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Effects of repeated investigator disturbance on corticosterone concentrations and the immune system in chicks of Black-Legged Kittiwake (*Rissa tridactyla*)

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Preface

The Masters thesis has been written at the Department of Biology, Norwegian University of Science and Technology, Trondheim. The fieldwork was conducted in Kongsfjorden, Svalbard in July-August 2016. The staining procedures were performed at Sverdrupstasjonen laboratories, Ny-Ålesund. The sexing procedures were performed at NTNU laboratories. The analyses of corticosterone were carried out at the *Centre National de la Recherche Scientifique* (CNRS) in Chizé, France. Arctic Field Grant of the Svalbard Science Forum supported the fieldwork and Kong Haakon den 7des utdannelsesfond for norsk ungdom supported the corticosterone analysis.

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Finally, I would like to thank Ingrid for your love, support and encouragement, and for taking care of our newborn son while I wrote the thesis.

Sammendrag

Repetert eksponering av forstyrrelse fra forsker kan øke kortikosteron nivået og gi skadelige effekter på både vekst og immunforsvar hos fugleunger. Fugleungene må de investere i ulike livshistoriekomponenter under utviklingen. Siden ressursene er begrenset, må investeringen være en trade-off mellom ulike komponenter som immunsystem og vekst. Immunforsvaret er delt inn i det medfødte immunsystemet som er første forsvarslinje, med ikke-spesifikk beskyttelse, og det ervervede immunsystemet som har spesifikk beskyttelse mot patogener. Det ervervede immunsystemet er mer kostbart å utvikle.

Denne studien undersøkte effektene av repetert forstyrrelse av ungene til Krykkje (*Rissa tridactyla*) under ungeperioden på Svalbard. Målet med studien var å undersøke om repetert forstyrrelse førte til økt basalnivå av kortikosteron, økt heterofil til lymfocytt (H/L) ratio eller endring i vekst hos fugleungene i eksperimentgruppen. Det ble tatt blodprøver av eksperimentgruppen og kontrollgruppen på dag 6 og 31 etter klekking. Fugleungene i eksperimentgruppen ble fjernet fra reiret sitt og det ble gjort biometriske målinger på ungene hver andre dag i totalt 25 dager, mens kontrollgruppen bare ble målt på dag 6 og 31. Fugleungene i eksperimentgruppen økte ikke sitt basalnivå av kortikosteron eller sin H/L ratio sammenliknet med kontrollgruppen. Det var ingen signifikante forskjeller i kroppskondisjon eller biometriske målinger mellom de to gruppene. Resultatene av studien indikerer at ungene til Krykkje er motstandsdyktig til forstyrrelse og at tilstedeværelse av forsker ikke målbart påvirker dem.

Det ble funnet en signifikant korrelasjon mellom H/L ratio og kroppskondisjon hos fugleungene, og dette viste at unger med lav kroppskondisjon har høyere H/L ratio. Det ble også bevist forskjeller i H/L med hensyn på kjønn, der hunn-ungene hadde høyere H/L ratio enn hann-ungene. Disse resultatene samstemmer med tidligere studier.

Abstract

Repeated exposure to investigator disturbance increases the corticosterone levels and can give detrimental effects on the growth and immune system in nestlings. As the offspring is developing, they need to invest in different life history components. Since the resources are limited, the investment has to be a trade-off between components like immune system and growth. The immunity is divided into the innate immune system with initial non-specific protection and the acquired immune system with specific pathogen protection. The acquired immune system is considered to be the more costly to develop.

The present study examined the effects of repeated investigator disturbance on the nestlings of the Black-Legged Kittiwakes during the nestling season at Svalbard. The objective was to test if repeated disturbance caused an increase in the baseline corticosterone levels, increased the heterophil to lymphocyte (H/L) ratio or altered the growth of the nestlings of the experimental group. Blood samples were taken of the experimental group and the control group at day 6 and 31 after hatching. The experimental nestlings were removed from their nests and the biometrics were measured every second day for 25 days, while the controls were only measured at day 6 and 31. The nestlings of the experimental group did not increase their baseline corticosterone levels or H/L ratio compared to the control group. There were also no significant differences on the body condition index (BCI) or the biometric measurement between the two groups either. The results of the present study suggest therefore that the nestlings of the Black-Legged Kittiwake are resilient to and do not perceive the investigator disturbance as a stressor.

A significant correlation between the H/L ratio and the body condition index (BCI) of the nestlings was found, proving a relationship between nestlings with low BCI having a high H/L ratio. Differences in H/L ratio between the sexes were also proven, with the females having a higher H/L ratio than the males. These results are consistent with previous studies.

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1 Introduction

1.1 The avian immune system

1.1.1 Function of the avian immune system

The mammalian and avian immune systems are in broad terms similar in both structure and response. Even though the mammalian immune system is better understood, there has been a dramatic progress in the understanding of the avian immune response due to the chicken genome sequence being fully discovered in 2004 (Kaiser and Balic, 2015). The immune system is an internal control mechanism acting as a defense against pathogens and parasites that affect the health, reproduction and survival of an animal (Sheldon and Verhulst, 1996; Hõrak et al., 1998). The avian immune system consists of five different types of leucocytes: lymphocytes, heterophils, basophils, eosinophils and monocytes with heterophils and lymphocytes making up the majority of leukocytes in birds (Newman et al., 1997; Davison et al., 2008). The leucocyte-related immune response can be divided into two separate parts of the immune system, innate and acquired immunity (Juul-Madsen et al., 2008). The two parts are working together with the innate immune system inducing and modulating the acquired immune response toward the pathogens (Juul-Madsen et al., 2008; Murphy, 2012).

The innate immune system forms the first line of defense and works in the general protection of the body against a variety of microbial pathogens (Juul-Madsen et al., 2008; Murphy, 2012). The innate immune system produces inflammatory cytokines that induce local inflammation together with the granulated heterophils (Harmon, 1998; Lee, 2006). The heterophils are phagocytic and are diverse in their antimicrobial activity (Harmon, 1998), while the eosinophils and basophils secrete proteins (histamins), toxins and prostaglandins (Demas et al., 2011). Macrophages are developed from monocytes and are phagocytizing pathogen-infected cells and produce cytokines, providing a non-specific defense against pathogens. They also induce the acquired immune system by activating the lymphocytes (Demas et al., 2011; Murphy, 2012).

The acquired immune system acts as a specific response to pathogens and serves as the additional line of defense (Juul-Madsen et al., 2008). The acquired immune response is slower, as it requires activation of the pathogen-specific response (Demas et al., 2011). The

system is made up of lymphocytes and divided in B and T cells that coordinate the acquired immune responses of the animal (Demas et al., 2011; Murphy, 2012). The B cells produce antibodies, while the T cells eliminate infected host cells by direct contact or function as complementary cells. The T cells also coordinate the production of antibodies by interacting with the B cells (Demas et al., 2011; Murphy, 2012). The acquired immune system is working with antigen-antibody interaction and can memorize and recognize new pathogens (Murphy, 2012). On the second encounter with the antigen, the memory cells will allow the immune system to mount a stronger response, as it is both faster and more effective (Male et al., 2006). The specialization of acquired immunity enables a lifelong immunity after an exposure to a certain pathogen (Delves et al., 2011). In birds, the lymphocytes originates from the stem cells in the bone marrow and are differentiated in the primary lymphoid organs, the thymus and the bursa of Fabricius before entering the circulation and colonizing the lymphoid tissue. The T cells develops in the thymus, while B cells develops in the bursa of Fabricius, and both type of cells learn to ignore self-antigens but respond to foreign antigens (Kaiser and Balic, 2015).

1.1.2 Costs of the immune system

The investment of the immune system is energy expensive and the development has to be a trade-off with other life history traits. Developing broods have to balance between growth and immune system as energetic investments (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000). After hatching, the chicks primarily use the innate immune system if infected by a pathogen (Bar-Shira and Friedman, 2006). There is evidence that the immune system in neonatal chicks is not fully developed and that it continues to improve after fledging (Stambaugh et al., 2011). An intact immune system is considered costly to develop and maintain, as the immunity has to be at its peak to resist infections from pathogens and their products (Sheldon and Verhulst, 1996; Lee, 2006; Murphy, 2012). Nutritional limitations in experimental work have shown trade-offs between immune responses with reproduction and growth rate (Brommer, 2004; French et al., 2007). Long-lived species reportedly need to make a major investment in their immune system. This might be the reason why they prioritize self-maintenance and survival in breeding seasons with low availability of food (Kitaysky et al., 2007; Sandvik et al., 2012). As the long-lived species have a greater chance to encounter a greater number and more diverse pathogens during their lifetime, they have to invest more resources in the acquired immune system than their short-lived relatives

(Ricklefs and Wikelski, 2002). While the total immune system is costly, the different parts of the systems are different in their expense. The constitutive innate defenses are considered less costly, while the innate induced inflammatory responses and development of lymphocytes are energy expensive for the host (Lee, 2006).

1.1.3 Modulation and ontogeny of the immune system

The avian immune system can be modulated by different factors, which include intrinsic factors, such as sex and age, and extrinsic factors such as environmental condition, exposure to toxicants, type of diet and social interactions. Other factors that directly or indirectly affect the immune system are stress hormones, sex hormones, metabolic hormones and other signaling molecules (Koutsos and Klasing, 2008). Change in the immune cell parameters has been shown in response to routine handling, and these changes can even be influenced by the type of handling as seen in the house sparrows (*Carpodacus mexicanus*; Davis, 2005).

Even as an embryo, the chick begins to develop it own immune defense mechanisms, but the immunocompetence first appears a few days after hatching (Mast and Goddeeris, 1999). Maternal immunoglobulins are transferred to the developing avian embryos and transiently protect against bacterial toxins, parasites, viruses and bacteria (Koutsos and Klasing, 2008). The maternal antibodies protect the neonatal chicks and can be present for up to one month after hatching (Hamal et al., 2006). The presence is vital for the chicks as they lack the antibody response due to immaturity of the T lymphocytes (Bar-Shira et al., 2003). The acquired immune response has been demonstrated to be age-dependent in a study on Broiler chicks (Gallus gallus domesticus), with a higher immune response in older chicks than in young ones. This might be due to the immune function not being entirely developed in the embryonic and neonatal spleen (Mast and Goddeeris, 1999). The lymphoid tissue in the gut has also been shown to contain T and B cells that are functionally immature at hatching and their function is attained within the two first weeks in Broiler chicks (Bar-Shira et al., 2003). Most of the studies on the ontogeny of the immune system in birds have been done on domesticated species, due to their economic importance in the industry (Ardia and Schat, 2008)

1.1.4 Leukocyte profiles

The amount of leukocytes, such as heterophils, monocytes and lymphocytes, can provide an important measure of nonspecific host immune function and health status (Harmon, 1998,

Norris and Evans, 2000). A leukocyte profile is the relative proportion of each type of leukocyte in the circulating bloodstream, and can be used to estimate infection status and indices of current stress of an animal (Vleck et al., 2000; Ruiz et al., 2002). Leukocyte profiles or blood cell counts from blood smears have been used to assess general stress and immunocompetence in wild birds (Figuerola et al., 1999; Davis et al., 2004). The main focus of the studies is the heterophil to lymphocyte (H/L) ratio, in which they assess the immune function in the birds (Davis, 2005). H/L ratio can also give an indicator of the overall stress levels in individual birds (Gross and Siegel, 1983; Vleck et al., 2000; Ruiz et al., 2002). The measurement error for the H/L ratio has been shown to be relatively small compared to the total variation and thereby implying the method to be acceptable for ecological research (Ots et al., 1998). A low number of heterophils, and thus a low H/L ratio, indicates that the animal mostly uses its acquired immune system, while a high number of heterophils indicate that the animal is primarily using its innate immune system (Masello et al., 2009). The H/L ratio values has been suggested to be species-dependent, as both relative high and low H/L ratios have been observed in the adults of different bird species (Work, 1996; Newman et al., 1997). A relationship between body condition and H/L ratio has been observed in Burrowing Parrots (*Cyanoliseus patagonus*), indicating a favored investment in immune function in well-nourished nestlings (Masello et al., 2009). With the use of leukocyte profiles to monitor overall immune function, it appears that the H/L ratio increases with stress (Gross and Siegel, 1983; Vleck et al., 2000), injury (Ots et al., 1998;Vleck et al., 2000) and disease (Davis et al., 2004), as well as with diet (Maxwell and Robertson, 1998), starvation (Ruiz et al., 2002), urbanization (Ruiz et al., 2002) and decreasing habitat quality (Mazerolle and Hobson, 2002). In contrast, the H/L ratio will decrease due to parasite infestation as the number of lymphocytes increase and become abundant (Figuerola et al., 1999). The acquired immune system acts effectively against bacteria, viruses and ectoparasites, but it comes with a high metabolic cost due to the rapid cell proliferation (Lochmiller and Deerenberg, 2000; Blount et al., 2003; Lee, 2006). The leukocyte count and H/L ratio varies due to exposure of various stressors (Gross and Siegel, 1983; Maxwell, 1993). A reduction of the circulating leukocytes due to stress may be a result of redistribution of lymphocytes from blood and into other body tissues, as well as recruitment of heterophils from the bone marrow to the blood circulation (Bishop et al., 1968; Dhabhar et al., 1994; Dhabhar et al., 1995; Dhabhar et al., 1996). The effects are mediated by corticosterone (CORT), the foremost glucocorticoid (GC) in birds, in response to the stressors. The elevated levels of GCs cause the lymphocytes to relocate to the bone marrow, spleen, lymph nodes and skin, which significantly reduces the number of

circulating lymphocytes. This process might be an adaptive response, with increase in the immune response in important organs and in preparing the immune system (Dhabhar, 2002). The H/L ratio can be used as a reliable indicator of stress in birds, and other vertebrate taxa, as the increase in H/L ratio reflects the increase of GCs in the blood (Gross and Siegel, 1983; Ots et al., 1998; Vleck et al., 2000; Ruiz et al., 2002).

1.2 Stress hormone

Stress hormones associated with chronic or acute stressors alter an animal´s homeostasis and coordinate an effort to remove the animal for the stressful situation or environment (Koutsos and Klasing, 2008). Glucocorticoids are hormones secreted in an increasing amount by activation of the hypothalamic-pituitary-adrenal (HPA) axis in vertebrates as a response to a stressor (Wingfield et al., 1998). The catecholamines epinephrine and norepinephrine are released from the adrenal medulla to the circulation within seconds as a response to an acute stressor. The hypothalamus releases corticotropin-releasing hormone (CRH) to stimulate adrenocorticotropic hormone (ACTH) release from the pituitary (Wingfield et al., 1997; Hill et al., 2008). ACTH in the bloodstream facilitates the release of GCs, mainly CORT in birds, from the adrenal cortex within three minutes of perceiving the stressor, and the effect of GCs can be detected in the target tissue after one hour (Hill et al., 2008). GC secretion can be advantageous in the short term by promoting behavior that enhances survival, like releasing usable sources of energy to the bloodstream by producing glucose in the liver and releasing fatty acids from lipolysis (Hill et al., 2008). Repeated exposure to a stressor results in repeated activation of the HPA axis, which can result in lifelong changes in body function (Romero, 2004). Chronic elevation of GC levels resulting from repeated or prolonged stressors has deleterious consequences for growth, including impairing the immune function, suppressing the reproductive system, causing severe muscle loss and impairing cognitive development (Johnson et al., 1992; Wingfield et al., 1998; Sapolsky et al., 2000; Kitaysky et al., 2003). CORT are considered to be a reliable parameter for measuring stress in birds, but has some confounding factors that may influence the circulating concentration. Examples of confounding factors are food supply (Kitaysky et al., 2007), circadian rhythms (Quillfeldt et al., 2007) and breeding experience (Lanctot et al., 2003). As human presence has increased in the habitats of birds, the importance of the physiological consequences, such as hatching

success, fledging success and stress, have become more evident (Müllner et al., 2004; Walker et al., 2005).

1.3 Investigator effect

The presence of humans close to wild animals may act as an unpredictable stressor and the animals react as they would to a potential predator (Frid and Dill, 2002). This results in an increase in the circulating concentration of GC in the animals (Romero, 2002). Understanding the effects of the investigator disturbance on the development of different animals is vital for designing proper sampling protocols. Many studies require animals being handled, have tracking devises attached, have morphological measurements and blood samples taken. Understanding the effect of handling and investigator disturbance is especially crucial for endangered species and species known to be sensitive to disturbance.

Ecological and behavior studies have a goal of unbiased observations of wildlife in their natural conditions, but it is difficult to determine the extent of the disturbance without experimental studies to explore the effects of the investigator effect. Birds are a well-studied taxa and until recently many studies did not take the activities of the investigator into account on the birds normal behavior (Nisbeth, 2000). The diverse avian taxa are different in their sensitivity to investigator disturbance and this might relate to their life history strategies, as well as the frequency and timing of the disturbance (Götmark, 1992). Contrasting results have been reported from different studies where repeated disturbance during incubation and chick rearing have shown both reduced breeding success and no effect on breeding. A negative effect on breeding success was shown in Tufted Puffins (*Fratercula cirrhata*; Whidden et al., 2007), Adelie Penguins (*Pygoscelis adeliae*; Giese, 1996) and in Leach´s Storm Petrels (*Oceanodroma leucorhoa*; Blackmer et al., 2004), while no significant effect was shown on Gould´s Petrels´ (*Pterodroma leucoptera*) hatching success when the parents were handled (O'Dwyer et al., 2006) or in Roseate Terns (*Sterna dougallii*; Nisbeth, 2000). Repeated handling on nestlings of European Storm-Petrel (*Hydrobates pelagicus*) did not show increase in GC levels, and there were no differences between numbers of handling (Watson et al., 2016). This goes along with a study by Fiske et al. (2013) on Leach´s Storm Petrels chicks, where repeated disturbance did not influence growth rates.

The effect of the investigator disturbance might vary significantly between years as shown by Sandvik and Barrett (2001), as the environmental factors between the years may influence the results. These results go a long with the hypothesis that long-lived birds desert their nests in relation to varying environmental factors in different years (Erikstad et al., 1998). Long-lived iteroparous birds have several breeding opportunities in their life and therefore takes less risk during their current breeding attempt than shorter-lived animal (Stearns, 1976; Watson et al., 2016). As the cost of reproduction is high, the parents have to adjust their investment in the current breeding and its success, to the cost of survival and future reproduction (Stearns, 1992). The increase in circulating CORT levels may direct behavior towards activities such as feeding, to support self-maintenance at the expense of reproduction (Astheimer et al., 1992). This can explain why long-lived birds may be particularly vulnerable to investigator disturbance and are more likely to reduce the parental effort in unfavorable breeding conditions (Blackmer et al., 2004). The vulnerability can also be one of the explanations of the decline of the many long-lived Procellariiform seabird populations in the world (Warham, 1990). The primary response of breeding failure is egg desertion, with 70% of the Short-tailed Shearwaters (*Puffinus tenuirostris*) adults abandoning their egg due to daily investigator disturbance within the first four weeks, but it did not affect the chick survival of the eggs that hatched (Carey, 2011). In Leach´s Storm-Petrels, egg desertion accounted for 91% of the breeding failures (Blackmer et al., 2004). Investigator disturbance might also lead to an extended incubation period and thus a later development in the chicks, due to the parents being away from the nests (Boersma and Wheelwright, 1979; Pierce and Simons, 1986; Warham, 1990). The disturbance might reduce the nest attendance of the parents and thus reduce the chick growth rates and thereby the chicks post-hatching chances of survival (Magrath, 1991). In short-lived passerine birds it has been showed that frequent absence of the parents can cause the mean temperature of the egg to be reduced, which significantly lowers long-time survival (Berntsen and Bech, 2016), as well as adverse the conditions in the early stages of development (Lindström, 1999). This might as well be the case for long-lived birds breeding in high latitude, as the cold climate may expose the egg to large temperature differences.

A study on Black-Legged Kittiwakes (*Rissa tridactyla*) showed vague, but not significant effect on the growth rate and survival of the chicks, despite frequent disturbance (Sandvik and Barrett, 2001). Brewer et al. (2008) showed that Black-Legged Kittiwake chicks in the Pacific did not elevate their CORT levels in response to investigator disturbance. The life histories of

the different species are important to take into account when performing a study and there could also be differences in life history between different populations. Population differences were shown by a study on the Pacific and Atlantic populations of Black-Legged Kittiwake with different life histories, where the reproductive success was reduced in Pacific kittiwakes when exposed to stress (Schultner et al., 2013). This indicates that the results from Brewer et al. (2008) may not be similar for the Atlantic population, because of the differences between the populations. The Atlantic population might experience reduced growth as an effect of the investigator and there may still be an undetected effect on the immune system as well. As the populations of the kittiwakes are in a decline and have reached status of threatened on the mainland of Norway, it is vital to get information on the species and to learn their life history. This is a good reason to do studies on the still large population at Svalbard.

1.4 Aim of study

The objective of the present study was to determine the effect of repeated observer disturbance on the nestlings of the Black-Legged Kittiwake in colonies at Svalbard. I wanted to study if the repeated disturbance induced any stress effects such as increased plasma CORT levels or change in the H/L ratio.

I predict that

- At the end of the growth period, the experimental and handled nestlings will be more stressed and have increased CORT levels in the blood, compared to the non-handled control group.
- The increased stress in the experimental group will cause an increase in the H/L ratio of the nestlings and the ratio will increase.
- The body condition index for the stressed nestlings will be reduced compared to that of the control nestlings.

2 Material and methods

2.1 Fieldwork and species

The Black-Legged Kittiwake is a long-lived, medium sized seabird with a circumpolar distribution. The birds breed in the arctic and sub-arctic parts of the northern hemisphere, in the North Pacific and North Atlantic Ocean. The Black-Legged Kittiwake is the most numerous gull species in the world with a population of over four million breeding pairs (Strøm, 2006; Coulson, 2011). At Svalbard they breed in the spring and the egg laying usually occurs at the beginning of June. The size of the clutch is normally two eggs, though both one and three eggs can occur. The Kittiwakes are monogamous and both parents incubate the eggs. The incubation period lasts approximately 27 days (Strøm, 2006). In nests with two eggs, the first egg usually hatches one or two days earlier than the other, and the first nestling (alpha) has greater chance of survival. The nestlings are semi-precocial and require a high degree of parental care (Golet et al., 1998). The first 15-16 days they are dependent on their parents because of their reduced thermoregulatory function and the parents have to protect the nestlings from the rain, due to their non-waterproof plumage (Gabrielsen, 1992). Both parents feed the nestlings, with a diet consisting mostly of fish and crustaceans. The nestlings fledge five to six weeks after hatching. They do not return to natal water until their third summer. The nestlings become sexually mature at four or five years of age (Strøm, 2006).

The fieldwork for the present study was conducted during the nestling period the summer of 2016 in Kongsfjorden, Svalbard. The birds studied were gathered from three different sub-colonies on the Island of Blomstrand (figure 1). The colonies hosts in total approximately 200 breeding pairs.

2.2 Experimental setup

The present study was conducted from 5th July to 10th August 2016. Nests with eggs and nestlings were chosen from reachable breeding sites. In the study, 61 nestlings were used at the end of the experimental period and they were distributed into one experimental group (N = 31) and one control group (N = 30). The groups were chosen randomly in the colony. The nestlings were clustered within the two separate groups to prevent biasing the control group

with investigator disturbance while handling the experimental group. The nestlings were collected by hand with the use of a ladder.

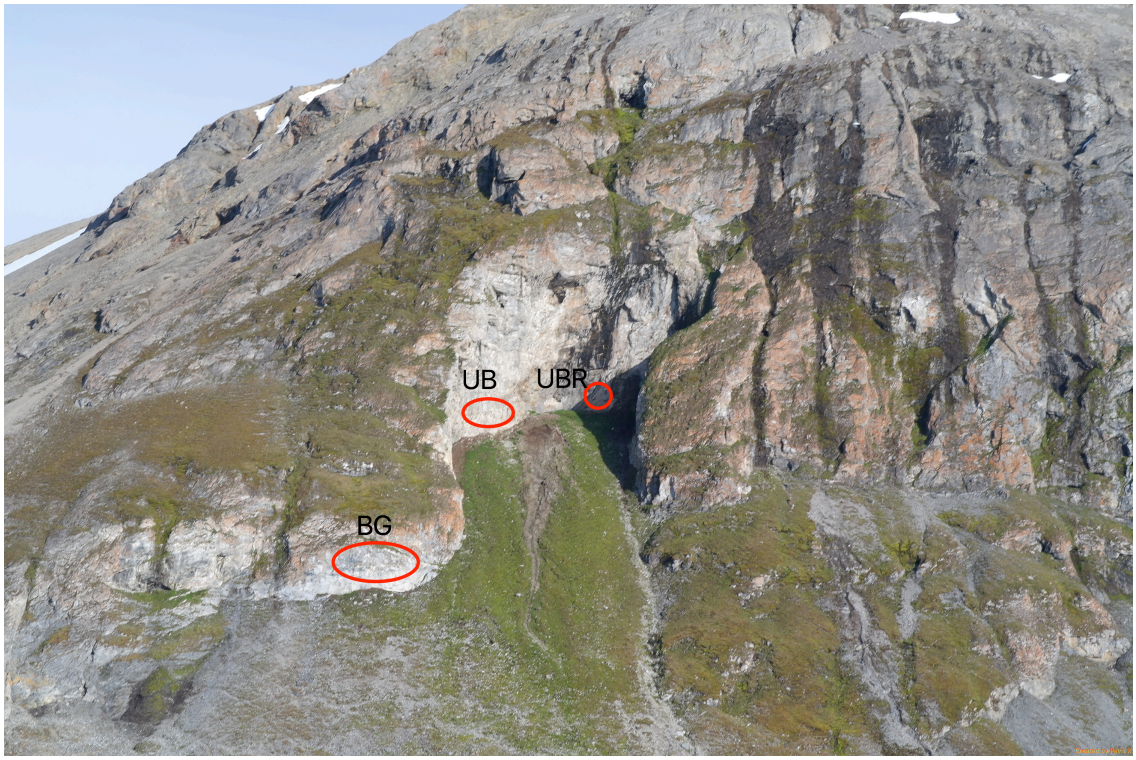

Figure 1: The three sub-colonies BG, UB and UBR at the Blomstrand Island used in the present study (Photo; Øyvind Gjønnes Tvedten).

Blood samples were taken from all used nestlings, as well as biometric measurements such as body mass, and tarsus and skull length. The body mass (g) was measured using a cloth bag and a spring weight (100g ± 1g, 200g ±2g and 500g ± 5g). The wing length (± 1mm) was measured by stretching out the right wing on a ruler. The skull and tarsus length (± 0.1mm) were measured by the use of a sliding caliper. The biometric measurements of the nestlings during the experimental period were used to create a growth rate for the nestlings (not presented). The growth rate was used to estimate the hatch date for the nestlings who had unknown hatching date.

All the nestlings were banded with color bands to distinguish between the siblings, being divided into alpha and beta chick with respect to hatching time. The bands were in addition used to distinguish between chicks from neighboring nests. The contents of all the nests in both the experimental and control group were checked every day at the beginning of the fieldwork. This procedure was done to find suitable nestlings for the study and to ascertain

knowledge about their age. After getting knowledge of the nest contents, nest checks were conducted regularly. This provided an overview of the nest content, the ability to monitor if the eggs hatched, and to check if number of nestlings remained the same or if the nests had been predated. Nest-checks were performed using a long pole with a mirror at the end, to reduce the amount of stress for the chicks.

The fieldwork was carried out together with Msc. student Øystein Hansgård Gjelsvik who used the same data on biometrics, BCI and CORT as the present study. In addition he also studied the telomere change of the nestlings.

2.3 Blood sampling and blood smears

The first blood samples were taken at day 6 (± 2.2 days) and the second blood samples were taken at day 31(± 2.2 days). The blood was sampled using a heparinized (5000 EI/ml) syringe at the brachial vein of the chicks. The blood was sampled within three minutes of capture (an average of 2 min and 10 seconds ± 27 seconds), as the CORT levels rise after three minutes (Romero and Reed, 2005). The total amount of blood taken was approximately 0.5 mL, and was split with a drop for the molecular sexing and a drop for each blood smear. The blood drops for molecular sexing analysis were stored in ethanol (96%). The rest of the blood was stored in the syringe and placed on ice until transferred to small centrifuge tubes at the laboratory. The tubes were centrifuged for four minutes at 9500 rpm to separate the plasma from the blood cells. The plasma samples were stored at -20 °C until they were shipped to Centre National de la Recherche Scientifique (CNRS) in Chizé France, for analyses.

The blood smears were made in the field accordingly to Clark (2009), by using a glass slide and smearing a drop of blood to create a blood smear with one cell layer. The smear was air-dried and rinsed in methanol (99.9%) for one to two minutes and stored in a box. The blood smears was set for drying at the laboratory and they were stained with Giesma-stain (Sigma Aldrich) for 20 minutes within three weeks after being sampled in the field (Houwen, 2000). Two blood smears were made from each chick handled.

2.4 Investigator disturbance

To induce the investigator disturbance, the nestlings in the experimental group were taken down from their nests at 6 (± 2) days of age, with the procedure of weighing them in a cloth bag and obtaining the biometric measurements. The procedure included a total of 17 minutes for each nestling, with approximately five minutes as the handling time when the biometrics were measured. The rest of the time included removing them from their nests and keeping them in a cloth bag on the ground. This procedure was repeated every second day for 25 days and consequently resulted in a total of 12 repeated handlings with the same procedure for every chick. The control group was only measured twice, at day 6 and 31 days, with both blood samples and biometrics.

2.5 Leukocyte counts

A Leica microsystems light microscope (1000x magnification) was used to scan the stained blood smears and to perform the leukocyte counts. The blood smears from each group, control group at day 6 and 31, and the experimental group at day 6 and 31, were randomly sorted into one group. The blood smears were randomly picked from this assortment group to be scanned. This procedure was done every day to ensure the identity of the sample was not known until the analyses were completed. The leukocytes were counted in the monolayer of the blood smears according to the criteria defined by Clark (2009). Approximately 100 leucocytes were counted in total per slide and they were distinguished as either heterophils or lymphocytes, and the resulting H/L-ratio was calculated.

2.6 Plasma corticosterone concentration

The plasma samples were sent to the CNRS in Chizé for CORT analyses. The analysis was performed accordingly to Lormée et al. (2003), and consisted of radioimmunoassay to measure the total amount (bound and free) CORT in the plasma. By adding three mL of diethyl-ether to 100 μL of each sample, the steroid was extracted before vortexing and centrifuging. The tube with the diethyl-ether phase containing the steroid was snap frozen and then decanted and poured off in an alcohol bath at 38°C. The solution was then evaporated and the dried extracts were re-dissolved in 300 μL of phosphate buffer and CORT was assayed in duplicate. A 100 μL of extract together with 5000 cpm of the appropriate ^{3}H-steroid (Perkin Elmer, Waltham, MA, US) and polyclonal rabbit corticosterone-21-

thyroglobulin antiserum (Sigma-Aldrich, St. Louis, MO, US) were incubated overnight. By adding dextran-coated charcoal, the bound fraction of CORT was separated from the free faction and the activity was counted on a tri-carb 2810 TR scintillation counter (Perkin Elmer, Waltham, MA, US). The CORT in the plasma samples were measured in ng/mL.

2.7 Molecular sexing

A small drop of blood for molecular sexing was obtained from each individual nestling in the field and then stored in 96% ethanol. The sexing procedure was performed at Department of Biology at NTNU, Norway. The avian erythrocyte contains cell nucleus and DNA, and can be used for sex determination. The molecular sexing technique is based on the method from Griffiths et al. (1998), with the female having the heterogametic sex chromosomes (ZW) and the male having the homogametic (ZZ). The heterogametic (ZW) female is carrying the CHD-1-W (chromo-helicase-DNA-binding) gene and the smaller CHD-1-Z gene on the sex chromosome, while the homogametic (ZZ) male is only carrying the small CHD-1-Z gene. The genes were exponentially amplified with polymerase chain reaction (PCR), by using specific primers (P2 and P8 as used by Griffiths et al. (1998)) that bind to specific sites the W and Z genes. By adding the primers to the four nucleotides of DNA of a sample, the desired amount of sequences were reproduced during 35 heat-cycles. Gel electrophoresis was then used to identify the different lengths of the sequences. The DNA-sequences were loaded in wells of agarose gel matrix. As the sequences are negatively charged, they were pulled towards the positive end of the gel when adding electricity. The length of the sequences determines the travel distance in the gel and the smaller Z gene moves more willingly than the larger W gene. This gives the heterogametic (ZW) female two distinct bands, while one band will be displayed for the homogametic (ZZ) male. The bands appear when the agarose gel matrix is exposed to UV light.

2.8 Statistical analysis

The statistical analysis was conducted using SPSS version 24.0 (SPPS Inc. 2016) and the plots were made with SigmaPlot 13 (Systat System, Inc. 2015). All variables and residuals were checked for normality (Shapiro-Wilk test, $P \leq 0.05$). All tests performed were two-tailed and $P \leq 0.05$ was set as the significance level with and tendencies assumed at $P \leq 0.10$. The means and parameter estimates are given with standard error (± SE).

A body condition index (BCI) was calculated for every individual in the present study at the age of 31 days. BCI was calculated by performing a principal component analysis (PCA) using skull length, wing length and age, as they had the best fit for the analysis. There was significant difference between the two sexes, showed by Bartlett´s test of sphericity (Kaiser-Mayer-Olkin (KMO) > 0.5) and independent t-test ($P < 0.001$). The standardized residuals were thus conducted by PCA with respect to the sex of the nestlings. The linear regression on PCA and body mass gave the values for BCI as residuals. The BCI was measured by a one-way ANOVA with the standardized BCI residuals with respect to group.

Independent t-test was used to check for significant difference of the basic biometric measurements between the start and the end of the experiment. The different parameters were age (days), wing (mm), tarsus (mm), skull (mm) and body mass (g). Independent t-test was also used to test the differences between the control group and the experimental group, with respect to the parameters above. BCI, H/L ratio and CORT were used in the t-test, as they were the most relevant parameters. The H/L ratio was also tested against sex to test if there was a significant difference between the sexes.

The CORT samples were tested for time of the sampling, to test if the CORT levels increased within the three-minute deadline before baseline levels increased (Romero and Reed, 2005). There was no significant effect of time on the levels of CORT in the samples ($P = 0.729$), which is in consensus with the set standard for CORT sampling (Romero and Reed, 2005). The CORT was dependent on body mass for the last sampling ($P = 0.039$), and thus all CORT data in the models were corrected for body mass and the residuals were used in the statistics.

General linear models (GLM) with an analysis of covariance (ANCOVA) were used to examine the variation in the BCI and H/L ratio for the nestlings. The first ANCOVA was conducted with BCI as the dependent variable with group, broodsize, chick type (alpha/beta/single) and sex as categorical variables, and CORT and H/L ratio included as covariates. In the second ANCOVA, the H/L ratio was set as a dependent variable, and CORT and BCI as covariates, with the same categorical variables. The models were conducted with the main effects of all factors and covariates, as well as 2-way interactions between them. The insignificant factors and covariates, as well as the interactions between them were removed until only the significant were left. The removal was done step by step with removing factor,

covariate or interaction with the highest P-value. The procedure resulted in a simplified model.

2.9 Permissions

The Governor of Svalbard provided the permission to conduct the fieldwork in Kongsfjorden (16/01043-2). The permission for blood samplings on the Black-Legged Kittiwake nestlings were provided by the Norwegian Food Safety Authority (2016/116563).

3 Results

The summer of 2016 proved to be a good year for the kittiwakes in Kongsfjorden. The mean clutch size in the colony was 1.90 (based on 160 eggs or chicks in 84 active nests observed the first day of the fieldwork). The breeding success, which is defined by number of hatched chicks per active nest at day 15, was 1.25 chicks per unit (105 chicks and 84 nests). These two ratios gave evidence for 2016 being a good year for the kittiwakes compared to other known values from Kongsfjorden in earlier years (Vihtakari et al., 2017). The high mean clutch size usually signals a high breeding success (Vihtakari et al., 2017). The high clutch size also indicates high fitness in the parents of the colony and that the winter and spring was good for breeding. A total of 84 nestlings were used in the present study during the first sampling, with 42 in each group. During the study period a total of 18 nestlings were lost due to predation or collapsing of nests. Two nestlings were removed from the study as they were injured and would not have representative CORT values, as injury has been shown to increase CORT levels (Ots et al., 1998; Vleck et al., 2000). Three nestling were removed due to measurement error and could not be used in the study.

3.1 Basic biometric measurements

Biometric measurements were done at first sampling day and continued for every second day for 25 days for the experiment group. The control group was only sampled at the beginning and at the end of the study. The biometric measurements such as body mass and wing, tarsus, and skull length showed no significant differences between the experimental group and the control group during the first sampling of the nestlings (table 1). The number of measurements is reduced on skull length and tarsus length due to measurement errors. No significant differences in the biometric measurements was shown between the groups for the last sampling either (table 1).

Table 1: Mean (± SE) basic biometric measurements and P-values from independent t-tests of the nestlings. N is the number of nestlings on the first and the last sampling of the control group (C) and the experiment group (E).

	First sampling				Last sampling			
Parameter	**n(C, E)**	**Control**	**Experiment**	***P***	**n(C, E)**	**Control**	**Experiment**	***P***
Age (days)	30, 31	6.0 ± 0.4	6.4 ± 0.4	0.538	30,31	31.1 ± 0.4	31.4 ± 0.4	0.675
Wing (mm)	30, 31	41.5 ± 2.0	43.7 ± 2.2	0.466	30,30	230.2 ± 2.4	234.2 ± 2.0	0.205
Tarsus (mm)	29, 31	26.0 ± 0.5	26.5 ± 0.4	0.449	30,31	34.6 ± 0.2	34.8 ± 0.2	0.253
Skull (mm)	29, 25	52.8 ± 0.6	53.0 ± 0.6	0.796	30,31	81.3 ± 0.5	82.2 ± 0.5	0.222
Body mass (g)	30, 31	109.3 ± 5.3	119.3 ± 5.7	0.203	30,31	374.2 ± 6.6	371.5 ± 7.1	0.786

3.2 Body condition index and corticosterone

There was no significant difference between the control group and the experimental group in the mean BCI for the last sampling (P = 0.110) (table 2), when tested against each other in an independent t-test. One of the BCI values for the experiment group was removed due to measurement error. The CORT analyses for the experimental group and the control group showed the CORT residuals did not differ compared to each other (P = 0.957) (table 2). The CORT residuals were corrected for body mass.

Table 2: Mean (± SE) BCI and CORT residuals of the nestlings (n) between the experimental group (E) and the control group (C) with P-values from independent t-tests.

	Last sampling			
Parameter	**n(C, E)**	**Control**	**Experiment**	***P***
BCI	30, 30	0.1938 ± 0.1749	- 0.1938 ± 0.1631	0.110
CORT	30, 31	0.0070 ± 0.1942	-0.0068 ± 0.1678	0.957

3.3 H/L ratio

No significant difference was found between the H/L ratios of the two groups during the first blood sampling of the nestlings (P = 0.973) (table 3). The same occurred for the last blood sampling, with no significant differences (P = 0.394). Both H/L ratios are reduced between day 6 and 31, with the control group being the most reduced (figure 3). T-test of the combined H/L ratio at day 6 and 31 shows a significant decrease between the start and the end of the study period (P = 0.001). The number of blood smears analyzed was reduced because of the bad quality of some of the blood smears.

Table 3: Mean (± SE) H/L ratio at day 6 and 31 between the nestlings (n) of the experimental group (E) and the control group (C) with p-values from independent t-tests.

		First sampling	Last sampling	
Parameter	**n(C, E)**	**Control**	**Experiment**	***P***
H/L ratio day 6	17,19	0.51 ± 0.07	0.53 ± 0.04	0.79
H/L ratio day 31	16,15	0.34 ± 0.02	0.40 ± 0.06	0.39

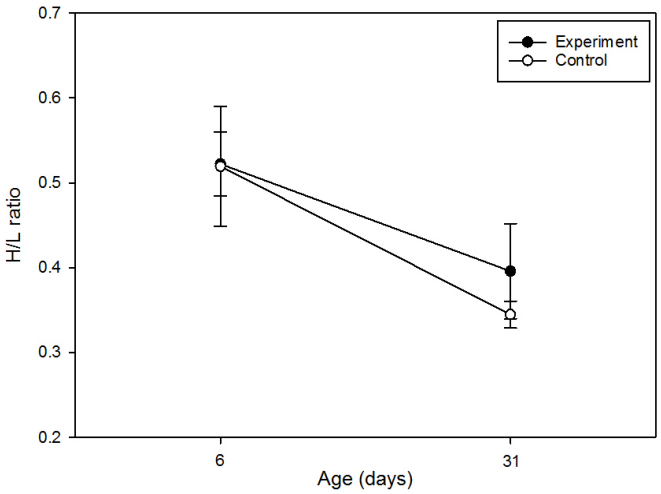

Figure 2: The H/L ratios for the experimental group and control group measured at day 6 and at day 31.

3.4 Determination of body condition index

The ANCOVA with BCI as dependent variable showed no significant variables. The model was simplified until the interaction group*CORT remained as the last explanatory variable. The model is not included as the interaction was insignificant with P = 0.565. The initial model is described in table A in the Appendix, with all the P-values, as they appeared when the variables were removed.

3.5 Determination of H/L ratio

When the H/L ratio was set as dependent variable in the ANCOVA, two variables appeared significant when the model was simplified. The explanatory variables sex and BCI were significant, explaining 22.3% of the variation in H/L ratio, and with P = 0.024 and P = 0.046 respectively (table 4). The model with the initial explanatory variables as they were when removed from the model, are included in the Appendix, table B.

Table 4: Summary of the final ANCOVA model explaining the variation in H/L ratio the dependent variable, with P- values for each explanatory variable and for the model in total. The variables explained 22.3% of the variation in H/L ratio. Details of all excluded explanatory variables included in the initial model are presented in table B in the Appendix.

Dependent	Explanatory	Df	F	P	r^2
Corrected model		**2**	**3.44**	**0.029**	**0.223**
H/L ratio	Sex	1	5.669	0.024	
	BCI	1	4.379	0.046	

The model describes that it is a significant relationship between the H/L ratio and the BCI for the nestlings (figure 3). An increasing BCI correlates with a decrease in the H/L ratio measured at day 31 and shows nestlings with low BCI have higher H/L ratio.

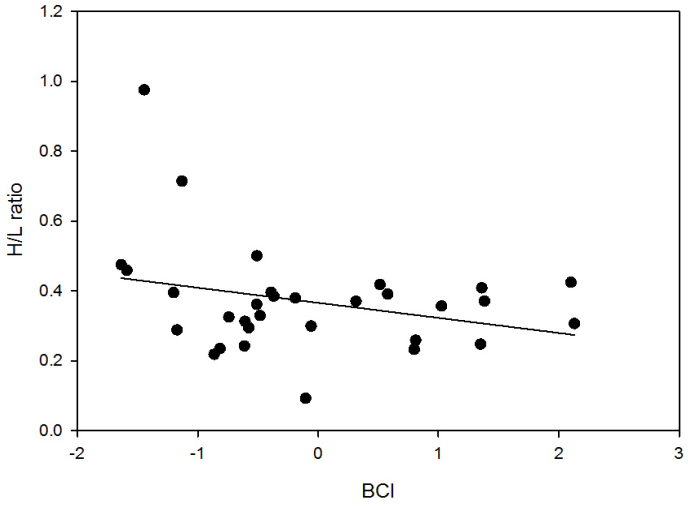

Figure 3: The relationship between H/L ratio and BCI for the nestlings at age 31.

The model also describes there is a significant relationship between H/L ratio and the sex of the nestlings. The model shows the H/L ratio measured at day 6 and day 31, with respect to sex (figure 4). A decrease in the H/L ratio from the start to the end of the study is shown in both sexes. The H/L ratio for the female nestlings is significantly higher than in the males during the study and describes a sex difference in the H/L ratio of the kittiwake nestlings. Results from testing the H/L ratio and the sex of the nestlings at day 31 shows there is a tendency of H/L ratio difference between the sexes (P = 0.081).

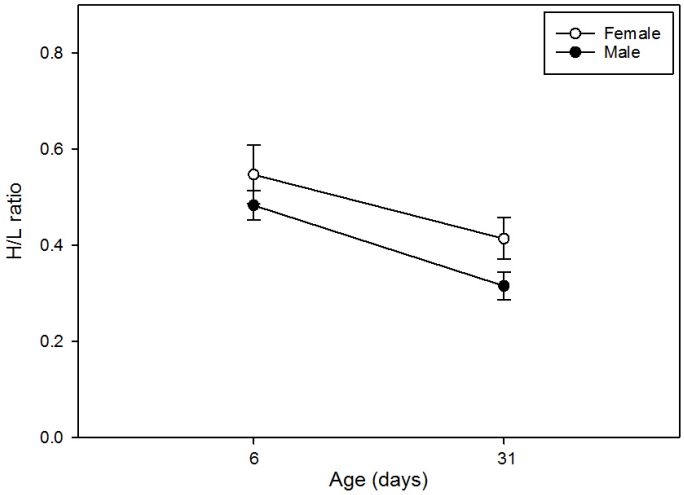

Figure 4: The relationship between H/L ratio and sex of the nestlings at 6 and 31 days of age.

4 Discussion

The aim of the present study was to investigate the effect of investigator disturbance on kittiwake nestlings and to measure the perceived stress by analyzing CORT levels and calculate H/L ratios between an experimental group and a control group. No significant increase was found in the plasma CORT levels of the experimental group. This was the case for the H/L ratio as well, with no difference between the two groups after the investigator disturbance of the experimental nestlings.

4.1 CORT

The present study presumed that the nestlings of the experimental group would be stressed by the investigator disturbance when removed from the nests and being subjects to biometric measurements every second day during the study period. No significant difference was found in the plasma CORT levels between the experimental group and the control group, which is somewhat surprising. Even as the nestlings in the experimental group were taken out of their nests and stressed every second day, they showed no increase in their baseline CORT levels at the end of the study. The handling procedure has presumably stressed them at the time of the handling as seen in previous studies on other birds (Blackmer et al., 2004; Whidden et al., 2007), but clearly had no effect on the baseline CORT levels between the experimental and control group in the present study. All the blood samples were taken within three minutes post-capture to ensure baseline or near baseline CORT levels (Romero and Reed, 2005). The increasing CORT levels after three minutes is well known, but it is also suspected to increase slowly even after the first two minutes (Romero and Reed, 2005; Quillfeldt et al., 2012). A weak but significant correlation between CORT and time of the blood sample was shown in a previous study in kittiwakes (Heggøy, 2013), but no correlation up to three minutes was found in the present study.

There may be several reasons why the experimental group did not differ from the control group in baseline CORT values. It has been shown that different species reacts to investigator disturbance differently. Some species are being stressed by the disturbance such as Leach´s Storm-Petrels (Blackmer et al., 2004) and Tufted Puffins (Whidden et al., 2007), while others are not significantly affected, as Gould´s Petrels (O'Dwyer et al., 2006), Short-tailed Shearwaters (Vertigan et al., 2012) and European Storm Petrel (Watson et al., 2016). As for

kittiwake nestlings, a previous study showed no significant effect on the baseline CORT levels as a response to investigator disturbance (Brewer et al., 2008). These results are consistent with the results of the present study and suggest there are differences in the species reaction to investigator disturbance.

To test if the nestlings perceived the investigator disturbance as stress, the present study could have included a CORT sample taken after the three first minutes. The blood could have been sampled for multiple nestlings after 10-15 minutes of capture to test if the nestlings actually were stressed. From the literature, the CORT levels are known to increase after the first minutes (Wingfield et al., 1998; Romero and Reed, 2005), but this is not directly measured in the present study. Previous studies has shown different bird species having increased CORT levels when sampled after the three minutes (Dawson and Howe, 1983; Romero and Reed, 2005), and there are reasons to belive the nestlings were stressed while handled. The nestlings also showed a clearly stressed behavior with vomiting, aggressive pecking and vocalization when removed from the nests, which is interpreted as a sign of stress.

The nestlings might also have been habituated to the investigator presence in the colony. The amount of stress induced may have decreased until the dates of the last sample when CORT was measured, as the nestlings were habituated during the period. The habituation is a well-known phenomenon and has been shown in Grey-Faced Petrel chicks (*Pterodroma macroptera gouldi*; Adams et al., 2005). With the kittiwake nests in an open colony, the presence of an investigator may cause widespread disturbance in the colony, and thus enhance the habituation of the nestlings. The stress impact on the nestlings in the nearby nests can reflect the behavior of the parents. As the stress reaction is individual for the parents in different nests, the nestlings may receive different amount of stress. This can happen even if the investigator is in a different part of the colony. The nestlings may also have been habituated with a reduced stress response, even if it did not show in the behavior of the nestlings. This phenomenon is called physiological desensitization, where the physiological response to the stimulus decreases but the nestlings do not learn to adapt to the stimulus (Cyr and Romero, 2009). The effect indicates decreased responsiveness of the HPA axis to ACTH from the pituary (Rich and Romero, 2005). The reduced ability to mount a stress response is presumably a reduction of fitness.

The birds may not have perceived handling procedure as a stressor and therefor experienced small amounts of stress during the handling. As there is a trade-off between costs and benefits of eliciting the stress response, the nestlings might have a regulation of the HPA axis to prevent potential harmful costs of increased GC levels (Watson et al., 2016). If the elicitation of stress has low benefits and high costs, down regulation of the HPA axis would be favored in early life, as the exposure to high CORT levels in the vulnerable period is reduced. Some bird species has shown to be hypo-responsive to stress during early development (Wada et al., 2007; Quillfeldt et al., 2009). The hypo-response is of great value for the nestlings, as postnatal stress has significant long-term effects on the physiological stress response in some birds (Spencer et al., 2009). There is also evidence that the CORT values increase with low abundance of food, resulting in low body fat stores in Eurasian Kestrel (*Falco tinnunculus*) nestlings (Müller et al., 2011). Food limitation was not the case for the year of the present study. As the breeding season of 2016 apparently had an abundance of food, the amount of food provided by the parents might have had some influence on the CORT levels of the nestlings.

4.2 Biometric measurements and body condition index

The present study predicted the nestlings of the experimental group to be stressed and show an increase their baseline CORT level, which also would affect the BCI and the biometrics negatively. The results showed this was not the case at the end of the present study. No differences in biometric measurements were shown between the groups at day 6, as expected. The same applied for 31, with no significant differences in the basic biometric measurements as age, wing, tarsus, skull and body mass between the experimental group and control group.

The BCI calculated from the skull length, wing length and age for every individual nestling, did not significantly differ between the two groups in the present study. The results of the BCI were not as predicted before conducting the present study, as there was no significant difference between the experimental group and the control group. The CORT measurement proved the same results and did not differ between the two groups. The experimental nestlings were apparently stressed as judged by the behavior and most of them vomited food when removed from the nests. As this happened every second they, the lost precious food was suspected to give the experimental nestlings a significantly lower body condition than the control nestlings. This was not the case in the present study. When stressed, the birds tend to

increase their begging for food, which results in an increased amount of food from the parents (Kitaysky et al., 2003). The increased food might mask the effects of the stress on BCI because the lost food from vomiting is replaced. The high clutch size and breeding success proved 2016 to be a good year for the breeding colony. As a good year comes with an abundance of food, the extra amount of food would be easier for the parents to give to the nestlings and thus strengthening the masking effect. The extra food would also positively affect the biometric measurement of the nestlings. The lack of effect on the BCI between the groups is consistent with previous studies on kittiwakes (Sandvik and Barrett, 2001, Brewer et al., 2008) and in other bird species as well (Fiske et al., 2013; Watson et al., 2016)

None significant explanatory variables where shown in the model with BCI as a dependent variable. The variation in the BCI could thereby not be explained by the variables. These results show there was no effect on the BCI of the nestlings by the variables sex, group, H/L ratio, CORT or if it was an alpha, beta or single nestling.

In bad years with little food available, long-lived seabirds are known to reduce parental effort and even abandon the nests as they prioritize self-maintenance (Erikstad et al., 1998; Blackmer et al., 2004). There are also big differences between individual pair of parents with high quality parents providing enough food to the nestlings and poor quality parents not giving enough food (Coulson and Porter, 1985; Moe et al., 2002). Both these factors may have a big effect on the BCI and biometric measurements of the chicks, but with an abundance of food, the effects can be masked. An abundance of food will also make it hard to observe a difference in high and bad quality parents. The BCI can also be heritable, with high BCI parents having high BCI chicks (Brinkhof et al., 1999). This may have an effect on the BCI on individual nestlings across the two groups and can affect the results of the study.

4.3 H/L ratio

4.3.1 CORT and H/L ratio

Hormones play a major role in the body and in modulating the immune system (Koutsos and Klasing, 2008). CORT is assumed to directly change the H/L ratio over time (Davis et al., 2008) and some suspect the H/L ratio to be a more reliable indicator of stress than the CORT levels in the plasma (Gross and Siegel, 1983). Even if the H/L ratio can be less variable and a more reliable indicator of stress, this only applies to mild or moderate stress (Maxwell, 1993).

The present study did not find any correlation between the CORT and the H/L ratio in the nestlings, which is somewhat surprising. The H/L ratio and CORT has been suggested to indicate different type of stress. Only three studies have studied the H/L ratio together with endogenous CORT levels and they only found weak or none correlations in Adelie Penguins, Song sparrows (*Melospiza melodia*) and Pied flycatchers (*Ficedula hypoleuca*) (Vleck et al., 2000; Clinchy et al., 2004; Ilmonen et al., 2003). Müller et al. (2011) showed that only exogenous administered levels of CORT increased the H/L ratio, while endogenous did not. The baseline of CORT increased due to human presence at the nests or when the body fat stores were low, while the H/L ratio increased due to low body fat store (Müller et al., 2011). The study of Müller et al. (2011) shows that different kind of stressors might influence differently on CORT and H/L ratio in some species, but the present study showed no effect of the handling on either the CORT levels or the H/L ratio between the groups.

4.2.2 Developmental changes in H/L ratio

As expected the H/L ratio at day 6 did not differ between the two groups of nestlings. The same result was found for the chicks at day 31, with no differences between the experimental and control group. Both groups have reduced their H/L ratio from day 6 to day 31, with the control group reducing the H/L ratio slightly more than the experimental group. The reduction of H/L ratio can result of a decreasing number of heterophils and/or an increasing amount of lymphocytes (Skomsø, 2013). The results might also suggest the chicks are replacing components of the innate immune system to components of the acquired immune system, and an alteration in the energy investment from innate immunity to acquired immunity (Skomsø, 2013). The changes in H/L ratio may be because of the maternal antibodies are being broken down around this age in birds (Hamal et al., 2006). The results of decreasing H/L ratio with age, agrees with previous studies in the colony (Skomsø, 2013). The decrease in H/L ratio is reduced in the experimental group, as the induced stress to a certain degree might be able to counteract the reduction of the H/L ratio at day 31.

4.2.3 H/L ratio models

There is a negative relationship between the H/L ratio and the BCI for the nestlings. The BCI significantly explains some of the variation in the H/L ratio. There is a correlation between the two variables, showing nestlings with low BCI having a higher H/L ratio. The results might indicate that some of the nestlings with low BCI actually were stressed and the stress is

seen in the H/L ratio and not the CORT levels, as the H/L ratio can be a more reliable indicator of stress (Gross and Siegel, 1983). The model shows a difference between the nestlings in the colony without correlating to the experimental group or the control group, which is somewhat surprising as the present study predicted the experimental nestlings to have reduced BCI and increased H/L ratio. The results show that even though the BCI between the groups not were significant, there are individual differences between nestlings in the colony. The effect might be because of different quality in the parents with not every nestlings getting sufficient amount of energy to make a strong investment in the immune system, as shown in previous studies (Coulson and Porter; 1985, Sheldon and Verhulst, 1996; Moe et al., 2002). The nestlings have to balance between investment in growth and immune system (Sheldon and Verhulst, 1996; Lochmiller and Deerenberg, 2000), but as the nestlings have low BCI they cant afford to invest in their immune system. The nestlings with high BCI might be able to afford a stronger investment in the innate immune system and in growth as shown in a previous study (Masello et al., 2009). The inflammatory responses of the innate immune system are considered costly and repeated initiation might lead to reduced growth and increased metabolism (Lochmiller and Deerenberg; 2000, Lee, 2006; Demas et al., 2011), which could be the case for the nestlings with low BCI. Maternal antibodies lingering during the development of the nestlings may counteract the inflammatory responses and thereby reduce the growth (Grindstaff, 2008), and thus could the condition of the mother and the maternal antibodies affect the results of the nestlings. The present study with high BCI giving lower H/L ratio is further consistent with the results of (Skomsø, 2013). The breeding season of 2016 proved to be a good year, and the present study may have detected stronger correlations in the H/L ratio and BCI in a year with less food.

Sex of the nestlings also explains some of the variation in the H/L ratio for the present study. The H/L ratio is significantly different in the females and males during the study period, with females having a higher H/L ratio. The results may indicate that the females of the study are more stressed than the males, as the H/L ratio can give an indication of stress (Gross and Siegel, 1983). The H/L ratios between the sexes show a tendency to be different at day 31, but the results are not significant. The results of the present study are consistent with a study of Ots et al. (1998) on Great tits (*Parus major*), where the females had higher H/L ratio than males. The differences can also possibly be a result of different endocrinology in the sexes (Ots et al., 1998), as there was no difference between the sexes in both BCI and CORT levels at day 31.

5 Conclusion

The present study suggests that the Black-Legged Kittiwake nestlings are resilient to the effects of investigator disturbance. The results show that the nestlings did not elevate their CORT levels or H/L ratio as they were removed from their nests and exposed to regular biometric measurements. The investigator disturbance did not affect the BCI or the biometrics of the nestlings either and implies that the nestlings are able to avoid short- and long-term deleterious effect of stress shown in other species, which is vital for a long-lived bird as the kittiwake. Even if the chicks showed no sign of being disturbed, the investigator should continue to be aware of possible effects of the investigator disturbance. Special sampling protocols and different types of disturbance might produce different results. Environmental factors may also affect the results in a different way, with the summer of 2016 proved to be a good year with abundance of food for the nestlings. The variation between different populations of kittiwakes may also influence the results, as the present study only was conducted in a certain colony at Svalbard.

The present study found in addition a significant correlation between the H/L ratio and the BCI, which showed the nestlings with low BCI also had a higher H/L ratio. The study discovered a relationship between H/L ratio and the sex of the nestlings, with the females having a higher H/L ratio during the study period. These results are consistent with previous studies.

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7 Appendix

The ANCOVA model was conducted with BCI as the dependent variable and with group, H/L ratio, sex, CORT and if the chick was alpha, beta or single as explanatory variables (table A). The two-way interaction between all the variables was also taken into the model. The second ANCOVA was conducted with H/L ratio as dependent variable and with the same explanatory variables and interactions, including BCI (table B). Table A and B are described with all the variables and interactions included, and the original P-values before the variables were removed by simplifying the model. The simplified models are presented in the results of the present study.

Table A: Results from the ANCOVA explaining the change in BCI of the Black-Legged Kittiwake nestlings after the experimental period. The explanatory variables are group, sex, CORT and H/L ratio, as well as the interaction between these variables. The values of the rejected variables are presented as they were before being excluded.

Dependent variable: BCI			
Explanatory variables	df	F	Sig.
Alphabetasingle	2	0.658	0.530
Sex	1	0.264	0.614
Group	1	0.017	0.898
HL_ratio	1	0.118	0.739
CORT	1	0.058	0.813
Alphabetasingle * CORT	2	1.118	0.346
Alphabetasingle * Group	5	2.152	0.097
Alphabetasingle * HL_ratio	2	0.324	0.731
Alphabetasingle * Sex	2	0.805	0.467
Group * CORT	2	0.582	0.565
HL_ratio * CORT	1	0.149	0.706
Sex * CORT	1	0.206	0.654
Group * HL_ratio	1	0.011	0.916
Sex * Group	2	0.953	0.404
Sex * HL_ratio	2	0.728	0.498

Table B: Results from the ANCOVA explaining the change in H/L ratio of the Black-Legged Kittiwake nestlings after the experimental period. The explanatory variables are group, sex, CORT and BCI, as well as the interaction between these variables. The values of the rejected variables are presented as they were before being excluded.

Dependent Variable: H/L ratio			
Explanatory variables	df	F	P
Sex	1	5.669	0.024
BCI	1	4.379	0.046
Rejected variables			
Alphabetasingle	2	2.608	0.093
Alphabetasingle * Group	3	1.479	0.246
Group * CORT	2	0.549	0.586
Sex * CORT	1	1.513	0.233
Alphabetasingle * Sex	2	1.298	0.297
Group * BCI	1	0.775	0.391
Alphabetasingle * BCI	2	0.559	0.583
Sex * BCI	1	0.356	0.560
Sex * Group	1	0.411	0.533
Alphabetasingle * CORT	2	0.225	0.802
CORT	1	0.055	0.818
Group	1	0.001	0.977