between sexes of animals caught around the PDV out-break, but very well between age-classes (Table 1). The PDV infected harbor seal females depicted an increase of TSP of 15%, which is reflected in a decrease in albumin and  $\alpha_1$ -globulins and an increase in  $\beta$ - and  $\gamma$ -globulins (Table 1-3). An increase in  $\beta$ - and  $\gamma$ -globulins has been described in anemia, acute phase response ( $\beta$ -globulins) and in respiratory infections ( $\gamma$ -globulins). A decrease of  $\alpha_1$ -globulins has been specifically seen in animals with emphysema. Hypoalbuminemia is present in many diseases, namely in renal and liver disorders (Kennedy 1990, Dierauf and Gulland 2001, Müller *et al.* 2004). All of the above mentioned afflictions were diagnosed in PDV infected harbor seals, dissected at the Research and Technology Center Westcoast in Büsum, Germany (Müller *et al.* 2004, Seibel *et al.* submitted).

Analysis of the densitograms of the wild catches proved to be rather difficult. The actual contamination was most likely caused by lipemia, which is a common phenomenon in marine mammals such as harbor seals (Tryland & Brun 2001). After finding the best voltage, ampere and separation time it was possible to distinguish several peaks, however. Still the densitograms look a lot different from the other groups. In Davis *et al.* (2001) the high molecular weight of the apolipoprotein E (apoE) in pinnipeds, especially in harbor seals, is mentioned. They found the molecular mass of apoE in cetaceans homologous to that of human apoE (35 kDa). In contrast, in the lipoproteins of pinnipeds (harbor seal, sea lion, walrus) there was no protein comparable in size to human apoE. Its molecular weight was measured to be 42 kDa in harbor seals. ApoE is known to be essential for the normal catabolism of triglyceride-rich lipoprotein constituents. It was initially recognized for its importance in lipoprotein metabolism and cardiovascular disease. None of the investigated marine mammals (bottlenose dolphin, killer whale, harbor seal, sea lion, walrus) showed high levels of LDL cholesterol or triglycerides, despite the fact that they consume a diet that is extremely rich in cholesterol and fats. Davis *et al.* (2001) also found high levels of HDL cholesterol in harbor seals. If HDL cholesterol is protective against atherosclerosis in these

animals, as it is in humans, it might help explain why atherosclerosis is rarely reported for pinnipeds.

Davis *et al.* (2001) only tested captive seals from Sea World, San Diego, CA, USA. One reason for the difference in the SPEP could be that the wild catches dispose a higher amount of lipoproteins, therefore also of apoE, that might interfere with the migration of serum proteins and blur the bands on the cellulose acetate. Another possibility is that the present lipoproteins do not separate due to their pH-value, but are dependent solely on the electroendosmose. In this case the lipid particles in harbor seals were bigger compared to other mammals, so that not even after the treatment with C<sub>2</sub>Cl<sub>3</sub>F<sub>3</sub> they were extractable. It is also possible that the pK-value of the protein fraction is only slightly acidic or even alkaline. Ultracentrifugation might bring clarification of the facts (pers. comm. Prof. Dr. Thomas). Why only the wild catches show this phenomenon is not clear yet.

#### 6.4.2 Rehabilitated Pups

An increase in TSP is only seen in male pups during their approximately 3 months rehabilitation period (Table 4). A difference between female and male pups is not reflected in the protein fractions, however (Table 5, 6). Especially in Table 5, but also in Table 6, the serum protein shift from albumin towards  $\alpha_2$ -,  $\beta$ - and  $\gamma$ -globulins is obvious. Pups admitted to the rehabilitation center had hardly any  $\gamma$ -globulins at all. This fraction mainly comprises the immunoglobulins, namely IgG. Neonates are not able to produce IgG themselves and are therefore temporarily dependent on the antibodies of their mothers, which are usually imbibed by the milk. Intrauterine the transfer takes place through the placenta (Tizard 2000, Kraft and Dürr 2005). It can be seen that after a period of about 3 months the pups have developed IgG to some extent (Table 5, 6). But still not as much as the pups born at the seal center and nursed by their mothers (Table 8, 9).

#### 6.4.3 Captive Harbor Seals

The TSP in the young male and female seals (*Hinnerk*, 3 weeks; *Lilli*, 2 weeks) differed from the older seals (*Mareike*, 19 months; *Hein*, 6½ years) (Tab.7). The former consort with the total protein of the young rehabilitated pups, whereas the wild catches show a slightly higher amount of TSP than the other groups.

In Hall (1998) differences in the serum chemistry of gray seals were clearly related to age and sex. The densitograms of the animals of the Seal Center Friedrichskoog show very little differences, however.

The  $\alpha_1$ -peak of the captive seals is very near the albumin fraction (Fig. 5-8). It seems that the acute phase proteins of this subunit are relatively more pronounced, whereas the fraction that contains LDL, haptoglobin and macro-globulins is less pronounced than in the other seal group or other mammals. The end of the graphs of some of the captive animals as well as of the wild catches shows a distinct peak of a protein that cannot be found in the pups (nor humans). It seems that an unknown stationary protein is present at the starting point of the electrophoresis.

In comparison with two terrestrial mammals (dog and cattle) the results show slightly more conformity with dogs than with cattle (Table 10, 11). Especially the percentage of albumin lies completely outside the bovine range. Other ranges of all three species are rather similar.

Reference values of serum proteins for many terrestrial animals (e.g. dogs, cats, cattle, horses) are known. In terrestrial mammals it is known that young animals have lower total serum proteins than adults (Kraft and Dürr 2005). This observation could be reinforced by this study. Free-ranging young harbor seals as well as captives show significant lower TSP than conspecific adults (Tab. 1, 7).

Table 10: Comparison of ranges of serum protein proportions [%] of different terrestrial mammals and adult free-ranging harbor seals.

%	Canine*	Bovine*	Harbor Seal (free-ranging)
albumin	47-59	51-59	39-50
$\alpha_1$ -globulin	4-7	2-7	6-10
$\alpha_2$ -globulin	5-12	4-8	11-19
$\beta$ -globulin	21-38	13-22	16-30
$\gamma$ -globulin	8-18	8-12	7-13

\*Reference values from Kraft and Dürr 2005



Table 11: Comparison of ranges of serum protein fractions [g/l] of different terrestrial mammals and adult free-ranging harbor seals.

<b>g/l</b>	<b>Canine*</b>	<b>Bovine*</b>	<b>Harbor Seal (free-ranging)</b>
TSP	54-75	60-80	70-91
albumin	25-44	30-42	27-36
$\alpha_1$ -globulin	2-5	1-3	3-8
$\alpha_2$ -globulin	3-10	3-6	9-15
$\beta$ -globulin	11-27	9-16	11-27
$\gamma$ -globulin	4-14	6-8	5-13

\*Reference values from Kraft and Dürr 2005

Hall *et al.* (1998) found out that the chance of survival of gray seal pups is decreasing with an increasing value of immunoglobulin G (IgG). This could not be confirmed by this study. IgG was almost none-existent in pups brought to the seal center but all were able to develop it during the rehabilitation period. The re-catch rate is almost 0% within the first year after release. Therefore it is presumed that the animals rehabilitated at the Seal Center Friedrichskoog are capable of producing further immunoglobulins once exposed to their natural habitat.

This study of the different harbor seal groups has supported the results of Hall *et al.* (2003) who found no sexual dimorphism in southern elephant and gray seals concerning early immunological development in pinnipeds. Just as described in the previous three chapters of this thesis the data have proven that it is important to differentiate between age-classes and groups. Even though in some cases sample size is still small, guidelines for further investigations could be given and baseline values presented. With the help of this study particularly renal and liver diseases but also chronic inflammation can be detected and treatment can be employed accordingly. Further sampling of mainly the captive group is necessary.

## **Final Conclusion**

Results of this thesis provide reference values not previously described for the applied assays (differential hemogram, Phagotest<sup>®</sup>, Phagoburst<sup>®</sup> and serum electrophoresis) for three different harbor seal groups (free-ranging, rehabilitated, and captive). High sample sizes allowed further sub-divisions of the seal groups for most values and additional differences between these sub-groups could be analyzed. A first important conclusion based on the used assays was that captive animals often differ extremely individually, so that pooling them might give a deceptive picture.

The different assays applied in this thesis proved to complement one another. They demonstrated that the differential hemogram is an important tool to gain a broad overview of the health status of the animals. Results of the phagocytosis and respiratory burst test are more sensitive and show deviant behavior of the immune system earlier than the differential hemogram might do. To get more detailed information additional tests of the used assays can help to analyze the cause of neutrophilia (increased neutrophil granulocytes) in the examined harbor seal groups.

Neutrophils play an important role in the defense against bacterial infections, which hold the largest proportion in diseases of marine mammals in the German North Sea (Siebert *et al.* 2001, Siebert *et al.* submitted).

Differences between captive and free-ranging seals, concerning physical and physiological conditions of latter animals, have been subject to some studies and were pointed out before (Boily *et al.* 2006, Dierauf and Gulland 2001, Ross *et al.* 1996, Swart *et al.* 1995). Some of these differences could not be supported by the work done for this thesis (e.g. higher hemoglobin values in free-ranging seals). Concerning the differential hemogram not much difference was found between free-ranging and captive animals. Even though one might expect a higher value of erythrocytes and their derivatives in free-ranging animals due to more activity and deeper dives, only rehabilitated pups show the expected lower values. Captive animals of the Seal Center Friedrichskoog seem to have the same opportunity for exercise as free-ranging animals. During rehabilitation pups are bound to limited space. This is a common and necessary procedure in order to be able to handle the animals. After a certain time, they are transferred to a bigger and deeper pool and are prepared for their release. Free-

ranging animals from California for example show higher HB values than animals held in human care (Dierauf and Gulland 2001). The waters at the Pacific coast are deeper than in the North Sea and animals have to dive more extensively. Thus different environments also play an important role when comparing hemogram values.

Eosinophilia (increased eosinophil granulocytes) was evident in free-ranging animals, as they are subject to parasite infested fish, but the high values in harbor seals of 1½ years of age have not been described before. These results were confirmed in pathological findings of harbor seals of that age group (Lehnert *et al.* submitted, Siebert *et al.* submitted). It seems that animals of that age have accumulated most parasites and either survive and build up a kind of immunity or die. Infestations of parasites of adult animals have been considerably lower than in young seals. Captive animals and rehabilitated pups at the seal center are fed pre-frozen and inspected fish and should therefore have no contact to parasites, thus no eosinophilia.

Seasonal changes in phagocytosis could be observed not only in free-ranging animals but also in captive harbor seals. These changes have never been reported before. The decrease of phagocytic activity of pups during rehabilitation has also never been mentioned before. To fully understand the phagocytic mechanism in harbor seals additional phagocytosis tests should be performed.

While results of the oxidative burst test were not as incisive as of the Phagotest<sup>®</sup>, results suggest that harbor seals can be identified relative to their immunological status. For neutrophil stimulation future examinations should focus on the use of *E. coli* as stimulus, as it seems to be more physiological than the also provided PMA. In order to apprehend the whole extent of the oxidative burst, future studies should be directed at a more extensive analysis of the events involved in respiratory burst of harbor seal neutrophils.

It was the first time serum electrophoresis has been conducted and densitograms are shown of free-ranging, rehabilitated and captive harbor seals. Comparisons of the three groups showed statistically significant differences. Especially the densitograms of free-ranging animals indicate the need for further handling of the serum before performing electrophoresis (e.g. ultracentrifugation). During rehabilitation pups were able to produce IgG but by far not as much as pups born in captivity, which were nursed. Hence, the importance of their mothers' milk and an adequate milk substitute for pups in rehabilitation has been

illustrated. Serum protein electrophoresis is an important complement to the previous tests as it looks at a different part of the immune system. It presents the different protein groups (albumin and globulins) operative in the immune response.

## Zusammenfassung

Die Ergebnisse dieser Arbeit liefern Referenzwerte für die verschiedenen angewandten Analysen (Differential Blutbild, Phagotest<sup>®</sup>, Phagoburst<sup>®</sup> und Serum Elektrophorese), die bisher noch nicht beschrieben wurden. Es wurden drei verschiedene Gruppen von Seehunden (freilebende, rehabilitierte und Stationstiere) untersucht. Eine große Datenmenge erlaubte oftmals eine weitere Aufteilung der Blutwerte, wobei zusätzliche Differenzierungen der Ergebnisse festgestellt werden konnten. Eine erste wichtige Erkenntnis aufgrund der Analysen ist, dass in Gefangenschaft gehaltene Tiere individuell sehr verschieden sind, sodass eine Zusammenfassung der Werte ein irreführendes Ergebnis bringen könnte.

Es wurde dargelegt, dass die verschiedenen Analysen sich gegenseitig ergänzen. Das Differential Blutbild erwies sich als gutes Hilfsmittel, um einen ersten Überblick über den Gesundheitszustand der Tiere zu gewinnen. Ergebnisse der Phagozytose- und Bursttests reagieren sensibler und zeigen Abweichungen des Immunsystems in einem früheren Stadium als das Differential Blutbild. Um genauere Informationen zu erhalten, könnten weitere Tests der angewandten Analysen helfen, unter anderem die Ursachen für Neutrophilie (erhöhte Anzahl der neutrophilen Granulozyten) in den Seehundgruppen zu ergründen.

Neutrophile Granulozyten spielen eine wichtige Rolle bei der Bekämpfung von bakteriellen Infektionen, die den größten Anteil an Erkrankungen von marinen Säugern in der deutschen Nordsee darstellen (Siebert *et al.* 2001, Siebert *et al.* eingereicht).

Unterschiede der physischen und physiologischen Kondition zwischen freilebenden und Stationstieren waren bereits Thema von früheren Untersuchungen (Boily *et al.* 2006, Dierauf and Gulland 2001, Ross *et al.* 1996, Swart *et al.* 1995). Einige dieser Ergebnisse konnten mit der vorliegenden Arbeit nicht unterstützt werden (z.B. erhöhter Hämoglobinwert in freilebenden Seehunden). Bezüglich des Differential Blutbildes konnten keine gravierenden Unterschiede zwischen freilebenden und Stationstieren gefunden werden. Obwohl man erhöhte Werte der Erythrozyten und deren Derivate in freilebenden Seehunden vermuten würde (höhere Aktivität, tiefere Tauchgänge), wurden nur in rehabilitierten Heulern die erwarteten niedrigeren Werte nachgewiesen. Tiere, die in der Seehundstation Friedrichskoog leben haben offensichtlich ähnliche Möglichkeiten sich zu bewegen wie ihre Verwandten in der deutschen Nordsee. Während der Rehabilitation steht Heulern nur ein begrenzter Raum

zur Verfügung. Dies ist üblich und erforderlich, um sich angemessen den Tieren widmen zu können. Untersuchte Seehunde vor der kalifornischen Küste beispielsweise, zeigten höhere Hämoglobinwerte als Tiere, die unter menschlicher Obhut stehen. Die Gewässer der pazifischen Küste sind jedoch sehr viel tiefer als die Nordsee und die Tiere sind gezwungen, bei ihren Beutezügen tiefer und länger zu tauchen. Dies verdeutlicht, dass auch verschiedene Umgebungen eine wichtige Rolle beim Vergleich von Blutwerten spielen.

Eosinophilie war evident in freilebenden Seehunden, da sie Zugang zu parasitiertem Fisch haben. Der sehr hohe Wert von 1½-jährigen Seehunden wurde bisher jedoch nicht beschrieben. Die Ergebnisse wurden von pathologischen Befunden von Seehunden dieser Altersgruppe bestätigt (Lehnert *et al.* eingereicht, Siebert *et al.* eingereicht). Es scheint, dass diese Tiere die meisten Parasiten akkumuliert haben und entweder dies überleben und eine Art Immunität aufbauen, oder versterben. Der Parasitenbefall von älteren Tieren war dagegen wesentlich geringer. Stationstiere und rehabilitierte Heuler werden mit vorgefrorenem und inspiziertem Fisch gefüttert und sollten daher keinen Kontakt mit Parasiten haben, daher auch keine Eosinophilie.

Saisonale Unterschiede bei der Phagozytose konnten nicht nur bei freilebenden, sondern auch bei Stationstieren beobachtet werden. Diese Abweichungen wurden bisher nicht beschrieben. Die Abnahme der Phagozytoseaktivität bei Heulern während der Rehabilitation wurde ebenfalls noch nie erwähnt. Um den Mechanismus der Phagozytose bei Seehunden vollständig zu verstehen, sollten weitere Tests durchgeführt werden.

Obwohl die Ergebnisse des oxidativen Bursttests nicht so prägnant waren wie die des Phagotests<sup>®</sup>, zeigen die Resultate, dass Seehunde relativ zu ihrem Immunstatus identifiziert werden können. Zur Stimulation von neutrophilen Granulozyten sollte sich in Zukunft auf *E. coli* konzentriert werden, da diese offensichtlich einen physiologischeren Stimulus darstellen als das ebenfalls angewendete PMA. Um das gesamte Ausmaß des oxidativen Bursts verstehen zu können, sollten zukünftige Untersuchungen auf eine intensivere Analyse der Vorgänge konzentrieren, die sich während des respiratorischen Bursts in neutrophilen Granulozyten von Seehunden abspielen.

Zum ersten Mal wurde bei freilebenden, rehabilitierten und unter Obhut lebenden Seehunden eine Serum Elektrophorese durchgeführt und deren Densitogramme beschrieben.

Vergleiche der drei Gruppen zeigten statistisch signifikante Unterschiede. Besonders die Densitogramme der freilebenden Tiere weisen darauf hin, dass eine weitere Bearbeitung der Probe nötig ist, bevor die Elektrophorese durchgeführt wird (z.B. Ultrazentrifugation). Während der Rehabilitation zeigte es sich, dass Heuler in der Lage sind IgG zu bilden, jedoch nicht in dem Maße wie Tiere, die in Gefangenschaft geboren und von ihren Müttern gesäugt wurden. Die große Bedeutung der Muttermilch und deren adäquater Ersatz für die Heuler wurde hiermit verdeutlicht. Serumprotein Elektrophorese ist eine wichtige Ergänzung zu den vorigen Tests, da sie einen anderen Part des Immunsystems beleuchtet. Sie repräsentiert die verschiedenen Proteingruppen (Albumin und Globuline), die im Immunsystem wirken.

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## ABBREVIATIONS

BD:	Becton Dickinson Biosciences
DHR:	dihydrorhodamine
DNA:	deoxyribonucleic acid
E. coli:	Escherichia coli
EDTA:	ethylenediaminetetraacetic acid
FACS:	fluorescence activated cell
FITC:	fluorescein isothiocyanate
fMLP:	N-formyl-methionyl-leucyl-phenylalanine
FSC:	forward scatter
granulo(s):	granulocytes
HB:	hemoglobin
HCT:	hematocrit
HDL:	high density lipoprotein
Hp:	haptoglobin
IgA/D/E/G/M:	immunoglobulin A/D/E/G/M
LDL:	low density lipoprotein
leuko(s):	leukocytes
LPS:	lipo-polysaccharide
MCH:	mean cellular/corpuscular hemoglobin
MCHC :	mean cellular/corpuscular hemoglobin concentration
MCV:	mean cellular/corpuscular volume
MELISA:	metal enzyme-linked immunosorbent assay (test for diagnosis of metal sensitivity)
MHC:	major histocompatibility
MINOS:	marine warm-blooded animals in the North and Baltic Seas
mono(s):	monocytes
neutro(s):	neutrophil granulocytes
NK cells:	natural killer cells
PBS:	phosphate-buffered saline
PDV:	phocine distemper virus
PLT:	platelets, thrombocytes
PMA:	phorbol 12-myristate 13-acetate

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pup_adm:	pups admitted to the rehabilitation center
pup_rel:	pups prior to release after rehabilitation
Pv:	<i>Phoca vitulina</i>
RBC:	red blood cells
RDW:	red cell distribution width
rehab_pups_last:	last blood withdrawal of rehabilitated pups prior to release
RNA:	ribonucleic acid
ROS:	reactive oxygen species
RT <sub>3</sub> U:	resin T <sub>3</sub> uptake
SPEP:	serum protein electrophoresis
SSC:	sidescatter
T <sub>3</sub> :	triiodothyronine
T <sub>4</sub> :	thyroxine
TMAP:	Trilateral Monitoring and Assessment Program
TSP:	total serum protein
wild:	free-ranging
WKK:	Westküstenklinikum
w_older_spr:	free-ranging animals > 2years caught in spring
w_older_su:	free-ranging animals > 2years caught in summer
w_older_win:	free-ranging animals > 2years caught in winter
w_pups_spr:	free-ranging pups caught in spring (not available)
w_pups_su:	free-ranging pups caught in summer
w_pups_win:	free-ranging pups caught in winter
w_yearlings_spr:	free-ranging yearlings caught in spring
w_yearlings_su:	free-ranging yearlings caught in summer
w_yearlings_win:	free-ranging yearlings caught in winter
WBC:	white blood cells
WF:	free-ranging

## APPENDIX A

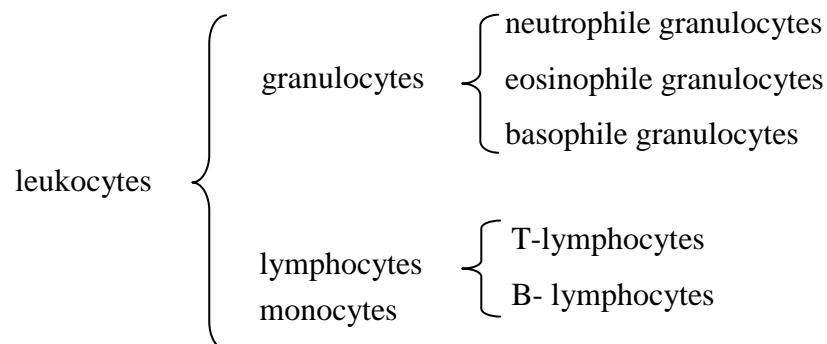
### Facts and Functions of Blood Components

Blood is the body fluid that carries an abundance of information about the physiological health status of an animal. The major tasks of the blood are

- transport of: nutrients, gas (O<sub>2</sub>, CO<sub>2</sub>), catabolism products, hormones, and enzymes
- regulation of the body temperature
- regulation of the inner environment, e.g. pH, osmotic pressure, ion synthesis, glucose level
- and defense of foreign substances

The blood consists of blood cells, thrombocytes and plasma. Blood cells are differentiated as follows:

erythrocytes



### A.1 Red Hemogram

The *red hemogram* describes the red blood cells (erythrocytes) and the connected blood values and thrombocytes.

*Erythrocytes* are developed in the bone marrow. They do not have a cell nucleus nor other cell components. They lose their components secondarily during their ontogenesis. The erythrocytes are responsible for the transfer of the respiration gases. The part of soluble

oxygen is very low and therefore plays only a minor role. The development of erythrocytes is mainly controlled by hormones. Lack of oxygen leads to increased distribution of a factor (REF) in the kidneys and a globulin in the liver. Combined they represent the Erythropoetin, which stimulates the development of erythrocytes in the bone marrow. Additionally, the development of erythrocytes can most likely be influenced by the central nervous system (Silbernagel & Despopoulos 1990).

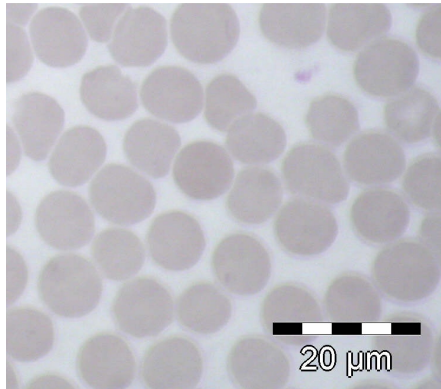


Fig. 1: Erythrocytes (+ thrombocyte) of *Phoca vitulina* in peripheral blood  
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Blood values that are connected to the erythrocytes are the hematocrit (HCT), the hemoglobin (HB), the mean corpuscular hemoglobin (MCH), the mean corpuscular hemoglobin concentration (MCHC), the mean corpuscular volume (MCV), and the red cell distribution width (RDW).

The hematocrit is usually presented in percentage and shows the ratio between the blood fluid and the corpuscles. The higher the value the more corpuscles are in the circulating blood (e.g. dehydration).

The hemoglobin is the main ingredient of the erythrocyte and serves as transport for oxygen. On each hemoglobin molecule four oxygen molecules can be bound. Due to the high MCV of harbor seals, more hemoglobin can be bound than in humans or many other terrestrial animals.

The mean corpuscular hemoglobin (MCH) represents the hemoglobin content of a single erythrocyte. The mean corpuscular hemoglobin concentration (MCHC) defines the medium cellular hemoglobin concentration. The mean corpuscular volume (MCV) describes the volume content of a single erythrocyte. In general, it represents the size of an erythrocyte. The larger the MCV the more HB can be bound the more oxygen can be transported. The red

cell distribution width (RDW) describes the distribution frequency of the erythrocyte volume. The MCH, MCHC, MCV and RDW were routinely calculated and serve for assessment of different impairments of the hemopoiesis.

The *thrombocytes* (200-500 t/ $\mu$ l; dog reference value, Knickel *et al.* 2002) also belong to the *red hemogram* although they are not directly connected to the red blood cells. They are cell fragments without a nucleus but are surrounded by a plasmalemma. Inside, they consist of pale basophilic cytoplasm and tiny azurophilic granules. Therefore they are not really cells but platelets. They are developed by separating from the cytoplasm of the megakaryocytes of the bone marrow. Their lifespan is about 12 days. Thrombocytes are the smallest particles in the blood. They carry the coagulation factors and are therefore responsible for the blood coagulation (Silbernagel & Despopoulos 1990, Barthels & von Depka 2002).

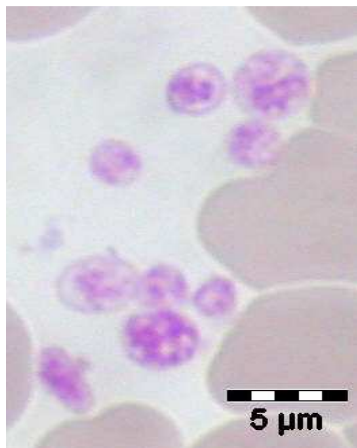


Fig. 2: Thrombocytes of *Phoca vitulina* in peripheral blood.  
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## A.2 White Hemogram

The *white hemogram* describes the white blood cells (leukocytes) and their derivatives. In all mammals, the *granulocytes* numerically form the majority (60-85%, dog reference value, Knickel *et al.* 2002) of the leukocytes. Within the granulocytes the *neutrophils* comprise again the majority (60-80%, dog reference value, Knickel *et al.* 2002). They are also produced in the red bone marrow. The mean lifespan is about one day and their function lies not only in blood and tissue but also on the mucous membranes (Roitt *et al.* 1993). The neutrophils contain a high proportion of granula, which can be divided into azurophilic and specific components. In order to defend the body against micro-organisms, the granula contain many different enzymes and proteins in different distributions (e.g. myeloperoxidase, lysosymes, neutral proteinase, acidic hydrolase). When, for instance, bacteria penetrate tissue,



the neutrophils are attracted by chemical foreign substances or endogenic complement factors (chemotaxis). In blood circulating neutrophils attach to the endothel of the blood vessel (margination) and passes through it (diapedesis). They move towards the damaged area of the tissue (migration), enclose the agent, and assimilate it endocytotically (phagocytosis) (Roitt *et al.* 1993, Janeway *et al.* 2002).

Even though bacteria can be directly bound by neutrophils, their “appetite” can be increased considerably, when the agent’s surface is marked with an unspecific complement factor, an antigene-specific immunoglobulin or both (opsonization). These processes result – together with increased blood circulation and an increased permeability of the capillaries for proteins – in an inflammation.

The destruction or degradation of the bacteria in the neutrophils is based on oxygen dependent as well as on oxygen independent mechanisms. Within the oxygen *independent* degradation lysosomes (= granula with lysosymes), together with phagosomes (= vacuoles with incorporated micro-organisms), form the so called phagolysosomes. Within these the pH-factor is reduced to an acidic environment. On one hand the decrease induces a direct damage to the bacteria and on the other hand the low pH represents an optimum for many lysosome enzymes (Roitt *et al.* 1993).

Within the oxygen dependent degradation of the bacteria reactive oxygen derivatives are created (e.g. superoxid anions, hydrogen peroxide, hydroxy radicals). These metabolites act microbicidal (Roitt *et al.* 1993). Usually the concentration of these oxidants is reduced to a certain level by enzymes like catalases and superoxide dismutase. This prevents self-damage of the granulocytes and the surrounding tissue. The protection is abandoned, however, when there is an invasion of agents, in order to fully develop the bactericidal effect of the oxygen compounds (respiratory burst). During this process the neutrophils and cells of the surrounding tissue can be destroyed, which results in what is widely known as pus. In the long run indigestible particles will be deposited in the body (Silbernagel & Despopoulos 1990, Tizard 2000). The respiratory burst will be further described in the respective part of this thesis (Chapter 3; Appendix C).

In summary it can be said that the main task of the neutrophils is the phagocytosis. They accumulate according to the unspecific immune reaction very fast and in high numbers. However, their chemical defense is quickly exhausted and their life span is short.

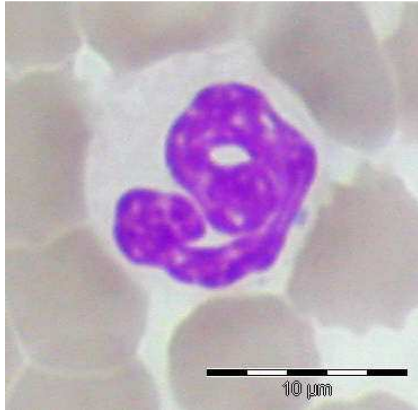


Fig. 3: Mature segmented neutrophil granulocyte of *Phoca vitulina* in peripheral blood.

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The second largest group of granulocytes are the *eosinophiles* (0.0-5.0%, dog reference value, Knickel *et al.* 2002). They are responsible for the disintegration of antigen-antibody-aggregate. They are playing a major role in the specific immune reaction. Their level rises, for example, with an allergic reaction, or with the existence of parasites (Tizard 2000, Janeway 2002). It was shown that the release of IL-5 by T cells, mast cells and macrophages stimulates the production of eosinophils (Begemann 1998, Mahlberg *et al.* 2004).

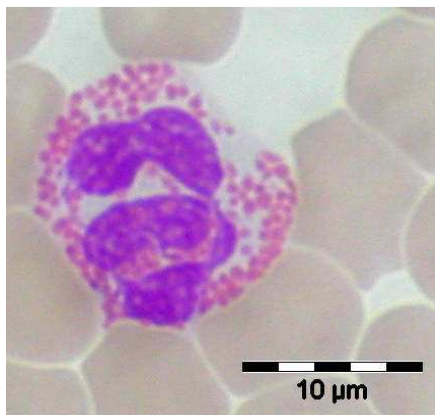


Fig 4: Eosinophil granulocyte of *Phoca vitulina* in peripheral blood.

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With only 0.0-1.0% *basophils* (dog reference value, Knickell *et al.* 2002), they are the minority of the granulocytes. They, too, play an important role with allergy reactions but also with the regulation of the fat blood value (Tizard 2000, Janeway 2002, Mahlberg *et al.* 2004).

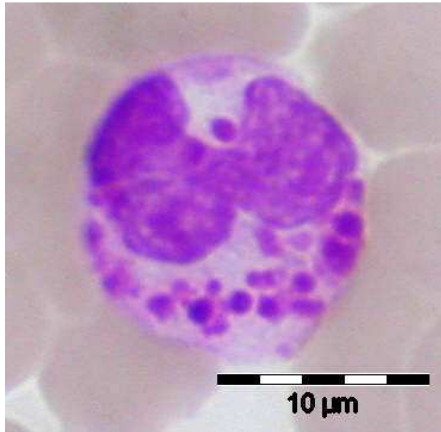


Fig 5: Basophil granulocyte of *Phoca vitulina* in peripheral blood.  
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All granulocytes have ameboid movability. They only stay within the blood stream for a few hours, to get from their place of development, the red bone marrow, to the places of their action in the tissues.

The *monocytes* are also developed in the red bone marrow and make out about 0.0-5.0% (dog reference value, Knickel *et al.* 2002) of the leukocytes. In a second, delayed defense wave the monocytes or macrophages, respectively (monocytes mature to macrophages in tissues), phagocytize the “left-overs” of the neutrophils, including the perished granulocytes themselves. Another central function of the macrophages is the activation of the antigen specific, lymphocidal part of the immune system, which they stimulate with the antigens represented on their surface. They place the necessary signal and messenger substances at disposal by producing and releasing cytokines and lymphokines, respectively (e.g. interleukins, Tumor Necrosis Factor [TNF]) (Roitt *et al.* 1993). Together with the neutrophil granulocytes they are carriers of the unspecific immune system. However, through their ability to express antigens on the cell surface after phagocytosis, and by doing so inform the B- and T-lymphocytes, they are a link between the unspecific and specific immune response. Monocytes are also not end cells but can be transformed further (Begemann 1998, Mahlberg *et al.* 2004).

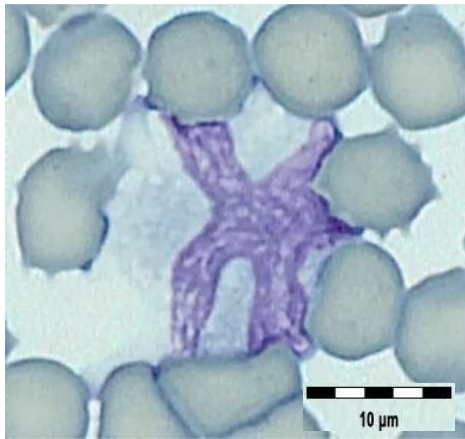


Fig 6: Monocyte of *Phoca vitulina* in peripheral blood.

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The *lymphocytes* also exist in different forms. Their proportion of the leukocytes is about 12-30% (dog reference value, Knickel *et al.* 2002). The place of action of the lymphocytes lies in the tissues, so that they only stay within the blood stream for a few hours. However, they do not have the advantage of ameboid movability. The lymphocytes represent the cellular, antigen specific immune reaction, but they also participate in the unspecific immune reaction. They have glucoproteins on their surface, which are called immunoglobulins and bind certain substances. The specific immune reaction includes the cellular immunity, which is taken care of by the T-lymphocytes, and the humoral immunity, which is carried out by B-lymphocytes. Both types of lymphocytes are developed in the red bone marrow, the completion of the T-lymphocytes is done by the thymus gland. After their activation through the monocytes and steered by T-helper and T-suppressor cells they act later, but infection specific. Their lifespan can be years. The lymphocytes are again no end cells, which means that they can always be transformed.

The humoral immune defense comprises in its entirety in the complement factors and the antigen-specific antibodies (Kress & Eberlein 1992).

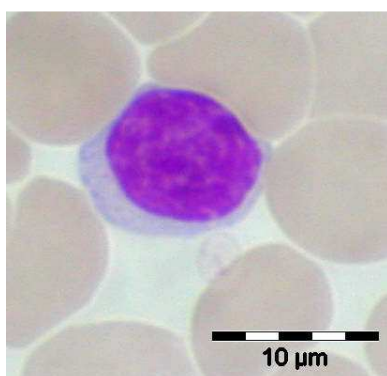


Fig 7: Small lymphocyte of *Phoca vitulina* in peripheral blood.

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## APPENDIX B

### General Overview of the Mechanism of Phagocytosis

“Phagocytosis is the most important defense mechanism in all phyla of the animal kingdom. Among protozoa phagocytosis is not only the means of defense against foreign cells and particles, but also the principle mode of ingesting food....Notwithstanding the increasingly sophisticated humoral and cellular immune defense components that have evolved in the vertebrates, which culminated in the complex immune system of birds and mammals, phagocytosis still remains the principal effector mechanism for the ultimate disposal of invading foreign, effete, or otherwise unwanted cells or particles.” (van Oss 1986).

The *neutrophil granulocytes* (or polymorphonuclear leukocytes, PMN) play the main role among the phagocytic cells. They form our first line of defense against invading particles, especially bacteria. The *monocytes*, which mature in the tissues to macrophages, are also able to perform phagocytosis and are therefore sometimes called *mononuclear phagocytes* (van Oss 1986). The main difference between these two important phagocytic cells is that the mononuclear ones have a much longer life and can proliferate locally (Bessis 1973, Daems 1980). They essentially represent the second line of defense against invading particles.

The trigger of the phagocytic process is the chemotaxis. By forming a concentration gradient, chemical attractants cause the respective cells to migrate into the direction of increased concentration of the attractant. Necrotactic substances from freshly lysed cells are known to attract phagocytic cells at a distance of up to 500  $\mu\text{m}$  (Bessis 1973). But by far the most important chemotactic agent seems to be the complement subfactor C5a (van Oss 1986). In addition, the presence of a cochemotaxin (or cocytotaxin) seems to be required (e.g. nucleotides like ATP or cyclic AMP) (Bessis 1973). The chemotactic role of the complement factor guarantees the migration of PMNs and of monocytic phagocytes to virtually all microorganisms that interact with the immunoglobulins (s. below). Chemotaxis itself seems to be triggered through chemoattractant receptors on the leukocyte surface (Snyderman & Pike 1984).

To move toward the chemotactic signal the phagocytes first throw a cytoplasmic veil (PMNs) or a frilly veil (monocytes and macrophages) forward, which attaches to a solid surface or object. Once the frontal veil is attached, it becomes rigid and the rest of the still quite fluid cytoplasm and nucleus streams toward it, leaving only a tail-like extension (uropod) behind (Bessis 1973).

The first step in the initiation of phagocytic ingestion is adhesion of the particle to the phagocyte's surface. The major physiochemical mechanism of that adhesion may be either a *van der Waals*-type attraction, a hydrophobic, and/or (rarely) an electrostatic attraction, or a receptor-mediated bond (Nagura *et al.* 1977). Once adhesion has ensued, endocytosis of the adhering particle can take place, starting with an invagination of the phagocyte's outer cell membrane which then proceeds to enclose the particle completely in a phagocytic vacuole, or *phagosome*. The phagosome then internalizes and fuses with one or more of the phagocyte's granules (*lysosomes*), which contain a variety of hydrolytic enzymes (van Oss 1986).

Once a phagosome with an ingested particle, has fused with a number of lysosomes, the particle (e.g. bacterium) is exposed to a large variety of hydrolases, which rapidly destroy and digest most bacteria (Sbarra & Strauss 1980). This lytic process does not affect the physical condition of the phagocytic cell itself, because all the enzymatic reactions take place inside the phagosome. The inner lining part of the phagosome used to be the outer membrane of the phagocyte, so that, topologically speaking, the digestion still takes place outside the phagocyte's cytoplasm.

## **B.1 Activation of phagocytes**

Various agents can stimulate or "activate" phagocytes, e.g. they can cause phagocytic cells to reduce their migration and enhance their capacity to spread on surfaces (i.e. make them stop), to increase their phagocytic activity, to become more hydrophilic (van Oss 1978), and to accelerate their metabolic and digestive process. Many such activating agents are produced by lymphocytes and have been known under the general name of *lymphokines* (Khan & Hill 1982). Even agents produced by macrophages themselves (*macrokines*), such as complement components, can also activate phagocytic cells (Bianco *et al.* 1980).

Apart from the influence of the usual lymphokines, phagocytic ingestion of very hydrophobic particles, such as mycobacteria, latex particles, or lipid droplets, also causes an increase in hydrophilicity and in phagocytic activity both in PMNs and in macrophages (van Furth 1980, Adams & Hamilton 1984).

## B.2 Cell-particle interactions

Classically, the interaction between particles and/or cells of various compositions and sizes is held to be the sum of their electrostatic repulsion (living cells and other biological particles generally carry a net negative charge), and their *van der Waals* attraction, also taking into account the interaction with and among molecules of the liquid medium (van Oss 1986). That total interaction is described by the “DLVO theory”, after the original Russian (Derjaguin and Landau) and Dutch (Verwey and Overbeek) authors of that theory (Verwey & Overbeek 1999). Due to the strong hydrophilicity of all blood cells, their mutual *van der Waals* attraction is extremely small. Therefore, their negative surface potential normally keeps the cells about 100 Å apart, which causes a very stable suspension (van Oss 1985). In the normal course of events, bacteria also will be repelled by circulating blood cells (including phagocytes), but that repulsion is proportional to the radius of curvature of the particle or cell, so that the inclination of phagocytes to extrude pseudopodia with extremities of small radii of curvature allows them to overcome the electrostatic repulsion and thus to establish contact with most bacteria (van Oss *et al.* 1972). And as soon as an interparticle or intercellular distance of less than 8 Å is reached, electrostatic repulsions tend to become less dominant and *van der Waals* attractions may locally become predominant and cause adhesion, or specific receptors may become sufficiently close to, e.g. Fc moieties, to attach to them via both electrostatic and *van der Waals* attractions. Generally speaking, in aqueous media, nonopsonized particles will be phagocytized the more actively, the lower their surface tension or the more hydrophobic their surface (van Oss 1979).

At first sight, such interactions should not take place at all in a biological fluid such as blood, but we know, of course, that they do occur all the same. Phagocytosis takes place quite actively in blood and in other biological media, and here also the more hydrophobic particles still are the ones to be most readily engulfed by phagocytes (van Oss 1978). The reason for this may appear somewhat complicated but is actually quite simple: in biological fluids containing plasma proteins, the most hydrophobic particles tend to absorb the most protein.

And IgG is one of the plasma proteins that is absorbed most (Absalom *et al.* 1982). Thus, even in the absence of specific antibodies to the bacterial surface, the more hydrophobic bacteria, when immersed in plasma, aspecifically absorb IgG (up to 3000 to 10,000 molecules per bacterium). A sufficient number of these absorbed IgG molecules appear to have their Fc moieties protruding outward to allow them to bind specifically to the Fc receptors on PMNs and monocytic phagocytes. Phagocytes have Fc receptors only for IgG<sub>1</sub> and IgG<sub>3</sub>. These two immunoglobulins also appear to absorb more strongly to hydrophobic surfaces than the other two isotypes (Absalom *et al.* 1982). In the presence of complement IgG<sub>1</sub> and IgG<sub>3</sub>, adsorbed particles also can trigger the complement cascade and cause additional interaction with phagocytes through their C3b receptors (van Oss 1986). Thus, both in aqueous and in plasma protein-containing media, the most hydrophobic particles are the most phagocytized. In the first case because of the direct hydrophobic and *van der Waals* interactions, and in the second case because of enhanced IgG adsorption, which allows interactions with Fc receptors for adsorbed IgG<sub>1</sub> and IgG<sub>3</sub>.

As by far the most frequent encounters between bacteria (and other particles) and phagocytes take place in biological fluids that comprise immunoglobulins, the interaction between Fc and C3b and their receptors (R) on the phagocytes' surface is of preponderant importance in the initiation of phagocytosis. This holds for aspecific interactions (IgG adsorbs only physically onto hydrophobic bacteria) as well as for specific binding of antibodies, of the IgG<sub>1</sub> and IgG<sub>3</sub> classes, for interaction with Fc-R, and of the IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, and IgM varieties plus complement for interaction with C3b-R.

### **B.3 Phagocytic recognition and pathogenicity**

Most if not all non-encapsulated bacteria are sufficiently hydrophobic to become spontaneously phagocytized, in aqueous fluids directly, and in plasma protein-containing liquids indirectly, because of their hydrophobicity, through adsorption of IgG and the subsequent phagocytosis via Fc and/or C3b receptors. Thus most non-encapsulated bacteria are non-pathogenic because of the ease with which they are phagocytized.

Bacterial capsules are very hydrophilic and as such resist attachment to and engulfment by phagocytes in aqueous liquids, on account of their hydrophilicity, and in plasma protein-containing liquids because any aspecifically adsorbed IgG attaches to the bacterial cell wall



and remains well within the outer limits of the capsule (Stinson & van Oss 1971), which obviates any possibility of contact with phagocytic Fc-R. However, capsular proteins, glycoproteins, or polysaccharides almost invariably are antigenic, so that in due course, i.e. within days after the first exposure to the capsular materials, the host starts producing specific antibodies to the capsule, which then attach to its outer periphery and trigger phagocytic ingestion, via Fc-R and, in the presence of complement, also via C3b-R interactions (Stinson & van Oss 1971).

#### **B.4 Anti-phagocytic strategies of micro-organisms**

Anti-ingestion strategies may be divided into short-term and long-term mechanisms. Among the short-term mechanisms is the envelopment with a hydrophilic capsule, which however prevents phagocytic engulfment only until specific anticapsular antibodies are produced by the host (Stinson & van Oss 1971). Typical micro-organisms that have adopted this approach are *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Streptococcus* spp. with M protein (Davis *et al.* 1973). A longer term mechanism for the prevention of phagocytic ingestion consists of the attachment of IgG molecules to the bacterial cell wall by the Fc moiety, so that the Fab part protrudes.

Anti-digestion strategies may consist of simple outright indigestibility of bacterial cell walls (e.g. *Mycobacteria* spp.) (van Oss *et al.* 1986), or of alterations brought about in the phagosomal membrane (e.g. various parasitic protozoa) (Aikawa & Kilejian 1979). Phagocytotoxic strategies depend mainly on lysing the phagocytes, or their granules. *Streptococcus* spp. can kill phagocytes by lysing their granules (and thus liberating their hydrolases intercellularly) (Davis *et al.* 1973).

## APPENDIX C

### General Overview of the Mechanism of Respiratory Burst

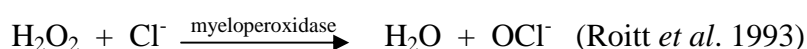
Within seconds of binding to a bacterium, neutrophils increase their oxygen consumption nearly 100-fold. This increase is a result of activation of the cell-surface enzyme NADPH oxidase (Tizard 2000). When the neutrophil is triggered by adherence of a bacterium to a cell receptor, the oxidase is activated. Activated NADPH oxidase converts into NADPH (the reduced form of NADP [nicotinamide adenine dinucleotide phosphate]) to NADP<sup>+</sup> with the release of electrons. One molecule of oxygen accepts a single donated electron, resulting in the generation of one molecule of superoxide anion (the dot in ·O<sub>2</sub><sup>-</sup> denotes the presence of an unpaired electron).



The NADP<sup>+</sup> accelerates the hexose monophosphate shunt, a metabolic pathway that converts sucrose to a pentose and CO<sub>2</sub>, and releases energy for use by the cell. The two molecules of ·O<sub>2</sub><sup>-</sup> interact spontaneously (dismutation) to generate one molecule of H<sub>2</sub>O<sub>2</sub> under the influence of the enzyme superoxide dismutase.



Because this reaction occurs so rapidly, superoxide anion does not accumulate but H<sub>2</sub>O<sub>2</sub> does. The hydrogen peroxide is converted to bactericidal compounds through the action of myeloperoxidase, the most significant respiratory burst enzyme in neutrophils (Roitt *et al.* 1993, Tizard 2000). Myeloperoxidase is found in large amounts in the primary granules. It catalyzes the reaction between hydrogen peroxide and intracellular halide ions (Cl<sup>-</sup>, Br<sup>-</sup>, I<sup>-</sup>, or SCN<sup>-</sup>) to produce hypohalides:



$\text{Cl}^-$  is probably used at most sites in vivo except in milk and saliva, where  $\text{SCN}^-$  is also employed.  $\text{OCl}^-$  is the major product of neutrophil oxydative metabolism (Tizard 2000). Because of its intense reactivity,  $\text{OCl}^-$  does not accumulate in biological systems but instantly disappears in multiple reactions. As long as  $\text{H}_2\text{O}_2$  is supplied (and neutrophils can generate  $\text{H}_2\text{O}_2$  for up to 3 hours after triggering), myeloperoxidase will use plasma  $\text{Cl}^-$  to generate  $\text{OCl}^-$ . It rapidly attacks many biological molecules.  $\text{OCl}^-$  kills bacteria by oxidizing their proteins and enhances the bactericidal activities of the lysosomal enzymes (Tizard 2000). ( $\text{HOCl}$  is the active ingredient of household bleach and is commonly used to prevent bacterial growth in swimming pools.)

There are minor quantitative differences in neutrophil activity between the domestic species, especially in the intensity of the respiratory burst. Sheep neutrophils, for example, appear to produce less superoxide than human or bovine neutrophils. Neutrophils also have defense mechanisms to detoxify oxidizing radicals. Thus they contain large amounts of glutathione, which reduces them. Redox active metals such as iron can be bound to lactoferrin to minimize  $\text{OH}\cdot$  formation, whereas antioxidants, such as ascorbate or vitamin E, interrupt these reactions (Tizard 2000).

## APPENDIX D

### Curriculum vitae

#### PERSÖNLICHE ANGEABEN:

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Name: **Ilka Hasselmeier**  
Staatsangehörigkeit: deutsch  
Geburtstag: 01.02.1966  
Geburtsort: Bremerhaven

#### AUSBILDUNG:

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**Juli '03 - Jan. '07** Doktorandin an der Christian-Albrechts-Universität Kiel;  
Durchführung der Doktorarbeit am Forschungs- und Technologie-  
zentrum (FTZ) Westküste in Büsum

**Febr. '01 - Jun. '03** Doktorandin an der Christian-Albrechts-Universität Kiel;  
Arbeit musste aus technischen Gründen abgebrochen werden.

**Okt. '95 - '00** Diplom-Hauptstudium an der Christian-Albrechts-Universität Kiel;  
Abschluss: Diplombiologin  
Datum: 01.08.2000

**Okt. '93-'95** Diplom-Grundstudium an der Christian-Albrechts-Universität Kiel

**Apr. '86 - Apr. '89** Ausbildung zur Krankenschwester  
Abschluss: Diplom-Krankenschwester

**Juni '85** Abitur am Fachgymnasium-Hauswirtschaft Schiffdorf, Schiffdorf  
Abschluss: Abitur

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#### BERUFSERFAHRUNG:

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**Feb. '01 - März '07** Wissenschaftl. Angestellte beim Totfund- und Lebendmonitoring am  
FTZ Westküste (unregelmäßig über mehrere Monate)

**Nov. '00 - Jan. '01** Koordinatorin des Totfund- und Lebendmonitorings am FTZ  
Westküste

**Juni '97 - Okt. '00** Hilfwissenschaftler beim Totfund- und Lebendmonitoring am FTZ  
Westküste

**Apr. '98 - Sept. '98** Praktikum beim Marine Mammal Research Program in Galveston,  
Texas, USA bei Prof. Bernd Würsig

**Juli '93 - dato** Krankenschwester auf der Internen Intensivstation des Universitäts-  
klinikums S-H, Campus Kiel

**Mai '89 - Juni '93** Krankenschwester am Bernhard-Nocht-Institut für Tropen- und  
Schiffskrankheiten und AIDS  
(Zusatzausbildung für Tropenkrankheiten: Diagnostik und Therapie)

## Erklärung

Hiermit erkläre ich, **Ilka Hasselmeier**, geb. 1.2.1966, die vorliegende Arbeit selbständig angefertigt, und keine anderen als die angegebenen Hilfsmittel verwendet zu haben. Diese Arbeit hat an keiner anderen Stelle eines Prüfungsverfahrens vorgelegen. Dies ist mein erstes Promotionsverfahren. Teile dieser Doktorarbeit sind zum augenblicklichen Zeitpunkt zur Veröffentlichung eingereicht. Die Promotion soll im Fach Zoologie erfolgen. Des Weiteren erkläre ich, dass ich Zuhörer bei der Disputation zulasse.

Kiel, 15.12 2006

(Ilka Hasselmeier)