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Black-legged kittiwakes (*Rissa tridactyla*) change their leukocyte profiles because of handling, repeated sampling and development

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Preface

This Master's thesis has been written at the Department of Biology, Norwegian University of Science and Technology, Trondheim. The fieldwork was carried out in Kongsfjorden, Svalbard in July-August in 2012 and 2013. Fieldwork in 2012 was supported by an Arctic Field Grant of the Svalbard Science Forum.

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Sammendrag

Slik som for andre arter med et langt liv så må krykkjer (*Rissa tridactyla*) investere i immunforsvaret sitt, men kun som et kompromiss med andre kroppsfunksjoner. Immunforsvaret er viktig for fremtidig overlevelse og det er spesielt viktig for avkom under utvikling. Ungene må balansere investeringene mellom vekst og immunforsvar, da tilgangen på resurser som regel er begrenset. Mens det medfødte immunforsvaret fungerer som en bred og uspesifikk beskyttelse mot ulike patogener, er det ervervede immunforsvaret mer spesifikt, men samtidig mer kostbart.

Selv om leukocyttprofiler fra blodutstryk, slik som heterofil til lymfocytt (H:L) raten, har blitt mye brukt i ulike vurderinger av blant annet immunitet hos fugler, så er effekten av håndtering ofte ikke medregnet i forskningen. I dette studiet ble 33 voksne krykkjer valgt for å undersøke effekten av generell håndtering og gjentatte blodprøver på leukocyttprofiler fra blodutstryk. Blod ble samlet inn (a) ved gjentatte prøvetakinger (ved 3, 30 og 60 min), samt etter 60 min til en kontrollgruppe for håndtering alene, og (b) ved èn gjentatt prøve (ved 3 og 60 min), tatt på samme eller motsatt vinge for å teste for *lokale* eller *helhetlige* prøvetakingseffekter. Resultatene viste at gjentatte prøvetakinger, og ikke håndtering alene, førte til en økning i H:L raten over tid. Blodprøvetaking forårsaket også en *helhetlig* immunrespons siden leukocyttverdiene var like etter 60 minutter, uavhengig av om prøven ble tatt fra samme eller motsatte vinge. Andre leukocyttparameter, som det totale antallet leukocytter, viste like endringer ved gjentatte blodprøver som ved håndtering alene. Dermed ser det ut til at effekten av de ulike formene for stress ved håndtering er parameterspesifikke. Forskere bør derfor være oppmerksom på de mulige effektene fra stress som følge av vanlig håndtering.

Utviklingen av leukocytt profiler ble undersøkt hos krykkjeunger 10 og 25 dager gamle og endringen av de samme profilene hos foreldrene fra klekking til 25 dager etter klekking. Foreldrene endret ingen leukocyttprofiler i den målte tidsperioden. Krykkjeungene hadde en nedgang i antallet heterofile celler og en økning i antallet lymfocytter uten en økning i total mengde leukocytter. I tillegg var H:L ratio til de 25 dager gamle ungene like foreldrenes. Ut i fra resultatene kan en anta at krykkjeunger går fra et generelt medfødt immunforsvar, til en investering i et mer spesifikt immunforsvar som er likt foreldrenes.

Abstract

Similar to other long-lived species, Black-legged kittiwakes (*Rissa tridactyla*) need to invest in the immune system to ensure future survival. However, since resources are considered to be limited, this investment constitutes a trade-off with other life history components. In the developing offspring an important balance is between their investments in growth and in immunity. The innate immune system is an initial, non-specific protection from a variety of pathogens, while the acquired immune system provides a more specific protection but is considered to be more costly.

Although obtaining immune parameters such as the heterophil to lymphocyte (H:L) ratio from blood smears is a standard approach in bird studies, effects of handling on these measurements are usually disregarded. In the present study, 33 adult kittiwakes were selected to study the effects of handling time and repeated sampling on leukocyte profiles from blood smears. To examine these effects, blood were collected (a) through repeated sampling (at 3, 30 and 60 min) and also after 60 min for handling alone (control), and (b) through one repeated sample (at 3 and 60 min of capture), alternatively sampling at the same or the opposite wing, to test for *local* versus *global* sampling effects. The results suggest that repeated sampling and not handling *per se* causes a significant increase in H:L ratio over time. Furthermore, sampling causes a *global* immune response within minutes, since leukocyte profiles at 60 minutes were similar irrespective of whether the same or opposite wing was sampled. However, since the total leukocyte counts changed significantly regardless of handling time alone or repeated bleedings, the effects of handling seem to be parameter specific. Researchers should therefore be aware of the possible effects from the stress which may follow normal handling.

The present study also examined leukocyte profiles and H:L ratios of kittiwakes from chicks under development, 10 to 25 days after hatching, and the change of the parental profiles from hatching to 25 days after hatching. While the parental leukocyte profiles did not change, the chicks experienced a decline in the numbers of heterophils and an increase of lymphocytes without any changes in the total numbers of leukocytes. In addition, the H:L ratio in the older chicks resembled their parents. These data suggests that younger kittiwake chicks have stronger innate immunity which generally protects them from pathogens, while investing more into a more specialized acquired immunity similar to their parental leukocyte status.

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List of abbreviations

$60_{opposite}$	Re-sampled on the opposite wing at 60 min
60_{same}	Re-sampled on the same wing at 60 min
Ad	Adults
Ad0	Adults at hatching
Ad25	Adults 25 days after hatching
BCI	Body condition index
BM	Body mass
Ch10	Chicks 10 days old
Ch25	Chicks 25 days old
CORT	Corticosterone
CV	Coefficients of variability
EFE	Estimates of fixed effects
EOS%	Percentage of eosinophils (of leukocytes)
EOS10	Eosinophils per 10,000 red blood cells
HET%	Percentage of heterophils (of leukocytes)
HET10	Heterophils per 10,000 red blood cells
H:L ratio	Heterophil to lymphocyte ratio
HTC	Hematocrit
LEU10	Leukocytes per 10,000 red blood cells
LME	Linear mixed effects
LYM%	Percentage of lymphocytes (of leukocytes)
LYM10	Lymphocytes per 10,000 red blood cells
MON%	Percentage of monocytes (of leukocytes)
MON10	Monocytes per 10,000 red blood cells
PCA	Principle component analysis
PCR	Polymerase chain reaction
RA	Regression analysis
RBCs	Red blood cells
RL	Radiolabeled
SE	Standard error
UL	Unlabeled

1 Introduction

1.1 Life history and trade-offs

Life history theory describes strategies that optimize survival and reproductive success. These strategies balance trade-offs between traits that enhance individual fitness, such as reproduction, growth, maintenance and immune system (Stearns, 2000; Ricklefs and Wikelski, 2002). In theory, longer lived animals should prioritize investments into traits critical for survival before other, less immediate ones, such as mating preferences and secondary sexual traits (Andersson, 1986). It is specifically important for developing offspring to balance their investments between growth and immunity (Sheldon and Verhulst, 1996; Lee, 2006), as resources often are considered to be limited (Martin, 1987; Norris and Evans, 2000).

Immunity is the body`s ability to resist infection from a pathogen or its products (Murphy, 2012). Responses and maintenance of the immune system involve costs such as energy, nutrients, potential tissue damage and host cell integrity (Klasing and Leshcinsky, 1999), which must be balanced against their advantages and disadvantages to the individual (Ricklefs and Wikelski, 2002). As for other long lived species (Klasing and Leshcinsky, 1999), Black-legged kittiwakes (*Rissa tridactyla)* need likely to invest strongly in their immune system. However, any investment will be a trade-off with other life history components (Sheldon and Verhulst, 1996; Lee, 2006) as developing and maintaining an intact immune system is considered costly (Sheldon and Verhulst, 1996; Norris and Evans, 2002; Lee, 2006).

In a breeding season with low food availability, long-lived birds such as the kittiwake prioritize self-maintenance and survival to increase lifetime reproductive success, rather than current reproductive effort (Kitaysky et al., 2007; Sandvik et al., 2012). Kittiwakes may therefore be selected to invest more resources in the acquired immune system to protect future reproductive success, while short-lived relatives may invest less because of the lesser chance of encountering diverse pathogens during their lifetime (Ricklefs and Wikelski, 2002). However, enhanced allocation of energy towards investments in the immune system might even suppress reproductive effort or certain physiological (endocrine) states of reproduction because of the fewer resources available (Møller et al., 1998; Lindström et al., 2001).

The development, maintenance and costs of use of the immune system may differ between the different immune system components (reviewed in Lee, 2006; table 1). While some parts of the of the immune system is considered less costly, such as constitutive innate defenses (Lee, 2006), other responses may give high costs to the host, such as innate induced inflammatory responses (Schantz et al., 1999; Lee, 2006) or the development and differentiation of lymphocytes (Lee, 2006).

Table 1. Costs and benefits of innate and acquired immune defense, modified freely from Lee (2006).

		Functions	**Relative costs**
Innate	Constitutive	First line of defense: ingest and destroy pathogens and infected host cells	Developmental, maintenance and use costs thought to be low
	Induced	Increases rates of many immunological processes and sequester nutrients from pathogens	Developmental costs: low Use costs: very high
Acquired	Constitutive	First line of defense: opsonize or neutralize pathogens	Developmental costs: thought to be low Maintenance and use costs: low
	Induced	Kill infected host cells, memory of intracellular pathogens and neutralize or destroy pathogens	Developmental costs: high Use costs: low to high

While there is some agreement that the immune system is costly, some disagreement exists to what degree the immune system participates in physiological tradeoffs which influence life-history variation (*e.g.* Ricklefs and Wikelski, 2002). Several authors have approached this subject (*e.g.* Lochmiller and Deerenberg, 2000; Lee, 2006), but it is emphasized that future studies are needed on wild animals designed to manipulate the immune system more specifically (Norris and Evans, 2000).

1.2 The avian immune system

As for other animals, the immune system in birds is fundamentally important for future survival (Apanius, 1998). Traditionally, the immune system of leukocytes (white blood cells) is divided into innate and acquired immunity (Juul-Madsen el al., 2008). The innate immune system works as initial, non-specific protection from a variety of pathogens (Murphy, 2012). Components of the innate immune system can also induce local inflammation (Lee, 2006), and accumulation of innate cells in the inflamed tissues (Harmon, 1998). Acquired immunity is a more pathogen-specific response (Juul-Madsen et al., 2008), generally considered more costly (Klasing and Leshchinsky, 1999; Lee, 2006; table 1) and typically has a slower

response time (Demas and Nelson, 2011; Murphy, 2012). The acquired immune system also recognizes and memorizes pathogens by antigen-antibody interactions (Murphy, 2012). However, the immune system is not clear-cut divided into two separate systems as the innate immune responses are also involved in induction and modulation of the acquired immune response towards a pathogen (Juul-Madsen el al., 2008; Demas and Nelson, 2011; Murphy, 2012).

Birds have five types of leukocytes: Lymphocytes, heterophils, eosinophils, monocytes and basophils (Davison et al., 2008). Heterophils and lymphocytes constitute the majority of leukocytes in kittiwakes (Newman et al., 1997; Oddvar Heggøy, unpublished results). Heterophils are granulated leukocytes involved in the acute inflammatory response (Harmon, 1998). They form the first line of (innate) defense against invading microbial pathogens (Juul-Madsen el al., 2008) and are highly phagocytic and capable of a broad spectrum of antimicrobial activity (Harmon, 1998). Basophils and eosinophils are also a part of the innate immune system together with heterophils. Each takes part by secreting proteins (*e.g.* histamine), toxins and prostaglandins. Monocytes develop into macrophages which provide nonspecific defense against pathogens by phagocyte pathogen-infected cells. These cells of the innate immune system take part in activation of the lymphocytes (Demas and Nelson, 2011; Murphy, 2012). Lymphocytes, made up of B and T cells, coordinate the organisms acquired immune responses (Demas and Nelson, 2011; Murphy, 2012) as well as immunological memory by antigen-antibody interactions. This enables the animal to sustain a lifelong immunity after the initial contact with a certain pathogen (Murphy, 2012). Lymphocytes are derived from stem cells in the bone marrow, which is differentiated in lymphoid organs. The B cells develop in the bursa of fabricus, and the T cells develop in the thymus (Oláh and Vervelde, 2008). While B cells produce antibodies, T cells eliminate infected host cells by direct contact or serve as complementary cells. These are protein producing cells where the proteins act together as a defense against pathogens in extracellular spaces (Murphy, 2012). T cells also interact with B cells to coordinate the production of antibodies (Demas and Nelson, 2011; Murphy, 2012).

1.2.1 Leukocyte profiles

Leukocyte counts from blood smears have been used extensively to assess different physiological functions and responses (Davis, 2005; Davis et al., 2008). Many of these studies

focus on the heterophil to lymphocyte (H:L) ratio, *i.e.* the cells that make up the majority of leukocytes involved in the innate and acquired immune system. Measurement errors have previously been found to be small in assessing H:L ratios, suggesting that this is an adequate method for ecological research purposes (Ots et al., 1998). Higher heterophil numbers (*i.e.* a higher H:L ratio) indicates that the animal primarily uses its innate immunity (Masello et al., 2009) while a low H:L value indicates that the animal in question is more reliant on the acquired immune system. Both relative high and low H:L ratio values have been observed in adults of different wild bird species (*e.g.* Work et al., 1996; Newman et al., 1997) which suggests this to be species-dependent. In addition, H:L ratios may even differ between populations of the same species, *e.g.* in kittiwakes at the Shumigan Islands, USA (Newman et al., 1997) and Hornøya, Norway (Oddvar Heggøy, unpublished results).

As leukocyte profiles have been used to assess innate immune function (Davis, 2005), it has also been used to monitor overall immune function, as H:L ratio appears to increase with disease (Davis et al., 2004), injury (Ots et al., 1998; Vleck et al., 2000), stress (Vleck et al., 2000; Davis et al., 2008; Cīrule et al., 2012), urbanization (Ruiz et al., 2002) and with decreasing habitat quality (Mazerolle and Hobson, 2002). In addition, H:L ratios have been shown to increase as a response to lower body condition in song sparrows (*Melospiza melodia;* Pfaff et al., 2007) and increased H:L ratio has also been linked to increased mortality in the Eurasian treecreeper (*Certhia familiaris*; Suorsa et al., 2004).

In previous studies, leukocyte profiles have been demonstrated to be heritable in different species of birds. In chickens (*Gallus gallus domesticus*) for example, the estimated heritability of the H:L ratio, and the numbers of heterophils and lymphocytes were high (Campo and Davila, 2002). Heat stress resistance, measured by the H:L ratio, have also been shown to heritable from parents to chicks in domestic fowls (Al-Murrani et al., 2007). Therefore, parental leukocyte values might predict chick levels of leukocyte investment.

1.2.2 Modulation of the immune system

Several factors may modulate the avian immune system. These include intrinsic factors such as age and sex of the individual, as well as extrinsic factors which include environmental conditions, social interactions, exposure to toxicants and type of diet (Koutsos and Klasing, 2008). For example, in wild American kestrels (*Falco sparverius*) environmental factors were the major determinants of offspring T cell mediated immune response (Tella et al., 2000). In

addition, a study on parent kittiwakes showed a decline in cell-mediated immune response during the breeding season (Broggi et al., 2010). Also, interactions between metabolic, immunological and neuroendocrine systems affect animal physiology and homeostasis. Stress hormones, metabolic hormones, sex hormones and other endocrine signaling molecules can directly or indirectly affect the immune system (Koutsos and Klasing, 2008).

Stress is a physiological condition defined as a state of threatened or perceived as threatened homeostasis (Charmandari et al., 2005), and physiological changes during the response to these pressures are aimed at ensuring survival (Wingfield et al., 1995). An important part of the stress response is the release of glucocorticoid hormones from the adrenal cortex (Sapolsky et al., 2000). In birds, the major glucocorticoid is corticosterone (CORT) and concentrations rise within minutes of the onset of a stressor. Elevation of CORT may facilitate short-time survival of individuals and is in fact an important regulator of daily homeostasis, although chronically elevated CORT levels may result in immunosuppression (Dhabhar, 2002; Charmandari et al., 2005; Koutsos and Klasing, 2008).

Leukocyte profiles seem closely associated with stress in birds and with CORT levels in particular (Vleck et al., 2000; Davis et al., 2008). A reduction in the number of circulating leukocytes, which is usually observed in stressed animals with elevated CORT levels, is due to changes in the distribution of lymphocytes from the blood to other body compartments (Dhabhar, 2002; figure 1). The observed changes in leukocyte distribution are likely mediated by changes in either the expression, or affinity of adhesion molecules on leukocytes and/or endothelial cells (Dhabhar et al., 1996). Elevated levels of CORT cause the lymphocytes to adhere to endothelial cells that line the walls of blood vessels, where they “migrate” from the circulating blood into other tissues, such as bone marrow, spleen, lymph nodes and skin (Dhabhar, 2002). This results in a significant reduction in the number of circulating lymphocytes (Dhabhar, 2002). At the same time, CORT causes both a flow of heterophils into the bloodstream from bone marrow and reduces the migration of heterophils from the blood to other compartments (Bishop et al., 1968). These redistributions are thought to be an adaptive response, preparing the individual`s immune system by increasing immune surveillance/response in important organs (Dhabhar, 2002). This response, which is observed in all five vertebrate taxa, results in an increased H:L ratio proportional to the level of glucocorticoids release (Davis et al., 2008).

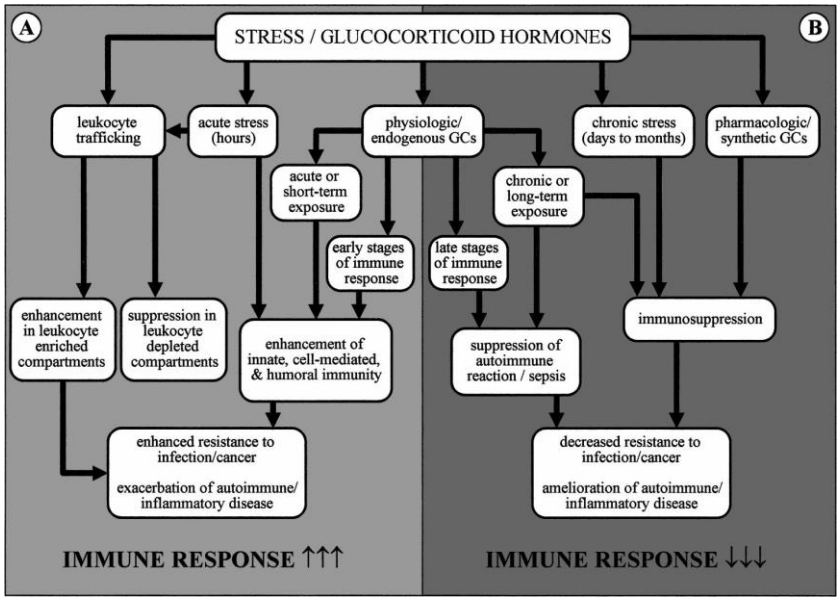

Figure 1. The responses of the immune function to acute (A) or chronic stress (B). A short or acute stress-induced reaction (A) may enhance immune function within certain compartments and suppress others. While acute stress may enhance leukocyte activation and effector function, harmful effects may be increased autoimmunity and/or inflammatory disorders. Chronic stress (B) suppresses immune function and may increase susceptibility to infections and cancer, but may also protect against autoimmune and inflammatory reactions. Variability in both acute and chronic stress on the immune function is likely due to factors such as genes, age and sex. In addition to CORT, catecholamine's and peptide hormones are also released during stress and influence immune responses. GC = Glucocorticoids. Rewritten from Dhabhar (2002).

In previous studies, immune cell parameters have been shown to change in response to routine handling (Davis, 2005) and transportation of different bird species (Parga et al., 2001; Scope et al., 2002; Groombridge et al., 2004; Huff et al., 2005). The leukocyte profiles may in some circumstances even change differently depending on the type of handling stress. In a study of house finches (*Carpodacus mexicanus;* Davis, 2005), the total amount of circulating leukocytes changed because of handling alone, while the H:L was only affected by repeated bleedings. Consideration of the effect of handling and repeated sampling in a study may be very important, because the leukocyte profiles might change within the interval which is considered to be normal handling time (Davis, 2005).

1.2.3 Ontogeny of the immune system

There are distinct differences in the immune systems between developing chicks and the adult individuals. The developing embryo and newly hatched chick rely mainly on the innate immune system (Klasing and Leshchinsky, 1999). The acquired immunity is then later

mediated by the development and differentiation of lymphocytes, which both take time and resources. Maturation of lymphocytes requires selection and differentiation to ensure that all possible non-self antigens can be detected and recognized with minimal errors which cause autoimmunity (Klasing and Leshchinsky, 1999). When "completed", this system of immunity reacts to specific pathogens with lower costs to the birds than the cellular components of the innate immunity (Klasing and Leshchinsky, 1999; Lee, 2006; table 1).

The H:L ratio and other leukocyte profiles are likely to differ between nestlings and adults. A study on broiler chicks demonstrated that their acquired immune response was age-dependent, with older chicks showing a higher response than young ones (Mast and Goddeeris, 1999). In addition, these chicks do not have functional intestinal lymphocytes until 14 days of age (Bar-Shira et al., 2003), but this lack of self-derived antibodies is somewhat compensated with maternal antibodies transferred via the egg yolk (Hamal et al., 2006; Fellah et al., 2008). These stay active for approximately two weeks after hatching (Klasing and Leshchinsky, 1999; Hamal et al., 2006) and are important for detecting and removing specific pathogens (Murpy, 2012). Other leukocyte profiles, such as the total amount of circulating leukocytes has been shown to decline from chicks to adults (*e.g.* Dehnhard et al., 2011a, 2011b). This decline has been shown to coincide with an increase in hematocrit (HTC) levels. Specifically, HTC levels in the blood has been shown to increase with age in several birds, from young to older chicks (Chapman, 1974; Merino and Barbosa, 1996; Howlett et al., 1998; Næss, 1999) and from chicks to adults (Work, 1996; Merino and Barbosa, 1996; Næss, 1999; Potti, 2007). Similar to the broiler chick experiments, previous studies on the ontogeny of the immune system in birds is mostly dominated by domesticated species (Ardia and Schat, 2008).

1.3 Aim of study

In the present study, I investigated whether an arctic breeding seabird, the kittiwake, changes their leukocyte investment in the immune system from hatching to adult age. Specifically, my aim was to test whether kittiwakes invest in acquired immunity from a basic innate immunity during the breeding season using leukocyte profiles such as H:L ratio, relative (%) leukocyte numbers and numbers of leukocytes (per 10,000 red blood cells). Secondly, I assessed the effect of stress on leukocyte profiles, by testing the effect of handling and circulating CORT. I predicted the following:

1. Chicks rely mainly on innate immunity during development, but older chicks rely mainly on the acquired component and body condition correlates with leukocyte investment.
2. Adults experience a reduced amount of total circulating leukocytes as well as reduced acquired immunity from hatching to the end of the chick-rearing period.
3. CORT levels and H:L ratios will correlate in both chicks and adults.
4. Handling and repeated sampling affects kittiwake leukocyte profiles negatively.

2 Materials and methods

2.1 Study area and model species

Fieldwork for the present study was conducted from July to August in 2012 and 2013 in Kongsfjorden, Svalbard (78° 59’ N, 12° 07’ E). Individuals were studied in a colony of kittiwakes on Blomstrand Island that hosts a few hundred breeding pairs (figure 2).

The kittiwake is a medium sized, long-lived, circumpolar seabird. With an estimated population size of 4.5 million annual breeding pairs it is the most numerous gull species in the world (Coulson, 2011), as well as the most common bird on Svalbard with approximately 270 000 breeding pairs (Mehlum and Bakken, 1994 in Barret and Tertitski, 2000).

Adult kittiwakes are socially monogamous and both sexes share parental duties about equally (Strøm, 2006). Kittiwakes usually lay their eggs in the first half of June, with a normal clutch size of two eggs, although clutches with one or three occur (Strøm, 2006). Eggs are incubated for approximately 25-32 days (Strøm, 2006), after which all eggs in a clutch usually hatch within three days (Braun and Hunt, 1983). The kittiwake is a semi-precocial species and nestlings require a high degree of parental care (Coulson and Porter, 1985; Golet et al., 1998). The chicks are completely dependent on their parents during the first 15-16 days after hatching because of their poor thermoregulatory function (Gabrielsen et al., 1992). Kittiwake chicks are normally brooded until they reach about 15 days (Moe et al., 2002). After 5-6 weeks the nestlings fledge (Strøm, 2006), and become sexually mature at 3-5 years of age (Coulson, 2011).

Figure 2. Colony (red dot) of Black-legged kittiwakes at Blomstrand, Svalbard. Freely edited from Norsk Polarinstitutt (2013).

2.2 Field procedure

Nests were selected from accessible breeding sites. Forty adult kittiwakes and their chicks were used for the development study, while an additional 33 breeding adults were tested for

the effect of handling. Adult birds were caught using a fishing rod with a nylon noose at the end, while chicks were retrieved from their nests by hand using a ladder. Blood was sampled from all captured birds and biometrical parameters were measured. Body mass (BM) was measured by weighing birds in a cloth bag using a spring balance (Pesola, accuracy to ± 0.1g). Both head and bill and tarsus length was measured using a sliding caliper (Starret, ± 0.1mm accuracy) and the stretched out right wing was measured to the nearest 1.0 mm using a ruler. Non-banded birds were banded with a three-letter, field readable plastic ring and a numbered metal ring. A drop of blood from non-sexed birds was used for later molecular sexing.

To measure kittiwake breeding parameters, nest checks were conducted every other day from 3rd to 15th of July in 2012 (median hatch date; 10th July) and between 2rd and 22th of July in 2013 (median hatch date; 9th July). After this period nest checks were carried out opportunistically. Adults were marked according to their sex with non-permanent ink that lasted for about one week, to assist in the selective recapturing of individuals. Chicks were banded with expandable plastic color rings early after hatching to distinguish siblings and individuals in neighbor nests. Biometric data and blood samples of adults were taken at hatching (Ad0 group; sampled at -1.8 ± 0.4 days, N = 40), as well as when the chicks were 25 days old (Ad25 group; sampled at 25.8 ± 0.2 days, N = 28). Chicks were sampled and measured twice, 10 (Ch10 group; sampled at 10.2 ± 0.2 days, N = 23) and 25 (Ch25 group; sampled at 25.2 ± 0.2 days, N = 23) days after hatching. Samples for the effect of handling on leukocyte profiles were taken from naïve (*i.e.* not sampled earlier in the season) adults with approximately 25 day old chicks (N = 33).

2.3 Blood sampling

Blood samples were obtained from the basilic (ulnar) vein with a heparinized syringe within 3 minutes of capture (on average 02:25 ± 00:03 min). Blood was split for use in replicated blood smears, for CORT analyses and for sex determination. A total of approximately 0.6 mL blood was sampled: about 0.4 mL blood for CORT analysis, a drop of blood for each blood smear and another for sexing.

The samples used for molecular sexing were stored in ethanol (96%). Blood smears were made immediately after sampling, following established protocols (*e.g.* Ruiz et al., 2002; Lobato et al., 2005; Masello et al., 2009). A drop of blood was smeared on a glass slide, then

air-dried and fixated for 1-2 minutes in methanol (99.9 %). Within two weeks, the slides were stained in Giemsa-stain (Sigma-Aldrich) for 20 minutes (Houwen, 2000).

Blood sampled for CORT was kept on ice until centrifugation (9500 rpm, 5 min) and separation of plasma and red blood cells later on the same day (within 6-8 hours). Plasma was stored in a freezer (-80 °C) until analysis.

2.4 Leukocyte counts

Leukocyte counts were carried out using a light-microscope (1,000x) in a monolayer-section of the blood smear. The identity of the sample was not known until after the completed analyses to avoid observer-expectancy effects. Leukocytes were counted in a section of the smear where blood cells had separated in a monolayer and the slide was scanned along the short-axis to minimize differences in the thickness of the smear (Moreno et al., 1998). Using criteria defined by Clark et al. (2009), a total of 100 leukocytes was counted per slide and distinguished as lymphocytes, heterophils, eosinophils, monocytes or basophils. In addition to H:L ratios, relative numbers of lymphocytes (LYM%), heterophiles (HET%) and the other cell types were calculated as the percentage of all leukocytes. The number of lymphocytes (LYM10), heterophils (HET10) and total leukocytes (LEU10) per 10,000 red blood cells (RBCs) was obtained by multiplying the average number of three visual fields of red blood cells with the number of microscopic visual fields scanned and finally divided by 10,000. This gave the relative amount of leukocytes in relation to red blood cell numbers (Moreno et al., 1998). Thrombocytes were excluded as they often tend to aggregate into variably sized clumps throughout the blood film (Clark et al., 2009). Repeatability for the leukocyte profiles were conducted using 12 recounted blood smears.

2.5 Plasma corticosterone concentrations

Total free CORT concentrations were measured for all birds in the development study. The procedure was performed using plasma from blood samples and conducted at the University of Alaska, Fairbanks, USA. The technique is based on a radioimmunoassay following established protocols (Wingfield et al., 1992; Kitaysky et al., 2010).

For each sample, blood plasma (20 µl) was first equilibrated with 2000 counts per minute of titrated CORT. Then, 4.5 mL distilled dichloromethane was added to the mixture and the organic phase extracted, dried and reconstituted by phosphate buffered saline. The solution was added to duplicate assays. Percent titrated hormone in the extracts from each individual sample was used to correct final values. Further, the samples with unlabeled (UL) antigens were combined with antibody and radiolabeled (RL) antigens in a radioimmunoassay. Since both the RL and UL antigens have equal binding affinity to the antibody, they are equal in the competition for antibody binding sites. Larger concentrations of CORT in blood samples cause more UL antigens to bind to the antibodies. Finally, after removing excess unbound RL antigen with charcoal, the CORT concentration in each plasma sample is measured by determining the remaining bound RL antigen (Murphy, 2012).

The cross-reactivity of antibody with different steroids was negligible because of the high antibody specificity to CORT (Wingfield and Farner, 1975; Shultz and Kitaysky, 2007). The sensitivity of CORT detectable was 7.80 pg/sample and average recovery was above 90 % (93.1 ± 3.22 %). Finally, intra-assay coefficients of variability (CV) was less than 2 % (1.15 ± 0.95 %) and inter-assay CV was also less than 2 % (1.75 ± 0.96 %).

2.5 Molecular sexing

Molecular sexing was conducted for all birds not sexed in connection with previous studies. The sexing procedure was performed using a drop of blood stored at 96 % ethanol and conducted at the Department of Biology NTNU, Norway. The sexing technique is based on the bird's sex chromosomes where females are the heterogametic sex (ZW) while the males are homogametic (ZZ; Griffiths et al., 1998). With polymerase chain reaction (PCR) the genes was exponential amplified with the use of specific primers (*ref.* P2 and P8 primers in Griffiths et al., 1998) binding to specific sites on the Z and the W gene. The primers together with the four nucleotides of DNA reproduced the desired amount of sequences during 35 heat-cycles. In a gel electrophoresis, the smaller Z gene moves more readily through the agarose gel matrix than the larger W gene. Since females are heterogametic (ZW), and males homogametic (ZZ) the gel will display a single band for males and two distinct bands for females (figure 3; Griffiths et al., 1998).

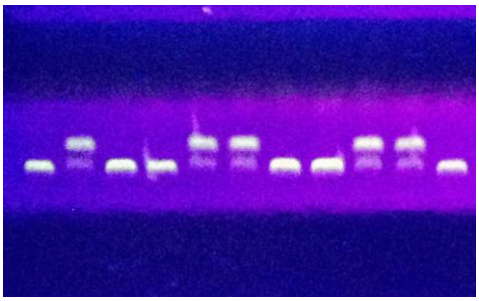

Figure 3. DNA sex identification. One band on the gel corresponds to a male (ZZ), while a female (ZW) is shown as two bands (Photo; Dagfinn B. Skomsø).

Extraction of DNA was done following a modified version of the Chelex extraction method, previously described elsewhere (Walsh et al., 1991). A small blood sample (< 1 µL) was added to 200 µL 5 % Chelex 100 resin solution (Biorad, Hercules, CA, USA). The solution were further heated to 56 °C (20 min), vortexed, heated to 96 °C (8 min) and then centrifuged (12000 rpm, 3 min) after which 20 µL of supernatant was extracted for further sex analysis.

The PCR analyses were done according to Griffiths et al. (1998). First, a stock mix containing primers and nucleotides were made (appendix, table A). Further, 8 µL of the stock solution was used together with 2 µL DNA supernatant resulting in a total reaction volume of 10 µL per sample. Each individual sample was then added to a well of a PCR plate, where the PCR process began with a 94 °C DNA denaturation step for 3 min. Following this initial step, 35 cycles of subsequent temperatures followed: 94 °C for 30 sec, annealing at 46 °C for 45 sec and elongation at 70 °C for 45 sec. The last step consisted of an elongation period at 70 °C for 10 min. The product samples were then stored at 4 °C until further analysis. Samples were finally analyzed using gel electrophoresis in a 1 % agarose gel containing SYBR® Safe gel-stain (Invitrogen) and a standard TAE (50 x) running buffer for 45 minutes. This completed the separation of the two bands which was visualized under UV-light. Duplicates and samples from previous sexed individuals were added to each gel for control.

2.7 Statistical analyses

All statistical procedures were conducted with SPSS (Version 20.0.0, SPSS Inc. 2011) and plots were made in SigmaPlot 12.5 (Systat System, Inc. 2010). All tests were two-tailed with a significance level set at $P \leq 0.05$. Collinearity between variables was checked before conducting the tests assessing variance inflation factors, where values below 3.000 were deemed acceptable (appendix, table B-C). Model data were assessed for normal distribution by plotting sample residuals against theoretical residuals. Means and parameter estimates are given with standard error (± SE). Data in figures are presented as mean value point-plots with standard errors (± SE). Data of different sampling times were pooled to obtain larger sample sizes whenever possible, i.e. when they were similar ($P > 0.05$; see appendix, table D-F and

table J for details). Leukocyte measurements were tested for repeatability and calculated as the intraclass coefficient based on variance components derived from a one-way ANOVA (analysis of variance) table (Lessells and Boag, 1987).

Body condition index (BCI) was calculated for each individual using general linear models. In both groups of chicks, morphological measures of skull and tarsus length were regression transformed with a principal component analysis (PCA) for body size. BCI was then calculated from the standardized residuals of a linear regression between the PCA and BM. BCI for adults was similar calculated, but included wing length in the PCA for body size as well.

2.7.1 Handling tests

Blood smears from adults were obtained at < 3 min (02:20 ± 00:09 min) and at 60 min (61:03 ± 00:20 min) after capture. To address whether sampling evoked a global or local response, I compared samples obtained from the same wing (60_{same}) with those from the opposite wing ($60_{opposite}$) for the 60-min measure (figure 4).

Further, blood smears obtained from adults at 3 min (02:20 ± 00:12 min), 30 min (30:22 ± 00:10 min) and 60 min (60:18 ± 00:14 min) after capture were analyzed to evaluate the effect of repeated sampling on leukocyte profiles (figure 4).

I also sampled a group of adult birds only after 60 (61:15 ± 00:54 min) min after capture (control) to evaluate the effect of handling alone, without repeated sampling. Linear mixed effect (LME) models were used with the leukocyte profiles as dependent variables, time as fixed categorical factor and individual identity as a random factor. Leukocyte profiles at each of the time series were then compared to the control group using estimates of fixed effects (EFE) table in LME models.

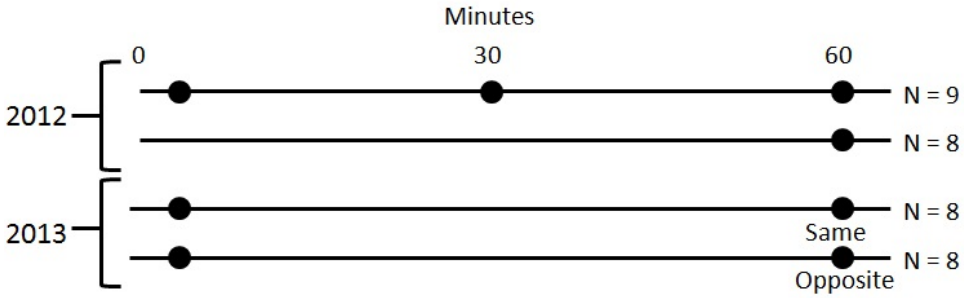

Figure 4. Time of measurements in the handling study. In 2012, one group of kittiwakes were sampled repeatedly (3, 30 and 60 min) in addition to a group of individuals which only was sampled at 60 min (control). In 2013, one group of kittiwakes was re-sampled from the same wing, while another group was re-sampled on the opposite wing (3 and 60 min). Effect of difference in repeated wing sampling was not assessed for in the 2012 group.

2.7.2 Development of the immune system

Blood smears from Ch10 and Ch25 were analyzed to evaluate the development of leukocyte profiles. Further, the changes of leukocyte profiles in the course of the breeding season were measured in Ad0 and in Ad25 (figure 5). LME models were used to analyze variation in leukocyte profiles in response to age, BCI, sex, CORT and the interaction of age with the other variables. Individual bird identity, nest identity and individuals nested in nest identity were used as random variables, in order to account for repeated measures of individuals and pseudoreplication within a nest.

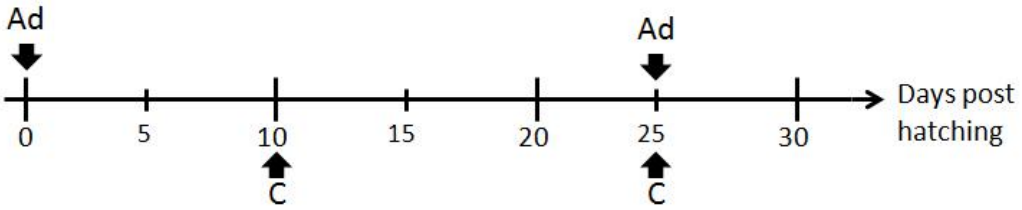

Figure 5. Time of measurements in the development study. The timeline represents days post hatching. Ad = adults and C = chicks.

A significant increase in CORT within 3 minutes were detected in Ch10 ($P = 0.031$) and Ad25 ($P = 0.011$) while the increase were non-significant in Ch25 ($P = 0.255$) and Ad0 ($P = 0.249$). For a time-independent value, CORT values were calculated using the standardized residual for each individual, and adding this value to the mean of CORT levels for each separate age group.

The full LME models (appendix, table J) were simplified by a backwards stepwise selection, where variables or interaction of variables were excluded when they did not significantly

predict leukocyte values (*i.e.* when $P > 0.05$). Presented values of rejected variables are those before model simplification (appendix, table J). Leukocyte profiles at each of the age groups were then compared to each other in the EFE included in the LME analyses.

Correlations between BCI and leukocyte parameters within age groups were examined using regression analysis (RA). Further, RA was also used to compare correlations with different combinations of adult data (*e.g.* Ad0, adult females 25 days after hatching) versus both chick groups (appendix, table M). Finally, RA was used to compare BM between chick groups and between adult groups.

2.8 Permissions

Permission for conducting the fieldwork was provided by the Governor of Svalbard (Sysselmannen; ref. 2011/00488-25), while blood sampling permission was given by the Norwegian National Animal Research Authority (Forsøksutvalget; ref. 2012/65070).

3 RESULTS

Heterophils and lymphocytes were the dominant types of leukocytes in both chicks and adults, each accounting for 30-65 % of all leukocytes. This was observed for the handling test (table 2-3) and development study (table 4). Eosinophils (< 3.5 %), monocytes (< 2.0 %) and basophils (< 2.5 %) accounted for only a small proportion of the leukocytes (table 2- 4).

H:L ratio, LYM%, HET%, LEU10, LYM10 and HET10 were the only leukocyte profiles with significant repeatability (table 5), and thus were further analyzed.

3.1 Handling tests

None of the leukocyte profiles differed significantly between the same and opposite wing (LME; $P \geq 0.525$, appendix, table D). Similarly, counts of the 60 min blood sample did not differ significantly from the 3 min count for any of the leukocyte profiles that were calculated per 10,000 RBCs (LME; $P \geq 0.254$) in repeated samples from 2013 (appendix, table D).

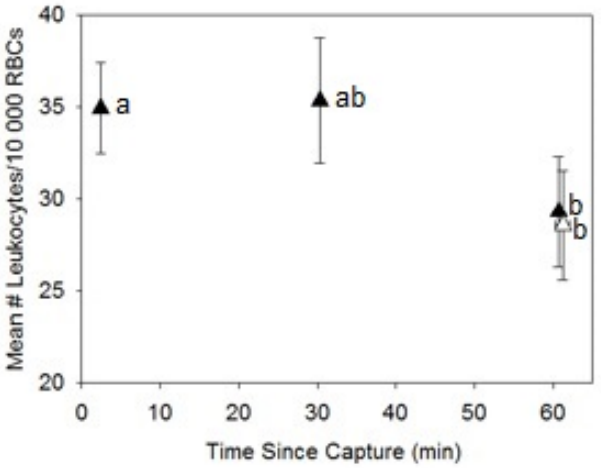

Figure 6. Mean (± SE) of total leukocyte numbers (#) per 10,000 red blood cells of adult Black-legged kittiwakes. Closed triangles are repeated samples 3 min (N = 9), 30 min (N = 9) and 60 min (N = 9) after capture. Open triangle is for birds sampled only at 60 min (N = 8; control). Values with different letters are significantly different from one another (LME; $P \leq .05$).

Repeated sampling of kittiwakes in 2012 (appendix, table G) resulted in a significant decrease in LEU10 (LME; $F_{2,16} = 4.286$, $P = 0.032$, figure 6) and LYM10 (LME; $F_{2,16} = 7.050$, $P = 0.006$), while HET10 (LME; $F_{2,16} = 0.548$ $P = 0.588$) did not change significantly over time. The changes was only significant from 3 to 60 minutes in LEU10 and LYM10 (LME; $P < 0.010$, appendix, table H). However, LEU10 and LYM10 at 60 min did not significantly differ from the 60 min control individuals, *i.e.* birds that were not sampled before 60 min of handling (LME; $P \geq 0.442$, appendix, table H), indicating that handling time and not repeated sampling caused the observed changes in LEU10 and LYM10.

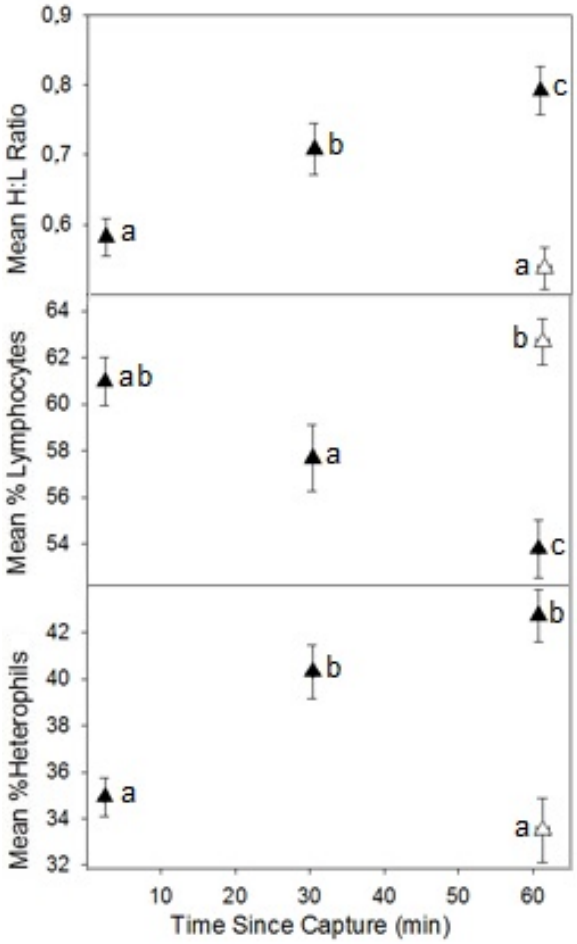

Figure 7. Mean (± SE) value of H:L ratios, LYM% and HET% of adult Black-legged kittiwakes. Closed triangles are repeated samples at 3 min (N = 25), 30 min (N = 9) and 60 min (N = 25) after capture. Open triangle is for birds sampled only at 60 min (N = 8; control). Values with different letters are significantly different from one another (LME; $P \leq 0.05$).

the observed changes (appendix, table I).

Repeated sampling of kittiwakes also caused a significant change in the leukocyte profiles of H:L ratio, LYM% and HET% (LME; $F \geq 5.610$, $P \leq 0.014$, appendix, table I, figure 7). A significant increase in H:L ratio was observed from 3 to 30 min, from 3 to 60 min and from 30 to 60 min (LME; $P \leq 0.043$). A difference in response time was observed for LYM% and HET%. A significant increase in LYM% was not observed from 3 to 30 min (LME; $P = 0.080$), although between 30 and 60 min (LME; $P= 0.023$). In contrast to LYM%, HET% increased significantly from 3 min to 30 min (LME; $P \leq 0.002$), although not between 30 and 60 min (LME; $P = 0.127$).

H:L ratios, LYM% and HET% did not differ between the 60 min control individuals and birds that were re-sampled at 60 min (LME; $P \geq 0.384$, figure 7). These results suggest that repeated sampling and not handling *per se* caused

Table 2. Mean values (± SE**)** of leukocyte profiles from the effect of repeated bleedings at 3, 30 and 60 min. Samples collected only at 60 min (control) are also included.

	3 min (N=9)		***30 min (N=9)***		***60 min (N=9)***		***Control (N=8)***	
	Mean ± SE	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**
LYM%	59.8 ± 2.0	45.8 - 66.1	57.7 ± 1.4	50.1 - 66.2	52.2 ± 1.9	41.4 - 63.4	62.7 ± 1.0	59.1 - 67.6
HET%	34.3 ± 1.7	30.0 - 45.8	40.3 ± 1.2	33.8 - 46.0	43.3 ± 1.8	31.4 - 50.0	33.5 ± 1.4	28.6 - 39.4
EOS%	3.5 ± 0.8	0.0 - 6.4	1.0 ± 0.5	0.0 - 4.3	2.7 ± 0.7	0.0 - 5.7	2.2 ± 0.6	0.0 - 5.7
MON%	0.7 ± 0.3	0.0 - 2.8	0.05 ± 0.05	0.0 - 0.5	0.0 ± 0.0	0.0 - 0.0	0.2 ± 0.2	0.0 - 1.4
BAS%	1.7 ± 0.5	0.0 - 4.3	1.0 ± 0.4	0.0 - 2.6	1.9 ± 0.4	0.0 - 4.3	1.5 ± 0.5	0.0 - 4.3
H:L ratio	0.59 ± 0.05	0.48 - 1.00	0.71 ± 0.04	0.51 - 0.92	0.85 ± 0.06	0.51 - 1.21	0.54 ± 0.03	0.44 - 0.67
LEU10	40.6 ± 3.7	29.0 - 65.2	35.4 ± 3.4	19.3 - 52.0	29.8 ± 3.3	17.9 - 50.3	28.6 ± 3.0	20.6 - 44.3
LYM10	24.5 ± 2.6	13.3 - 41.9	20.2 ± 1.7	11.2 - 27.9	15.7 ± 1.8	9.2 - 26.5	18.0 ± 2.0	12.3 - 28.5
HET10	13.6 ± 1.0	11.0 - 20.5	14.4 ± 1.6	7.4 - 23.8	12.8 ± 1.4	6.5 - 20.9	9.4 ± 0.8	7.1 - 12.7

EOS% = percent eosinophils (of leukocytes)
MON% = percent monocytes (of leukocytes)
BAS% = percent basophils (of leukocytes)

Table 3. Mean values (± SE**)** of leukocyte profiles from the effect of repeated bleedings at 3 and 60 min. Repeated samples on the same wing (60_{same}) and on the opposite ($60_{opposite}$) are specified.

	3 min (N=16)		***60_{same} min (N=8)***		***$60_{opposite}$ min (N=8)***	
	Mean ± SE	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**
LYM%	61.9 ± 0.9	55.0 - 67.1	54.3 ± 2.3	44.3 - 61.0	55.0 ± 1.6	48.6 - 60.6
HET%	35.4 ± 0.8	31.4 - 40.7	42.2 ± 2.1	36.9 - 52.9	43.0 ± 1.4	38.0 - 49.3
EOS%	1.7 ± 0.2	0.0 - 2.9	2.3 ± 0.2	1.4 - 2.9	1.2 ± 0.2	0.0 - 1.4
MON%	1.0 ± 0.2	0.0 - 2.9	1.1 ± 0.3	0.0 - 2.7	0.6 ± 0.5	0.0 - 4.3
BAS%	0.0 ± 0.0	0.0 - 0.0	0.2 ± 0.2	0.0 - 1.4	0.2 ± 0.2	0.0 - 1.4
H:L ratio	0.58 ± 0.02	0.47 - 0.76	0.80 ± 0.08	0.60 - 1.19	0.79 ± 0.05	0.63 - 1.00
LEU10	30.6 ± 2.4	19.2 - 49.7	26.1 ± 2.5	16.6 - 35.5	30.6 ± 7.1	16.7 - 78.2
LYM10	19.0 ± 1.5	11.5 - 31.2	14.2 ± 1.5	7.3 - 19.8	17.2 ± 4.5	8.8 - 47.4
HET10	10.7 ± 0.8	6.9 - 17.0	11.0 ± 1.3	8.3 - 18.8	12.9 ± 2.6	7.1 - 29.8

Table 4. Mean values (± SE) of chick (10 and 25 days old) and adult (at hatching and 25 days after hatching) leukocyte profiles in the development study.

	Ch10 (N=23)		***Ch25 (N=23)***		***Ad0 (N=40)***		***Ad25 (N=28)***	
	Mean ± SE	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**	**Mean ± SE**	**Range**
LYM%	55.2 ± 1.0	47.9 - 64.5	63.7 ± 1.6	47.0 - 90.2	62.8 ± 0.7	52.3 - 74.5	60.0 ± 1.0	43.1 - 66.1
HET%	40.6 ± 1.1	27.9 - 49.8	32.4 ± 1.7	5.9 - 50.5	33.3 ± 0.6	23.6 - 43.4	35.3 ± 0.7	30.0 - 45.8
EOS%	1.8 ± 0.3	0.0 - 5.6	1.6 ± 0.2	0.0 - 4.0	1.5 ± 0.2	0.0 - 4.3	3.3 ± 0.7	0.0 - 19.8
MON%	0.2 ± 0.1	0.0 - 2.0	0.7 ± 0.1	0.0 - 1.9	0.6 ± 0.1	0.0 - 4.1	1.6 ± 0.2	0.0 - 4.3
BAS%	2.2 ± 0.4	0.0 - 6.1	1.7 ± 0.3	0.0 - 4.2	1.9 ± 0.2	0.0 - 5.5	0.3 ± 0.1	0.0 - 2.8
H:L ratio	0.75 ± 0.03	0.43 - 1.07	0.53 ± 0.04	0.07 - 1.08	0.55 ± 0.02	0.32 - 0.91	0.59 ± 0.02	0.48 - 1.00
LEU10	65.2 ± 5.8	16.6 - 136.1	70.5 ± 6.1	31.0 - 163.5	43.9 ± 3.3	13.9 - 107.7	37.0 ± 2.4	20.6 ± 75.2
LYM10	35.8 ± 3.0	8.9 - 73.5	44.1 ± 4.0	23.1 - 102.3	27.5 ± 2.2	9.6 - 80.3	22.0 ± 1.3	12.6 - 41.9
HET10	26.9 ± 2.7	6.5 - 58.5	23.3 ± 2.4	2.0 - 54.3	14.6 ± 1.06	4.4 - 35.2	13.0 ± 0.8	7.0 - 26.6

Table 5. Test of repeatability between blood smears for leukocyte profiles (N = 12).

Measurement	Repeatability	F	df	P	Measurement	Repeatability	F	df	P
H:L ratio	.886	10.773	11	**<.001**	EOS%	-.024	.976	11	.515
LYM%	.859	8.342	11	**.001**	MON%	-.198	.848	11	.606
HET%	.824	7.042	11	**.002**	BAS%	.285	1.367	11	.307
LEU10	.705	3.384	11	**.027**	EOS10	.053	1.057	11	.464
LYM10	.744	4.160	11	**.013**	MON10	.226	1.268	11	.350
HET10	.813	5.173	11	**.006**	BAS10	.456	1.800	11	.172

EOS10 = Eosinophils per 10,000 RBCs
MON10 = Monocytes per 10,000 RBCs
BAS10 = Basophils per 10,000 RBCs

3.2 Development of the immune system

The body mass of the adults decreased significantly (RA; $P < 0.001$) from 416.8 ± 5.9g at hatching to 369.1 ± 5.8g when chicks were 25 days old. Chick BM increased significantly (RA; $P < 0.001$) from 10 days of age (160.4 ± 7.5g) to 25 days of age (341.8 ± 10.4g).

Body condition correlated overall poorly with most leukocyte parameters, despite a few notable exceptions (appendix, table J-L). Firstly, BCI correlated with H:L ratios significantly (LME; $F_{3,99} = 2.822$, $P = 0.043$, figure 8) for 10 day old chicks (RA; $P = 0.012$) and adults sampled at hatching (RA; $P = 0.025$). Another exception was HET10 which correlated positively with BCI (LME; $F_{1,95} = 6.333$, $P = 0.014$), more specifically in 10 day old chicks (LME; $F_{3,95} = 4.447$, $P = 0.006$). Even though not reaching significance (RA; $P = 0.054$), a tendency towards a positive correlation between BCI and LEU10 was observed in 10 day old chicks.

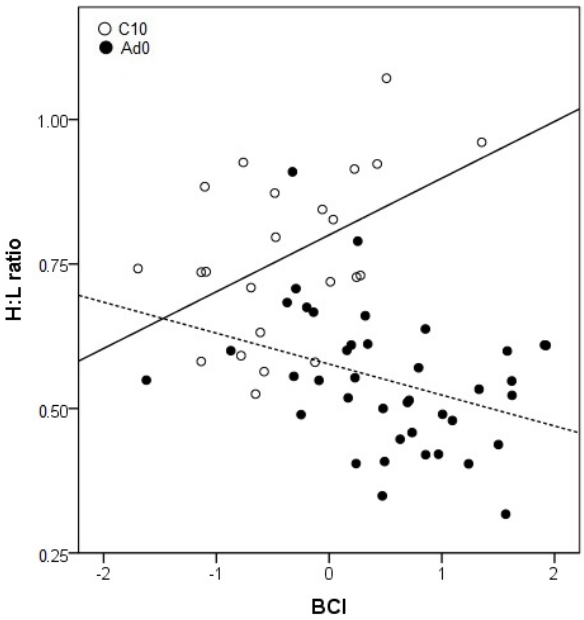

Figure 8. Correlation between H:L ratio and BCI in 10 day old chicks (RA; $P = 0.012$, $R^2 = 0.219$) and adults at hatching (RA; $P = 0.025$, $R^2 = 0.126$). H:L ratios in chicks and adults 25 days after hatching did not correlate with BCI and (RA; $P > 0.100$) are not shown.

Corticosterone (CORT) or the interaction of CORT with age correlated not significantly with any leukocyte profiles (LME; $P \geq 0.183$, appendix, table J).

The interaction between age and sex was significant for HET10 (LME; $F_{3,83} = 3.016$, $P = 0.035$); additionally, sex or the interaction of sex and age was also not found to be significant for any leukocyte profiles (LME; $P > 0.073$, appendix, table J).

Age as a predictor was significant for each of the tested leukocyte parameters (LME; $P < 0.001$, appendix, table J). The total overall number of leukocytes declined significantly from chicks to adults (LME; $P \leq 0.001$), but not between the groups of chicks (LME; $P = 0.368$) or between the adult groups (LME; $P = 0.242$, appendix, table L, figure 9). The numbers of lymphocytes per 10,000 RBCs declined from chicks to adults (LME; $P \leq 0.049$, appendix, table L), where the 10 day old chicks had the lowest value (LME; $P \leq 0.049$) and 25 day old chicks had the highest value (LME; $P \leq 0.036$) of all age groups. The two adult groups did not differ significantly in LYM10 (LME; $P = 0.129$). Higher numbers of HET10 were observed in chicks than adults (LME; $P > 0.021$, figure 9), although there was only a tendency towards a significant difference between chicks and adults at 25 days after hatching (LME; $P = 0.055$). HET10 counts did not differ between chick age groups (LME; $P = 0.538$) or adult age groups (LME; $P = 0.793$).

The youngest chicks had significantly higher H:L ratio, HET% and lower LYM% than the other age groups (LME; $P \leq 0.002$, figure 10). The two adult groups and older nestlings did not differ significantly from each other (LME; $P \geq 0.052$), indicating that 25 day old chicks had reached adult H:L ratio, HET% and LYM% levels.

Chick leukocyte profiles correlated poorly with the leukocyte profiles of their parents (appendix, table M). On average Ch25 ($R^2 = 0.038 \pm .008$) showed a somewhat stronger correlation with adults than Ch10 ($R^2 = 0.028 \pm .008$) (appendix, table M).

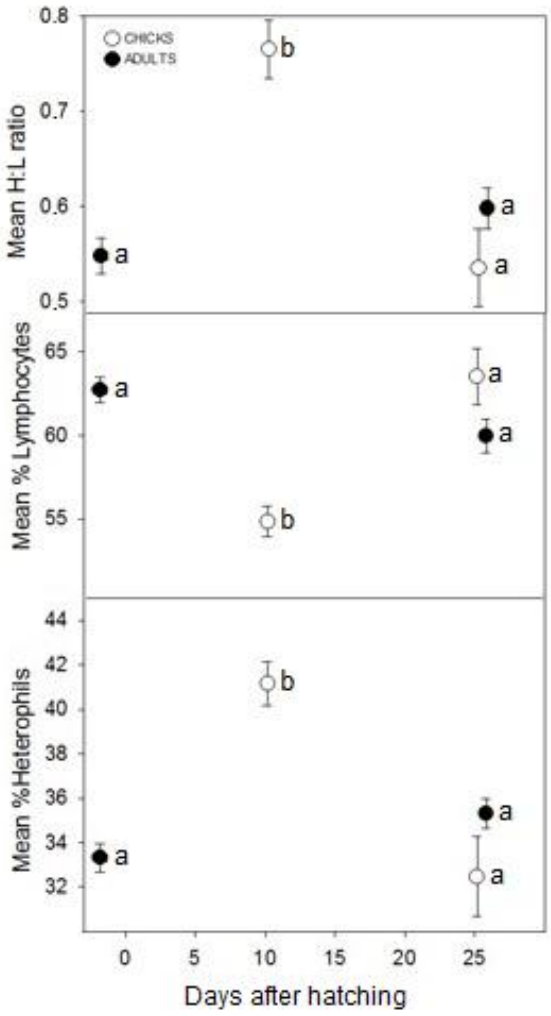

Figure 9. Mean (±SE) leukocyte numbers (#) per 10,000 red blood cells for different age classes in Black-legged kittiwakes. Values with different letters are significantly different from one another (LME; $P \leq 0.05$).

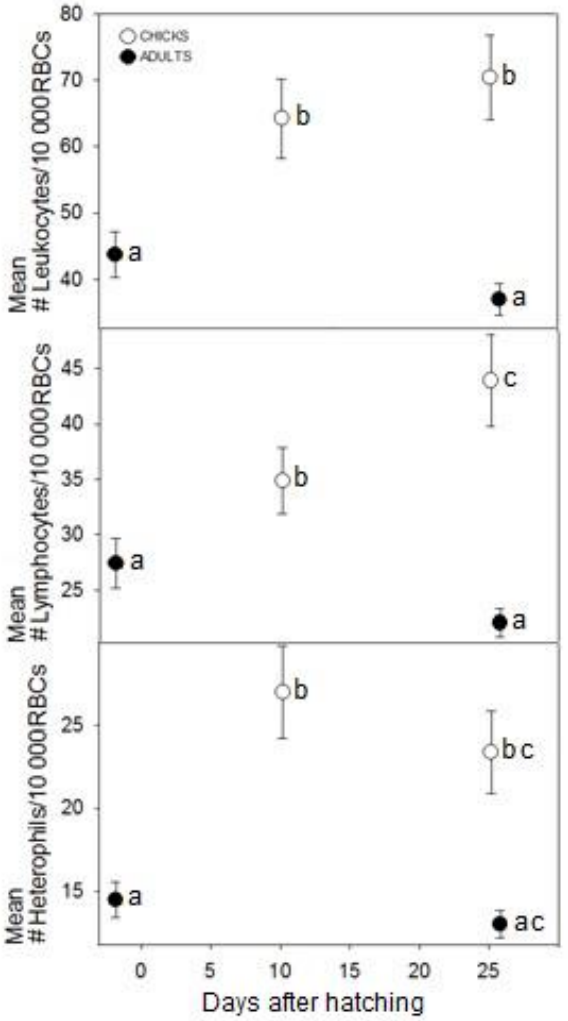

Figure 10. Mean (±SE) values of H:L ratios, LYM% and HET% of different age classes of Black-legged kittiwakes. Values with different letters are significantly different from one another (LME; $P \leq 0.05$).

4 Discussion

4.1 Limitations of study

In the present study it was assumed that the main circulating cells of the innate and acquired immune system, the heterophils and lymphocytes respectively, are indicative of the investment level and immune status of kittiwakes. However, as no tests for actual immune responses were conducted, leukocyte profiles from blood smears are only to be regarded as an index of immune investment levels. To obtain a better measurement of the overall immune function of kittiwakes, multiple assays which challenge specific immune system components should preferably have been performed in addition (Norris and Evans, 2000). The different leukocyte profiles only measures the level of circulating cells in the bloodstream and the relative presence of each leukocyte cell type in the blood may be altered by *e.g.* infection (*e.g* Ots et al., 1998) or stress (Davis et al., 2008). Nevertheless, the blood is an important immune cell compartment which works as a duct where leukocytes are transported between tissues (Mackay et al., 1990). Measurements of leukocyte profiles in the blood might therefore provide a good indication of the investment status in kittiwakes as the cells of the innate and acquired immune system has different costs (*e.g* Lee, 2006).

4.2 Handling tests

4.2.1 Handling time

How the immune system changes in the form of circulating leukocytes during development for chicks and during parts of the breeding period for adults, were the main issues of the present study. However, as transportation (*e.g.* Parga et al., 2001), routine handling and data collection procedures (Davis, 2005) have been shown to affect leukocyte profiles, handling stress may also invalidate the results of the main study.

The effect of handling alone, without repeated sampling, showed that the values of H:L ratio, LYM% and HET% at three min were similar to the control sampled after 60 min (figure 7). This finding implies that the time from initial capture is not crucial when considering these leukocyte profiles; at least not within the measured time scale of 60 min. For the relative amount of leukocytes, a different response was observed. Leukocytes and lymphocytes per 10,000 RBCs declined significantly from the baseline levels (measured at three min)

regardless of the type of handling (figure 6) while heterophils per 10,000 RBCs did however not change over time (appendix, table H). This overall decline in leukocytes after 60 min of handling has been argued to be attributable to a significant reduction in lymphocyte traffic in the bloodstream over time (Dhabhar, 1996). However, heterophil cell traffic in the blood may be *increased* as a response to stress (Dhabhar, 1996). The lymphocytes dominated the leukocyte composition in kittiwakes with approximately 60% of total leukocyte numbers (Newman et al., 1997; Oddvar Heggøy, unpublished results; table 2-4) and as the heterophils did not change significantly, any potential effect of an increased number of heterophils in the blood is likely to be small. Even though the total leukocyte numbers declined, they probably did not undergo destruction or apoptosis (Dhabhar, 1996). It is more likely an active response where the heterophils form the first line of defense and the lymphocytes are redistributed to lymph nodes where they receive antigens from a possible infection in the nearby future (Dhabhar, 2002).

Similar findings to the present study were seen in house finches (Davis, 2005) where a negative response was detected in LEU10, LYM10 and HET10 after 60 min, regardless of whether or not the birds were previously bled. These birds also had a high baseline amount of lymphocytes (over 70%, Davis et al., 2004), which may explain the findings. These results are further consistent with stressful handling of racing pigeons (*Columba livia domestica*; Scope et al., 2002) and broilers (Wang et al., 2003) all of which have in common a larger proportion of lymphocytes relative to heterophils. However, another study on broilers showed an *increase* in leukocyte numbers (Mitchell and Kettlewell, 1998). The form of handling may explain the contradictive results from the two studies on broilers; while Mitchell and Kettlewell (1998) tested heat stress and transportation, Wang et al. (2003) tested housing conditions and exposure to intravenous endotoxines. Other studies in peregrine falcons (*Falco peregrinus*) and Harris's hawks (*Parabuteo unicinctus*) by Parga et al. (2001) have shown the *opposite* response to that found in kittiwakes; *i.e.* that the response to handling alone caused an *increase* in total leukocyte numbers. These results may be explained by their high heterophil counts and possible behavioral differences (Parga et al., 2001). The previous studies together with the current study, suggests that the effect of handling time should be considered when interpreting results from leukocyte profiles.

4.2.2 Repeated sampling

Repeated sampling from the same or opposite wing may potentially also affect leukocyte profiles. It was hypothesized that re-sampling on the same wing in adults would give rise to a higher heterophil count and consequently increased H:L ratio. This is because inflammations are known to cause a *local* accumulation of heterophils at the infection site (Harmon, 1998), and that the same reaction might be detectable with a small wound inflicted by a needle. However, repeated sampling on the same and opposite wing did not result in any difference in leukocyte profiles. The small wound caused by the needle may not have caused a significant infection and potentially accumulated heterophils may therefore have been bound in the local inflamed tissue. Thus, the response appeared to be a global, whole-body reaction.

On the other hand, repeated bleedings, but not handling time *per se* changed the H:L ratios and relative (%) leukocyte parameters (figure 7). The H:L ratio increased significantly already after 30 minutes as a result of increased HET% and reduced LYM% over time. A similar response has also been shown in house finches (Davis, 2005), where the H:L ratio reached significance only after 60 minutes.

Because the present study shows that repeated sampling of kittiwakes changed the H:L ratio significantly already after 30 minutes, one should be aware of possible bias from repeated samples obtained even earlier than shown by Davis (2005). On the other hand, other studies have shown the H:L ratio to change with handling time. Transportation caused a significant increase in H:L ratio in the wild Hawaiian honeycreeper (*Paroreomyza Montana;* Groombridge et al., 2004), in racing pigeons (Scope et al., 2002) and in domestic turkeys (*Melagris gallopavo*; Huff et al., 2005). While the honeycreepers changed H:L ratios within one hour, the pigeons and turkeys only changed the H:L ratio after an evidently longer handling time (three and 12 hours respectively). Even though the experimental set up and handling time was different in the latter studies, one should still be aware of potential species specificity to handling.

The tests for repeated sampling on the kittiwakes were conducted in two different field seasons. In 2012 adult kittiwakes were sampled at 3, 30 and 60 min, while in 2013 the kittiwakes were sampled at only 3 and 60 min. While repeated sampling gave significant change of LEU10, LYM10 and HET10 in 2012, this was not seen in 2013. The responses of these particular leukocyte profiles may need several repeated samples (more than one) in order to elect significant changes within one hour. Another explanation may be that there are

differences between breeding seasons in different years in *e.g.* food abundance (Koutsos and Klasing, 2008). Testing one group with two repeated samplings and the other with one repeated sample within the same breeding season would enlighten this subject further. The response of H:L ratio, LYM% and HET% was similar in 2012 and 2013 for the initial three min and 60 min repeated samples, which may suggest that the three min samples triggered a full response in both tests. These results also suggest that the response of these leukocyte profiles to this particular type of handling did not differ between the two breeding seasons. However, even though the response seemed to be fully triggered by the initial sampling, the duration effect may be prolonged by the repeated sample at 30 min (in 2012). In that case, this would probably have been shown as a longer duration time before leukocyte values returned back to baseline levels after the stressor was removed. A stress test on horses (Cardinet et al., 1964 in Davis et al., 2008) might have shown this as horses forced to strenuous activity had lymphocyte values returning back to baseline levels 14 hours later compared to controls (non-forced activity). The results from previous and the current study suggest that the way leukocyte profiles respond to a stressor may vary among species, and may even depend on the type of handling and should thus be taken into consideration when interpreting results of leukocyte profiles obtained from blood smears.

4.3 Development and leukocytes

4.3.1 Corticosterone and Leukocytes

It is known that several hormones play a major role in modulating the immune system (Koutsos and Klasing, 2008). In the present study, corticosterone did not correlate with any leukocyte profile in either chicks or adults, which is somewhat surprising since it is often assumed that CORT directly causes a change in H:L ratio over time (Davis et al., 2008). However, there might be indications that the H:L ratio and circulating CORT do not indicate the same types of stress. As pointed out by Müller et al. (2011), only three studies have studied endogenous CORT levels and the H:L ratio together. In these studies, none or only weak correlations were found. In their own study of Eurasian kestrel nestlings (*Falco tinnunculus*; Müller et al. 2011), only the exogenous administered levels of CORT correlated with suppressed immune defense (increased H:L ratio), while endogenous CORT did not. Instead, H:L ratio correlated better with environmental factors such as hatching date. Baseline CORT was only elevated as a reaction to human presence at the nests, or when nestlings had very low body fat stores. Thus, the study of kestrels together with the present study supports

that the levels of CORT and leukocyte profiles should not be used interchangeably as indicators of stress, but rather together to provide a more complete picture of the current stress status (Müller et al., 2011).

4.3.2 Developmental changes in leukocyte profiles

Leukocyte profiles have been used extensively to assess different physiological aspects in birds (Davis, 2005; Davis et al., 2008) and have proved to be valuable indicators of health and condition (Masello et al., 2009). Sex correlated overall poorly with the leukocyte profiles in the present study. This was somewhat surprising as several immunological sex differences are observed in birds (Fellah et al., 2008). However, sex differences in immune function have been connected to mating systems (Klein, 2000), where monogamous species, such as kittiwakes, are expected to invest similar levels of immune function (O`Neal and Ketterson, 2012).

Surprisingly little is known about the development of the immune system in wild birds (Ardia and Schat, 2008). In addition, studies have often been conducted on domestic species selected for increased production which has been shown to change the immune development (Leshchinsky and Klasing, 2001). Of the two chick groups in the present study, leukocyte profiles correlated with body condition only in the 10 day old chicks (figure 8). The individuals with better body condition showed higher H:L ratios because of higher heterophil levels. Thus, the results show that young chicks with better body condition also had more cells of the innate immune system. Although the immune system of young chicks is considered immature and inefficient (*e.g.* Fellah et al., 2008), chicks may still benefit from increased investments in the innate components. The young chicks with higher BCI may afford to invest in a stronger innate immune system for non-specific protection, in addition to prioritizing growth. Although the innate immune system generally is considered to cost less than the acquired immune system (Klasing and Leshchinsky, 1999; Lee, 2006), the *inflammatory responses* of innate immunity are considered quite costly (table 1) and often lead to reduced growth (Lochmiller and Deerenberg, 2000). Maternal antibodies may reduce these costs as they can block the stimulation of potential inflammatory responses and therefore reduce growth suppression (Grindstaff, 2008). The results of the present study are further consistent with a study on nestling burrowing parrots (*Cyanoliseus patagonus*;

Masello et al., 2009) where a high BCI was correlated with higher H:L ratios also suggested to be favorable in terms of innate immune investment.

When chicks hatch, they hatch with a certain level of immune function (Fellah et al., 2008). Kittiwake chicks in the present study experienced a decrease in H:L ratio from 10 to 25 days after hatching as a result of decreased amount of heterophils and increased numbers of lymphocytes (figure 9-10). These results suggest that the chicks at the age of 25 days, have at least partially replaced the innate components with an increased acquired immune system. Thus, the relative decrease in heterophils and increase in lymphocytes from young to older chicks may display a shift in the energy investment from innate to acquired immunity with age. Now they may produce their own antibodies, since maternal antibodies are most likely completely broken down (King et al., 2010), while at the same time they may down-regulate the need for innate immunity.

A correlation between H:L ratio and BCI was found also in the adults sampled at hatching. Contrary to the young chicks however, this correlation was negative, *i.e.* individuals with higher BCI had lower H:L ratio (figure 8). Hence, individuals in better condition prioritized the acquired immune system in a larger degree than individuals with lower body condition. This result is in accordance with the previous statement that the acquired immune system in general is considered more costly to the host than the innate components (Klasing and Leshchinsky, 1999; Lee, 2006).

While the 10 day old chicks and parents at hatching showed correlations between leukocyte profiles and BCI, kittiwake chicks and adults 25 days after hatching did not (figure 8). Results in the latter kittiwake groups are similar to the results in other studies of birds such as Thin-billed prions (*Pachyptila belcheri*; Quillfeldt et al., 2008) and Red-tailed tropicbirds (*Phaethon rubricauda westralis*; Dehnhard et al., 2011b) where a lack of correlation between BM/BCI and leukocyte profiles was found. Even though these results showed no apparent correlation between conditional and immunological data, nutrients may still be an important regulatory factor. The BCI was measured using biometric measurements and body mass, whilst the status of specific nutrients was not examined. This may be important, as for example a lack of vitamin E leads to a decrease in certain acquired immune responses (Fellah et al., 2008). Therefore, nutrient levels may still affect the immune system, but are not necessarily connected directly with BCI or BM in particular.

Young kittiwake chicks with high BCI also had correspondingly higher levels of heterophils which previously have been interpreted as a symptom of infection, diseases and stress (*e.g* Davis et al., 2008). However, these symptoms also include a simultaneous decrease in lymphocytes (Dhabhar, 2002) which was not seen in these kittiwakes as lymphocytes did not correlate with BCI. In addition, CORT was not correlated with any leukocyte parameter, suggesting this indicator of stress to be absent or minimal. Further, mounting an immune response has proven to be costly and to reduce body mass and increase metabolism (reviewed in Demas et al., 2011). For example, heavier infestations of mite have been correlated to increased levels of heterophils, although with a correspondingly lower body mass (Lobato et al., 2005). Young kittiwake chicks on the other hand showed higher levels of body condition with higher levels of heterophils, suggesting the heterophilic profiles to reflect investment status and not infection. A heterophilic response to infection is also seen together with an increase in total leukocyte numbers (*e.g.* Ots et al., 1998). This was not seen in the present study, although there was a tendency to significance in the youngest chicks. One might argue that the observed high values of heterophils and H:L ratio should not be related to diseases or inflammation as the high heterophil counts would then be expected to stronger affect the nestlings with *weaker* body conditions (*see* Masello and Quillfeldt, 2002), rather than the kittiwake chicks with higher body conditions. Therefore the tendency of higher leukocyte numbers and levels of heterophils in the young kittiwakes could rather be interpreted as a favored investment in a robust innate immunity.

4.3.3 Species specific strategies?

During chick development, birds might change their H:L ratio according to their species´ specific investment strategy as similar species often have different H:L ratios (*e.g.* Hawkey et al., 1983; Newman et al., 1997). The degree of change in innate or acquired immune system could be indicative on the investment levels if the cost of the immune system components is similar for different species. A difference between initial "newly-hatched" H:L ratio and adult H:L ratio could then describe the *degree of investment* in a particular species. For example, Red-tailed tropicbirds (Dehnhard et al., 2011b) showed similar patterns as the kittiwakes in the present study. The young chicks (approximately 14 days old) had the highest H:L ratio (1.4), which later (approximately 46-90 days old) declined to a level similar to the adult tropicbirds (0.8). These results suggest that these birds invest in an acquired immune system from a basic level of innate immunity. However, the *degree* of reduction in H:L ratio from the

young (0.75) to the older (0.53) kittiwake chicks was *lower* (ca 29%) than the tropicbirds (ca 43%). These results may be explained by different investment strategies in an arctic breeding, versus a tropic breeding seabird. Compared to individuals in the tropics, species from the high arctic are exposed to a low parasitic pressure, because insect vectors and ectoparasites are uncommon at high latitudes (Coulson et al., 2009; Morand and Krasnov, 2010). Therefore, the tropicbirds might invest in a larger degree into their specific acquired immune system compared to the kittiwakes because of a potential larger pathogenic pressure (Møller, 1998). In addition, tropicbirds develop slower (fledge after 67-91 days; Orta, 1992) than the kittiwakes, a difference which may have a profound impact on their immunological needs as a prolonged developmental period may allow for greater diversification of the immune system (Apanius, 1998).

A decreasing H:L ratio such as in the tropicbirds and the current study is not a common strategy of birds. The opposite direction of development is seen in seabirds such as the Thin-billed prions (Quillfeldt et al., 2008), where the H:L ratio was higher in older chicks (pre fledgling) compared to younger chicks (about three weeks old), suggesting an overall innate immunity investment. These birds live most of their non-breeding life at the marine sea (Prince and Morgan, 1987), an environment hypothesized to be relatively pathogen-free (Piersma, 1997; Mendes et al. 2005). Therefore, prions might experience even less pathogenic pressure than the arctic breeding kittiwakes. The different strategies may be indicative that different species rely on different combinations of innate and acquired immunity, which is not surprising since adult leukocyte profiles in different wild seabirds are shown to be markedly different (*e.g.* Newman et al., 1997).

4.3.4 Adult leukocyte profiles

Adult kittiwakes have been shown to suppress the immune system *response* in one costly acquired immune system component, the cell-mediated immunity, during reproduction (Broggi et al., 2010). Additionally, both BCI and BM of adults are often lowest at the end of the breeding season (Bech et al., 2002). In the present study, while the chicks increased BM, the parent kittiwakes also showed similarly reduced BM from hatching until the end of the breeding season. Therefore it was expected that leukocyte profiles would change accordingly with increased innate and lowered acquired components of the immune system. However, none of the adult leukocyte profiles changed significantly during the course of the breeding

season in the present study. Whether this is caused by a recovery from a period of immunosuppression earlier during chick-rearing, remains unclear as the previously observed reduced immune system was measured only 14 days after hatching (Broggi et al., 2010), compared to 25 days in my study. Another explanation is that not leukocyte profiles, but the immune system`s ability to *respond* is suppressed. Reducing the responsiveness of one immune system component such as acquired cell-mediated immunity, without reducing cell numbers, may be a temporary strategy in a time with little resources. In conclusion, the results from the present study combined with previous ones imply that even though parent kittiwakes may experience important immunosuppression and lowering of BCI and BM, this does not necessarily lead to changes in leukocyte profiles.

4.3.5 Heritability

Previous studies on birds such as domestic fowls, have shown the H:L ratio to be highly heritable (Al-Murrani et al., 1997) and to respond heavily to selection (Campo and Davila, 2002). Adult kittiwake leukocyte profiles were therefore expected to significantly correlate, or at least partially correlate, with their chick leukocyte profiles. However, parental leukocyte values did not correlate with any corresponding leukocyte values in either 10 day old chicks or 25 day old chicks (appendix, table M) although the average value of all correlation tests revealed that chicks correlated better when they were older.

The above results are somewhat surprising, although it is known that many post egg-laying factors may modulate the immune system (Koutsos and Klasing, 2008). However, while chick H:L ratio changed markedly from 10 to 25 days after hatching, the oldest chicks did not differ from the parental values (figure 10). These similarities suggest that 25 day old chicks have reached adult levels of immune investment. Even though there were no correlations between the parental and chick leukocyte profiles, a genetic component may still be affecting the given level of immune investment (*e.g.* Benedict et al., 1975; Bayyari et al., 1997). Therefore, the results might suggest that the level of investment is determined before the chicks fledge, giving them the "package" needed for the rest of their life. This being said, the immune system does not stop to develop or adapt as the acquired immune system memory continuously learn from new challenges (Murphy, 2012). On the other hand, the relative amount of each cell type might stabilize on a certain level. The size of bursa for example, regresses at the onset of sexual maturation (Glick, 1991) probably reflecting reduction of cell

number differentiation. In addition, the measured antibody levels in Andean condors (*Vultur gryphus)* have been shown to be similar in fledglings and in adults (Bruning et al., 1981 in Apanius, 1998). The results of the present study suggests that the H:L ratio of kittiwakes is somewhat predisposed and is similar between the parents and chicks.

The relative amount of leukocytes in relation to total cell numbers decreased from chicks to adults in kittiwakes (figure 9). These results may be explained by higher HTC levels in adults compared to chicks. Studies on HTC levels in birds have shown it to increase with age in birds such as chinstrap penguins (*Pygoscelis antarctica*; Merino and Barbosa, 1996), black vultures (*Aegypius monachus*; Villegas et al., 2002), pied flycatchers (*Ficedula hypoleuca*; Potti, 2007) and kittiwakes (Næss, 1999). Similar connections of increased HTC values and/or lowering of leukocyte numbers have been found in other studies such as in Red-tailed tropicbirds, where HTC values were higher in adults than in chicks (Work, 1996), while the chicks have the higher relative amount of leukocytes (Dehnhard et al., 2011b). These findings suggest that the increased HTC values from chicks to adults are a general pattern among birds, and that lowered amount of leukocytes is consistent with this.

4.4 Conclusions

The two measured forms of handling, handling time and repeated bleedings, affected the adult kittiwake leukocyte profiles differently. Specifically in the present study, repeated sampling increased the H:L ratio significantly at 30 minutes. On the other hand, handling time did not change H:L ratios, although total leukocytes and lymphocytes per 10,000 RBCs decreased. This implies that one should take precaution when interpreting results from blood smears if one suspect that they may be biased by handling. Handling alone likely did not affect the kittiwake developmental data, at least not the H:L ratio. The parent kittiwakes do not seem to change their leukocyte profiles during the breeding season, but could still regulate the ability of the immune system to respond. Plasma CORT levels did not correlate with adult or chick leukocyte profiles which support the theory that CORT and leukocyte profiles are two different indicators of stress. Furthermore, different leukocyte profiles in young and older kittiwake chicks points to different investment strategies. While the young chicks prioritize growth and development together with the innate immunity as a more general protection, older chicks invests more heavily in the acquired and specific immune system to such a degree that their leukocyte profiles resembles those of their parents.

4.5 Future studies

Future studies should test immune *responses* as they might not reflect the same conditions in immunity as leukocyte profiles. Further, comparing measurements of HTC and leukocyte parameters would possibly reveal correlations as HTC is known to vary with factors such as infection (Natt and Herrick, 1955), moulting (Dein, 1986), diseases and parasites (*e.g.* Johnson et al., 1991; Potti, 2007). The time of which leukocyte numbers (after handling) and H:L ratios (after blood sampling) return to baseline levels in kittiwakes is to my knowledge not known and should be addressed by future research to prevent bias in immune measures of *e.g* seasonal time series. In terms of costs, the exact value for each immune cell component is not known and might be different in different species. The exact definition of costs is a disputed subject (*e.g.* Ricklefs and Wikelski, 2002) and therefore the actual cost of investment may only be suggestive. Additionally, researching the *degree* of investment in a given species might reveal new correlations with factors such as parental – chick immune investments, environmental variations etc. However, by looking at the levels of change in leukocyte profiles within one breeding season, one might get a good indication of the investment levels in the studied species.

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Appendix

For molecular sexing, a stock mix containing primers and nucleotides were made for PCR (table A).

Table A. Total reaction volume per PCR mixture/ per individual sexing

Solution	Volume (µl)	Solution	Volume (µl)
Taq DNA polymerase[1]	0.05	Q solution[1]	2.00
ddH_2O	1.95	10 µM Primer (2710)[2]	1.00
dNTP Mix[1]	0.40	10 µM Primer (2550)[2]	1.00
MgCl[1]	0.60		
10x PCR Buffer[1]	1.00	**Total stock**	**8.00**

[1] PCR Core Kit (Qiagen, West Sussex, UK)
[2] Invitrogen

Collinearity between all variables was checked in both the developmental study models (table B) and handling test models (table C).

Table B. Variance Inflation Factors (VIF) of variables tested in the final developmental models.

		Collinearity statistics				Collinearity statistics	
Model	**Variable**	**Tolerance**	**VIF**	**Model**	**Variable**	**Tolerance**	**VIF**
1	H:L ratio	.832	1.201	**4**	LEU10	.737	1.356
	Identity	.383	2.612		Identity	.367	2.725
	Nest identity	.950	1.053		Nest identity	.955	1.047
	Sex	.967	1.034		Sex	.966	1.035
	BCI	.814	1.229		BCI	.865	1.156
	CORT	.801	1.249		CORT	.796	1.256
	Age group	.398	2.510		Age group	.418	2.391
2	LYM%	.858	1.165	**5**	LYM10	.762	1.312
	Identity	.383	2.612		Identity	.363	2.756
	Nest identity	.955	1.047		Nest identity	.955	1.047
	Sex	.968	1.033		Sex	.964	1.038
	BCI	.818	1.222		BCI	.846	1.182
	CORT	.795	1.258		CORT	.799	1.251
	Age group	.404	2.477		Age group	.424	2.358
3	HET%	.838	1.194	**6**	HET10	.713	1.403
	Identity	.383	2.612		Identity	.372	2.685
	Nest identity	.949	1.054		Nest identity	.955	1.047
	Sex	.968	1.033		Sex	.968	1.033
	BCI	.810	1.235		BCI	.888	1.126
	CORT	.801	1.249		CORT	.798	1.253
	Age group	.403	2.483		Age group	.404	2.475

Table C. Variance Inflation Factors (VIF) of variables tested in the final handling test models.

		Collinearity statistics				Collinearity statistics	
Model	**Variable**	**Tolerance**	**VIF**	**Model**	**Variable**	**Tolerance**	**VIF**
1	H:L ratio	.924	1.083	**4**	LEUK10	.938	1.066
	Identity	.998	1.002		Identity	.938	1.066
	Time	.924	1.083		Time	.952	1.051
2	LYM%	.930	1.075	**5**	LYM10	.929	1.076
	Identity	.989	1.011		Identity	.959	1.042
	Time	.989	1.011		Time	.929	1.076
3	HET%	.920	1.087	**6**	HET10	.938	1.067
	Identity	.998	1.002		Identity	.938	1.067
	Time	.920	1.087		Time	.991	1.009

In the handling test, estimates of H:L ratio, LYM% and HET% between samples from 60_{same} and $60_{opposite}$ (60 vs 60) were significantly less than between 3 min and 60 min (table D) and therefore pooled together.

Table D. Comparisons of repeated sampling in left-left and left-right wing (60 vs 60) bleedings and comparisons between 3 min and 60 min pooled wing data (3 vs 60). LME model include individual identity as random variable.

Parameter	**Comparison**	**Estimate**	**d.f**	**t**	**P**	**Comparison**	**Estimate**	**d.f**	**t**	**P**
H:L ratio	60 vs 60	.010	1,14	.107	.916	3 vs 60	-.219	30	4.327	**<.001**
LYM%	60 vs 60	-.764	1,14	.273	.789	3 vs 60	7.273	30	4.542	**<.001**
HET%	60 vs 60	-.818	1,14	.322	.752	3 vs 60	-7.206	30	4.931	**<.001**
LEU10	60 vs 60	-4.554	1,14	.605	.555	3 vs 60	2.229	30	.527	.606
LYM10	60 vs 60	-3.060	1,14	.651	.525	3 vs 60	3.267	30	1.187	.254
HET10	60 vs 60	-1.860	1,14	.636	.535	3 vs 60	-1.205	30	.788	.443

Further, H:L ratio, LYM% and HET% from both handling test groups were also pooled together because of the lack of significant difference between the 3 min groups and the 60 min groups (table E). The estimates were less between the two 3 min groups and two 60 min groups than between 3min and 60 min in total (table F).

Table E. Comparisons between the two 3 min groups (3 vs 3) and between the two 60 min groups (60 vs 60) from 2013 and 2012. LME model include individual identity as random variable.

Parameter	**Comparison**	**Estimate**	**d.f**	**t**	**P**	**Comparison**	**Estimate**	**d.f**	**t**	**P**
H:L ratio	3 vs 3	.009	23	.170	.867	60 vs 60	.055	23	.729	.473
LYM%	3 vs 3	-2.086	23	1.120	.274	60 vs 60	-2.471	23	1.065	.298
HET%	3 vs 3	-1.098	23	.679	.504	60 vs 60	.658	23	.312	.758

Table F. Comparisons between pooled 3 min data and pooled 60 min data from 2012 and 2013 and pooled 60 min data from 2012 and 2013. LME model include individual identity as random variable.

Parameter	Comparison	Estimate	d.f	t	P
H:L ratio	3 vs 60	-.236	48	5.438	**<.001**
LYM%	3 vs 60	7.411	24	5.225	**<.001**
HET%	3 vs 60	-7.838	48	6.249	**<.001**

Repeated sampling (table G) caused a significant change in H:L ratio, LYM% and HET% (table H), while LEU10, LYM10 and HET10 changed significantly only in 2012 (table I).

Table G. The effect of repeated sampling (2012 and 2013) on leukocyte profiles. In 2012 repeated sampling (3, 30 and 60 min) caused a significant positive change in all leukocyte profiles with the exception of the numbers of HET10. In 2013 repeated sampling (3 and 60 min) caused a significant positive change in H:L ratio, LYM and HET, but not in the other leukocyte profiles. LME models include individual identity as random variable.

	Repeated				Repeated			
Parameter	**Sampling**	**d.f**	**F**	**P**	**Sampling**	**d.f**	**F**	**P**
H:L ratio	2013	2, 29	9.066	**.001**	2012	2, 16	7.442	**.005**
LYM%	2013	2, 20	10.360	**.001**	2012	2, 16	5.610	**.014**
HET%	2013	2, 29	11.888	**<.001**	2012	2, 16	9.977	**.002**
LEU10	2013	2, 20	.392	.681	2012	2, 16	4.286	**.032**
LYM10	2013	2, 29	.996	.382	2012	2, 16	7.050	**.006**
HET10	2013	2, 20	.542	.590	2012	2, 16	.548	.588

Table H. The effect of repeated sampling and the effect of handling time (60*). Comparisons include complete pooled data from 2012 and 2013. LME models include individual identity as random variable.

	Min	Estimate	SE	df	t	P
LEU10	3 vs 30	-5.242	3.605	18	-1.454	.163
	3 vs 60	-10.807	3.605	18	-2.998	**.008**
	30 vs 60	-5.565	3.605	18	-1.544	.140
	3 vs 60*	12.002	4.810	26	2.495	**.019**
	30 vs 60*	6.760	4.810	26	1.405	.172
	60 vs 60*	1.195	4.810	26	.249	.806
LYM10	3 vs 30	-4.326	2.336	18	-1.852	.081
	3 vs 60	-8.871	2.336	18	-3.798	**.001**
	30 vs 60	-4.545	2.336	18	-1.946	.068
	3 vs 60*	6.535	2.995	26	2.182	**.038**
	30 vs 60*	2.209	2.995	26	.738	.467
	60 vs 60*	-2.335	2.995	26	-.780	.442
HET10	3 vs 30	.711	1.415	19	.502	.621
	3 vs 60	-.849	1.415	19	-.600	.555
	30 vs 60	-1.560	1.415	19	-1.103	.284
	3 vs 60*	4.199	1.830	27	2.295	.073
	30 vs 60*	4.910	1.830	27	2.684	.082
	60 vs 60*	3.350	1.830	27	1.831	.078

Table I. The effect of repeated sampling and the effect of handling time (60*). Comparisons include complete pooled data from 2012 and 2013. LME models include individual identity as random variable.

	Min	**Estimate**	**SE**	**df**	**t**	**P**
H:L ratio	3 vs 30	.123	.054	52	2.262	**.028**
	3 vs 60	.236	.039	39	6.027	**.000**
	30 vs 60	.113	.054	52	2.073	**.043**
	3 vs 60*	.045	.058	63	.755	.441
	30 vs 60*	.170	.069	63	2.431	**.018**
	60 vs 60*	.280	.058	63	4.859	**.000**
LYM%	3 vs 30	-3.198	1.790	50	-1.787	.080
	3 vs 60	-7.411	1.273	38	-5.822	**.000**
	30 vs 60	4.213	1.789	50	-2.354	**.023**
	3 vs 60*	-1.514	1.937	62	-.781	.438
	30 vs 60*	-4.711	2.310	63	-2.040	**.046**
	60 vs 60*	-8.924	1.937	62	-4.607	**.000**
HET%	3 vs 30	5.294	1.641	52	3.226	**.002**
	3 vs 60	7.838	1.183	38	6.626	**.000**
	30 vs 60	-2.544	1.641	52	-1.550	.127
	3 vs 60*	1.520	1.735	63	.876	.384
	30 vs 60*	6.814	2.075	63	3.285	**.002**
	60 vs 60*	9.359	1.735	63	5.394	**.000**

In the developmental study, did sex not predict any of the leukocyte profiles (table J) and these data were therefore pooled (figures 9-10). Regression analysis of leukocyte profiles comparing adult and chick values revealed no apparent correlation in any combinations (table M).

Table J. Full and simplified LME models used in the developmental study. Values of rejected variables are those before model simplification. All predictor variables included as fixed effects. All models include Nest identity, individual identity and Individual identity (Nest identity) as random factors. Age = age group Ch10, Ch25, Ad0 and Ad25.

Dependent	**Predictor**	**d.t**	**F**	**P**		**Predictor**	**d.t**	**F**	**P**
H:L ratio	Age	3, 91	1.062	.369	*Simplified model*	Age	3, 85	14.031	**<.001**
	Sex	1, 63	.444	.507		BCI	1, 99	.453	.502
	Age*Sex	3, 80	2.342	.079		Age*BCI	3, 99	2.822	**.043**
	BCI	1, 89	1.041	.310	*Excluded*	CORT	1, 88	1.080	.302
	Age*BCI	3, 84	2.954	.037		Age*CORT	3, 88	1.625	.189
	CORT	1, 88	1.080	.302		Sex	1, 61	.022	.883
	Age*CORT	1, 88	1.625	.189		Age*Sex	3, 83	2.032	.116
LYM%	Age	3, 93	2.158	.098	*Simplified model*	Age	3, 76	11.609	**<.001**
	Sex	1, 63	1.417	.238	*Excluded*	CORT	1, 93	.842	.361
	Age*Sex	3, 83	2.309	.082		Age*CORT	3, 93	.131	.942
	BCI	1, 83	1.017	.316		BCI	1, 94	.445	.507
	Age*BCI	3, 88	1.456	.232		Age*BCI	3, 97	.887	.451
	CORT	1, 93	.842	.361		Sex	1, 61	.626	.432
	Age*CORT	3, 93	.131	.942		Age*Sex	1, 82	2.408	.073
HET%	Age	3, 92	.810	.491	*Simplified model*	Age	3, 74	14.408	**< .001**
	Sex	1, 61	.462	.499	*Excluded*	Sex	1, 61	.462	.499
	Age*Sex	3, 81	1.527	.214		Age*Sex	3, 81	1.527	.214
	BCI	1, 87	.308	.580		BCI	1, 93	.009	.926
	Age*BCI	3, 85	2.397	.074		Age*BCI	3, 93	1.589	.197
	CORT	1, 91	.913	.342		CORT	1, 96	1.797	.183
	Age*CORT	3, 91	1.919	.132		Age*CORT	3, 101	1,56	.204
LEU10	Age	3, 95	3.257	.025	*Simplified model*	Age	3, 81	11.635	**<.001**
	Sex	1, 95	3.804	.054	*Excluded*	CORT	1, 95	.355	.553
	Age*Sex	3, 95	2.841	.042		Age*CORT	3, 95	.789	.503
	BCI	1, 95	8.097	.005		Sex	1, 65	.884	.351
	Age*BCI	3, 95	3.870	.012		Age*Sex	3, 86	1.946	.128
	CORT	1, 95	.355	.553		BCI	1, 99	3.104	.081
	Age*CORT	3, 95	.789	.503		Age*BCI	3, 102	1.320	.272
LYM10	Age	3, 95	2.310	.081	*Simplified model*	Age	3, 81	11.126	**<.001**
	Sex	1, 95	2.853	.094	*Excluded*	CORT	1, 95	.045	.833
	Age*Sex	3, 95	1.677	.177		Age*CORT	2, 95	.508	.678
	BCI	1, 95	4.432	.038		Sex	1, 102	.776	.380
	Age*BCI	3, 95	2.011	.118		Age*Sex	3, 102	2.402	.294
	CORT	1, 95	.045	.833		BCI	1, 98	1.654	.202
	Age*CORT	3, 95	.508	.678		Age*BCI	3, 102	.609	.611
HET10	Age	3, 94	4.479	.006	*Simplified model*	Age	3, 84	14.599	**<.001**
	Sex	1, 68	4.377	.040		Sex	1, 62	.625	.432
	Age*Sex	3, 83	4.899	.003		Age*Sex	3, 83	3.016	**.035**
	BCI	1, 87	11.532	.001		BCI	1, 95	6.333	**.014**
	Age*BCI	3, 88	7.539	.000		Age*BCI	3, 95	4.447	**.006**
	CORT	1, 92	.904	.344	*Excluded*	CORT	1, 92	.904	.344
	Age*CORT	3, 93	1.610	.192		Age*CORT	3, 93	1.610	.192

Table K. Testing for differences in H:L ratios, lymphocytes (%) and heterophils (%) between age groups Ch10 (N=23), Ch25 (N=23), Ad0 (N=40) and Ad25 (N=28). LME model include Nest identity, individual identity and Individual identity (Nest identity) as random factors.

	Age group	Estimate	SE	df	t	P
H:L ratio	Ch10 vs Ch25	.272	.046	71	5.863	**<.001**
	Ch10 vs Ad0	.222	.042	97	5.349	**<.001**
	Ch10 vs Ad25	.206	.064	96	3.200	**.002**
	Ch25 vs Ad0	-.050	.043	98	-1.152	.252
	Ch25 vs Ad25	-.067	.066	99	-1.017	.312
	Ad0 vs Ad25	-.017	.062	104	-.270	.788
LYM%	Ch10 vs Ch25	-11.181	2.164	56	-5.166	**<.001**
	Ch10 vs Ad0	-10.249	1.965	90	-5.217	**<.001**
	Ch10 vs Ad25	-7.482	2.118	96	-3.532	**.001**
	Ch25 vs Ad0	.931	1.965	90	.474	.637
	Ch25 vs Ad25	3.699	2.118	96	1.746	.084
	Ad0 vs Ad25	2.767	1.823	66	1.518	.134
HET%	Ch10 vs Ch25	8.693	1.487	53	5.848	**<.001**
	Ch10 vs Ad0	7.797	1.374	87	5.675	**<.001**
	Ch10 vs Ad25	5.777	1.481	94	3.901	**<.001**
	Ch25 vs Ad0	-.896	1.374	87	-.652	.516
	Ch25 vs Ad25	-2.916	1.481	94	-1.970	.052
	Ad0 vs Ad25	-2.020	1.481	63	-1.608	.113

Table L. Testing for differences in leukocytes, lymphocytes and heterophils per 10,000 RBCs between age groups Ch10 (N=23), Ch25 (N=23), Ad0 (N=40) and Ad25 (N=28). LME model include Nest identity, individual identity and Individual identity (Nest identity) as random factors.

	Age group	Estimate	SE	df	t	P
LEU10	Ch10 vs Ch25	-6.166	6.797	54	-.907	.368
	Ch10 vs Ad0	20.512	6.181	109	3.319	**.001**
	C10 vs Ad25	27.245	6.646	109	4.100	**<.001**
	Ch25 vs Ad0	26.678	6.181	109	4.316	**<.001**
	Ch25 vs Ad25	33.411	6.646	109	5.027	**<.001**
	Ad0 vs Ad25	6.733	6.646	64	1.181	.242
LYM10	Ch10 vs Ch25	-9.040	4.191	54	-2.157	**.036**
	Ch10 vs Ad0	7.478	3.759	109	1.989	**.049**
	Ch10 vs Ad25	12.879	4.042	109	3.186	**.002**
	Ch25 vs Ad0	16.517	3.759	109	4.394	**<.001**
	Ch25 vs Ad25	21.919	4.042	109	5.423	**<.001**
	Ad0 vs Ad25	5.401	3.509	64	1.539	.129
HET10	Ch10 vs Ch25	2.179	3.520	54	.619	.538
	Ch10 vs Ad0	10.252	3.305	97	3.102	**.003**
	Ch10 vs Ad25	11.405	4.647	88	2.454	**.016**
	Ch25 vs Ad0	8.073	3.427	97	2.356	**.021**
	Ch25 vs Ad25	9.225	4.739	90	1.947	.055
	Ad0 vs Ad25	1.153	4.376	93	.263	.793

Tabell M. Regression analysis predicting leukocyte parameters from various adult groups (predictors) to 10 and 25 day old chicks (dependent).

Predictor		**Ad day 0**		**Ad day 25**		**Ad total**		**Ad females day 0**		**Ad females day 25**		**Ad females total**	
Dependent		Ch10	Ch25	Ch10	Ch25	Ch10	Ch25	Ch10	Ch25	Ch10	Ch25	Ch10	Ch25
H:L ratio	R Square	.039	.018	.064	.023	.000	.008	.027	.050	.163	.069	.003	.001
	Sig. F Change	.406	.578	.365	.591	.967	.706	.492	.342	.193	.411	.826	.925
LYM%	R Square	.004	.003	.159	.084	.003	.059	.001	.062	.221	.099	.031	.009
	Sig. F Change	.779	.830	.141	.294	.826	.301	.682	.288	.123	.320	.461	.686
HET%	R Square	.032	.005	.002	.001	.000	.028	.012	.042	.007	.006	.002	.011
	Sig. F Change	.454	.774	.878	.924	.094	.479	.649	.389	.802	.812	.838	.654
LEU10	R Square	.027	.037	.000	.005	.017	.004	.003	.071	.001	.009	.000	.065
	Sig. F Change	.490	.416	.976	.795	.589	.779	.818	.255	.758	.764	.980	.279
LYM10	R Square	.031	.113	.050	.009	.028	.041	.002	.208	.011	.004	.000	.184
	Sig. F Change	.455	.147	.423	0.74	.477	.395	.837	.043	.746	.533	.928	.059
HET10	R Square	.018	.002	.004	.007	.009	.007	.001	.000	.026	.000	.001	.002
	Sig. F Change	.572	.854	.827	.765	.691	.722	.919	.958	.617	.990	.915	.867