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The effect of repeated handling on telomere dynamics, body condition, and short-term survival in chicks of the Black-legged Kittiwake (*Rissa tridactyla*)

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I Preface

This Master's thesis was written at the Department of Biology, Norwegian University of Science and Technology, Trondheim. The fieldwork was conducted in Kongsfjorden, Svalbard, in July-August of 2016. Telomere and corticosterone analyses were conducted at *Centre National de la Recherche Scientifique* (CNRS) in Chizé, France, while the sex determination procedure was performed at NTNU laboratories. The project was supported by an Artic Field Grand of the Svalbard Science Forum, and received funding by Kong Haakon den 7des Utdanningsfond for Norsk Ungdom.

The thesis was supervised by Professor Claus Bech, who provided more help and support than could ever be expected. In addition to patient and constructive guidance to academic matters, he was an important part of what made this thesis an interesting and enjoyable experience. Øyvind, with whom I conducted all the fieldwork on Svalbard, fought off polar bears, and cooperated throughout the entire thesis, also has my gratitude for always having a humorous perspective on things and for having impressive ladder climbing skills. Big thanks should also be given to Solveig, who helped with analyses at NTNU, and to Frederic Angelier and Olivier Chastel for their help with the telomere and corticosterone analyses.

Finally, I would like to thank my family and friends for your encouraging support.

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II Summary

Det er ofte et mål i forskning å hindre at forskeren utilsiktet påvirker forskningsobjektet. Forskning på populasjoner av ville dyr er en viktig kilde til vitenskapelig kunnskap, men kommer alltid med en risiko for at forskeren kan påvirke både resultatene og velferden til de studerte dyrene. De potensielle negative effektene av slik «investigator disturbance» har blitt undersøkt i en del studier, men de bakenforliggende mekanismene er ikke godt forstått. Nylige studier viser at korte perioder med forhøyede kortikosteronnivå i fugleunger kan få livslange konsekvenser for fitness og overlevelse. I dette studiet ble effekten av «investigator disturbance» i unger av Krykkje undersøkt ved å se på telomerdynamikk, vekst, og korttidsoverlevelse. En eksperimentgruppe ble utsatt for håndtering annenhver dag i 25 dager, og sammenlignet med en kontrollgruppe. Både kontroll- og eksperimentfugler viste en statistisk signifikant forkortning av telomerlengde i løpet av eksperimentet, men ingen forskjell ble funnet mellom de to gruppene til tross for visuelle tegn på stress i eksperimentfuglene. En hyporesponsiv stressperiode i Krykkje-ungene er foreslått som en mulig forklaring på mangelen på forskjeller mellom de to gruppene. Selv om all forskning på populasjoner av ville dyr burde forsøke å redusere påvirkningen av menneskelig tilstedeværelse på forsøksdyrene både for velferds- og vitenskapelige hensyn, antyder resultatene i dette forsøket at Krykkje-unger er svært motstandsdyktige mot «investigator disturbance» i ungeperioden.

Krykkje-unger fra større kull hadde i dette studiet lengre telomerer ved en alder av 31 dager enn unger uten søsken. Forskjeller i foreldrekvalitet er foreslått som en mulig forklaring på den observerte sammenhengen. Videre ble det funnet en positiv korrelasjon mellom telomerlengde ved første måling og kroppsmasse 25 dager senere, noe som stemmer overens med tidligere studier på sammenhengen mellom telomerlengde og fitness.

III Abstract

Most research aims to reduce the unintentional impact of the investigator on the study object. Research on populations of wild animals is an important source of scientific knowledge, but contains the risk that the investigator may influence both the results and the welfare of animals. Although the potential negative effect of such investigator disturbance has received some attention, the physiological mechanisms behind it are not well known. Recent studies show that short periods of elevated corticosterone levels in chicks may lead to life-long effects on fitness and survival. In the present study, effects of investigator disturbance on chicks of the Black-legged Kittiwake were investigated by looking at telomere dynamics, growth, and short-term survival. An experiment group was exposed to a handling procedure every second day for 25 days, and compared to a control group. Although both experimental and control chicks showed a statistically significant reduction in total telomere length, no difference was detected between the two groups despite visual signs of stress in the experimental chicks. A hyporesponsive stress period is suggested as a possible explanation for the lack of differences between the two groups in the present study. Although all research on populations of wild animals should continue to be conscious of the impact of human presence on the studied animals both for welfare and scientific reasons, the results strongly suggest that chicks of the Black-legged Kittiwake are resistant to investigator disturbance during the nestling period.

In the present study, chicks from larger broods had longer telomeres at age 31 days than chicks without siblings. Differences in parental quality are suggested as a possible explanation for the observed relationship. Furthermore, a positive correlation was found between telomere length at first sampling body mass 25 days later, which is consisted with previous findings on the relationship between telomere length and fitness.

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1 Introduction

1.1 Investigator disturbance

Handling of wild birds occurs regularly as an integrated part of many research projects in order to obtain physiological and morphological data, collect blood samples, or for tracking purposes. Commonly investigated parameters such as growth and biometry require repeated interaction between researcher and chicks or adult birds. Such activity has the potential to disturb not only the handled birds, but also other birds in the vicinity of the researcher (Götmark, 1992). In addition to affecting the welfare and survival of the studied animals, such stress may bias sampled data and lead to questionable results.

The consequences of investigator disturbance are studied as either 1) short-term physiological consequences or 2) long-term consequences. Documented consequences of the first kind in birds includes acute, physiological effects such as increased baseline and induced levels of stress hormones (Wingfield et al., 1995; Herborn et al., 2014), increases in body temperature and breathing rate (Carere & van Oers, 2004), and myopathies (Nicholson et al., 2000). In the second category, effects range from desertion of nests (Blackmer et al., 2004), reduction in fledging success (Whidden et al., 2007) and decreased reproductive success (Carey, 2011) to life-long effects such as reduced survival and lowered productivity (Sharpe et al., 2009; Barron et al., 2010).

Despite such findings, the evidence for a consistent pattern in the effects of investigator disturbance is far from conclusive. For example, studies with repeated disturbance during the nestling period have shown reduced breeding success in Tufted Puffins (*Fraterula chirrata*; Whidden et al., 2007) and Leach's Storm-Petrels (*Oceanodroma leuchora*; Blackmer et al., 2004), but no significant impact in Roseate Terns (*Sterna dougallii*; Nisbet, 2000) and only very small effects in Black-legged Kittiwake (*Rissa tridactyla*; Sandvik & Barrett, 2001). Vertigan et al. (2012) found no effect of investigator disturbance on egg laying, chick survival or growth rate in Short-tailed Shearwaters (*Puttinus tenuirostris*), and only very small effects in the Little Penguin (*Eudyptula minor*). Overall, investigator disturbance has been shown to adversely affect measures like reproductive success in some bird species, while having no effect in other species (Reviewed by Carney & Sydeman, 1999). It is likely that several other factors other than species influence to what extent human presence is perceived as disturbing

by birds, including the nature and duration of disturbance, seasonal timing, and habitat characteristics. Of special note, long-lived species with the possibility of many future clutches may be expected to more easily surrender the current reproductive effort and allocate resources to prioritize their own survival in order to increase overall fitness, in line with life-history theories (Stearns, 1992). Indeed, there appears to be a trend of more adverse effects of investigator disturbance in long-lived species compared to short-lived species (Carney & Sydeman, 1999).

It is important to note that long-term measures such as reproductive success or survival measured in natural populations are susceptible to any number of confounding factors if not investigated carefully. The studies mentioned above highlight the need for increased knowledge of how research methods in field studies may affect the species investigated. The idea that the presence of researchers may impact wildlife is not new, and has received much attention in avian research (Götmark, 1992). For the last two decades however, an increasing focus has been given to the *physiological* consequences of human disturbance on birds (Romero & Wikelski, 2002; Müllner et al., 2004; Brewer et al., 2008a). This is important, as it may reveal the biological mechanisms behind the observable long-term effects. Such studies may also reveal effects of investigator disturbance that exist without being uncovered by parameters such as fledging- or reproductive success.

The increased focus on physiological mechanisms emerged together with studies investigating disturbance in chicks (Müllner et al., 2004; Walker et al., 2005; Sharpe et al., 2009; Brewer et al., 2008a). Previously, the main trend in studies on investigator disturbance has been to focus on adult birds, or on the reproductive success of adult birds (Rodway and Montevecchi, 1996; Blackmer et al., 2004; Hall & Kress, 2004). It is however well known that animals are sensitive to disturbances early in life, and that phenotypic development may be greatly affected by experiences in this period (Lindstrøm, 1999; Monaghan, 2008).

Studies on chicks have demonstrated lower body mass, higher mortality rates, increased secretion of corticosterone, and behavioral and physiological habituation in response to human disturbance (Müllner et al., 2004; Walker et al., 2005; Sharpe et al. 2009). A now well established approach is to look at the levels of stress hormones in response to handling, alone or in addition to reproductive metrics such as fledging or hatching success. This is

investigated with the perspective that high levels of stress may have detrimental effects on development (Wingfield et al., 1998; Kitaysky et al., 2003).

1.2 Stress, corticosterone and early development

Stress is defined as a stimulus that requires an emergency energetic response and thus increases the output of the Hypothalamic-pituitary-adrenal axis (HPA axis) above levels for normal physiological maintenance, as formulated by Wingfield and McEwen (2003). A stress response is triggered by a stressor, defined as an "unpredictable or noxious stimuli" (Romero, 2004). When a bird is exposed to a stressor, catecholamines are immediately released into the circulation from the adrenal medulla (Hill et al., 2012). In addition, all vertebrates react to acute stressors by increased secretion of adrenal hormones through the activation of the HPA axis (Wingfield et al., 1995; Wingfield & Kitaysky, 2002).

This activation of the HPA axis is initiated with the secretion of corticotropin-releasing hormone from the hypothalamus, which stimulates the release of adrenocorticotropin hormone (ACTH) from the pituitary gland into the blood (Hill et al., 2012). ACTH in turn stimulates the adrenal cortex to release glucocorticoids (GCs), and these are present in circulation within a few minutes of the stressor. The main GC and mediator of the stress response in birds is corticosterone (CORT) (Scanes, 2014). CORT is also involved in the regulation of normal functioning of several processes necessary for survival, including regulation of metabolism, immune system functioning, and gluconeogenesis (Astheimer et al., 1992; Sapolsky et al., 2000; Wingfield & Kitaysky, 2002). Circulating levels of GC are also involved in preventing malnutrition by promoting begging behavior in chicks (Quillfeldt et al., 2006), and in maintaining biological rhythms and the timing of life events (Quillfeldt et al., 2007).

When faced with a stressor, a short-term increase in CORT secretion is generally considered beneficial for the bird, allowing for a proportional response to the challenge at hand. CORT-secretion above intermediate levels promote survival by triggering a state of fight-or-flight (emergency life history stage) (Wingfield et al., 1998). This is achieved through mobilization of energy and an increase in heart rate and vigilance, concurrent with a short-term suppression of optimistic long-term activity such as reproduction, digestion and growth (Sapolsky et al., 2000; Wingfield and Kitaysky, 2002).

As detailed above, CORT regulation is important for the normal functioning and survival of birds, and necessary for adjusting behavior to environmental conditions. However, the stress response can become maladaptive in the face of a persistent or repeated stressor. Consequences of chronically or repeatedly elevated levels of CORT include suppression of the immune system and reproductive behavior, fatigue and depletion of energy reserves, muscle loss, and neuronal cell death (Wingfield et al., 1998; Sapolsky et al., 2000). CORT also influence cognitive functioning, and chronically elevated CORT levels are documented to have long-term effects on cognitive abilities in seabird chicks (Black-legged kittiwake; Kitaysky et al., 2003) and to lead to structural and chemical changes in the brain of humans (Teicher et al., 2003; McEwen, 2008).

Both a single exposure and chronic exposure of GC has been shown to increase oxidative damage in birds (Lin et al., 2004; Haussmann et al., 2012). This may occur by different mechanisms, as GCs have direct effects on the amount of free radicals and can affect both antioxidant defenses and antioxidant damage repair systems (Haussmann & Marchetto, 2010). The connection between GC and oxidative damage is well established in many species, and in birds an increase in markers for oxidative damage following chronic exposure to GCs is demonstrated in at least two species (Lin et al., 2004; Costantini et al., 2008). Furthermore, GCs may disable enzymatic or dietary antioxidants and thus promote oxidative stress (Haussmann & Marchetto, 2010). Oxidative stress is thus a significant consequence for organisms exposed to long-term or repeated stress.

Any unpredictable situation, such as increased predation pressure, food limitation, or human disturbance may constitute a stressor and hence elicit a stress response (Wingfield et al., 1998; Frid & Dill, 2002). A likely explanation for the stressing impact of human presence on animals is their perception of humans as predators, and an activation of anti-predator responses (Frid & Dill, 2002, Beale & Monaghan, 2004). These responses consist of behavioral and physiological adaptations to maximize fitness and survival in the event of encountering a predator, and are achieved partly through secretion of GCs (Romero, 2002). Interestingly, many of the negative consequences of mounting a prolonged stress response may not be apparent until long after the actual disturbance, as has been observed in many bird neonates (Kitaysky et al., 2003; Gil et al., 2004). Repeated exposure to a stressor may lead to chronically elevated GC levels and persistent changes in HPA axis functioning, possibly due to effects on negative feedback mechanisms (Meaney et al., 1988; Romero, 2004). Naturally

this will enhance the aforementioned negative effects on growth, reproduction, cognitive development and immune system (Wingfield et al., 1998; Sapolsky et al., 2000). Previous investigations of HPA axis responsiveness have shown that regular handling induce a *reduction* in the stress response capacity in young American Kestrels (*Falco sparverius*; Whitman et al., 2011) and eastern bluebird (*Sialia sialis*; Lynn et al., 2013). In a study of European Shag (*Phalacrocorax aristotelis*), on the other hand, increased sensitivity without a change in baseline levels has been shown as a result of handling (Herborn et al., 2014). Yet other studies have failed to find any effect on baseline or stress-induced levels in Black-legged Kittiwake (Brewer et al., 2008a), Thin-billed Prion (*Pachyptila belcheri*; Quillfeldt et al., 2009), or Leach's Storm Petrel (Fiske et al., 2013).

Even if there is no long-term change in HPA-programming occurring, and even in the absence of direct adverse effects of high GC-levels that are phenotypically observable, there may be more subtle consequences of stress exposure during early life. One proposed link between early stress and long-term effects on fitness and survival is the attrition of telomeres, possibly accelerated by high levels of GC. This has been investigated in some recent studies (Haussmann et al., 2012; Herborn et al., 2014; Hausmann & Heidinger, 2015; Watson et al., 2016), and is the focus of the present study. This approach has the potential to discover long-term effects of investigator disturbance that are not phenotypically obvious but may still bias research results and affect long-term fitness. Telomere dynamics are closely linked to survival, which naturally is a critical determinant of fitness.

1.3 Telomeres and telomere dynamics

1.3.1 Telomeres and telomerase

Telomeres are short, repetitive nucleotide sequences of non-coding DNA that form caps at the terminal end of the linear chromosomes of eukaryotes, and thus protect chromosomic information (Reviewed by Blackburn, 2000). Despite differences in telomere length among species and chromosomes (Aubert & Lansdorp, 2008), the sequence and structure of telomeric DNA appears to be well conserved amongst eukaryotes (Blackburn, 2005). The length of telomeres ranges from a few hundred base pairs in yeast to several thousand in vertebrates (Blackburn, 1991).

The DNA of telomeres is built up of tandemly repeated sequences that form a scaffold with binding sites for telomeric proteins. This produces a dynamic DNA-protein complex that readily exchanges proteins on and off individual telomeres within minutes (Mattern et al., 2004). In vertebrates, the telomeres are made up of the nucleotide sequence "TAGGG" (Moyzis et al., 1988; Meyne et al., 1989). Telomeres have a guanine-rich single-strand at their distal end, and this strand forms a t-loop by bending back into the double-stranded sequence. If the telomere is sufficiently long, it will "cap" the chromosome. This prevents the end of the chromosome from being recognized as double-stranded break, and protects it from end-to-end fusions (Blackburn, 2005). A cell with chromosomes that are uncapped for too long will go into cell cycle-arrest or have other detrimental responses. Capping status is thus an important factor for telomere function in addition to telomere length. It appears that regular uncapping occurs, and that telomere length, active telomerase and other molecular components work together to determine capping and uncapping of cells (Steensel et al., 1998; Smith & Blackburn, 1999).

Without telomeres, chromosomes are vulnerable to DNA-degradation and loss of genetic information (Blackburn, 1991). In addition to this protective role, telomeres provide a solution to "the end replication problem": conventional replication works in the 5`to 3`direction, and requires cellular DNA polymerase as an RNA primer. This means that while the leading strand is replicated in full, the lagging strand is not since DNA polymerase is unable to fill the gap of the RNA primer. In the absence of telomeres, successive rounds of replication would leave shorter and shorter daughter chromosomes due to incomplete end replication (Makarov et al., 1997). Thus, telomeres allow the complete replication of the linear DNA without loss of important genes at the 5`end of each DNA strand. As proposed by Olovnikov (1972), telomeres function as buffers allowing the DNA coding for genes to remain its original length. Instead, the telomeres shorten each round of replication and in somatic cells this will normally result in cell senescence when the telomeres reach their critical length (Bodnar et al., 1998).

In stem cells or "immortal" cell lines, telomeres are constantly elongated as cell replication occurs. This is made possible by telomerase, a specialized ribonucleoprotein enzyme that allows RNA-templated addition of repeated telomeric sequences (Blackburn, 2005). In general, long telomeres shorten more rapidly than short telomeres (Salomons et al., 2009), suggesting that telomere maintenance preferentially occurs at the shortest telomeres to prevent degradation beyond a critical length. While telomerase ensures that telomeres do not become

too short, protein interactions among the telomere-associated proteins prevents over-extension of telomeres (Levy & Blackburn, 2004).

1.3.2 Oxidative damage and telomeric DNA

As previously mentioned, high levels of GC increases levels of free radicals and oxidative damage (Finkel & Holbrook, 2000). Telomeric DNA is more susceptible to oxidative damage than normal DNA due to high guanine content and a reduced repair capacity (Zglinicki, 2002; Gomes et al., 2011), which may explain why oxidative damage due to stress can have significant impact on telomere shortening rate (Haussmann et al., 2012). The high guanine content makes telomeric DNA much more exposed for strand breakage (Henle et al., 1999). Studies of human cells show telomere shortening by 20-200 base pairs at each cell division. Only about 10 % of this is considered to be due to the end-replication problem, suggesting that other processes such as attrition due to oxidative damage has significant impact (Houben et al., 2008). While double-stranded DNA breaks may be responsible for some telomere shortening, it appears that most telomere shortening is stress dependent and is caused by single-strand breaks in the telomeric DNA. This idea is strengthened by the fact that single-strand breaks are present at much higher density in telomeric DNA than elsewhere in the genome (Zglinicki et al., 1995; Sitte et al., 1997; Zglinicki et al., 2000). Mitocondria status may also be an important factor for oxidative damage, as damage to mitochondrial DNA increases the production of reactive oxygen species, and improvement in mitochondrial function is documented to reduce telomere damage (Passos et al., 2007).

In addition to direct DNA damage, stress communicated through GCs may affect telomere attrition by increased cell division, and by effects on telomere maintenance (Houben et al., 2008). This is an area that requires more research. It has however been demonstrated that oxidative stress dramatically decreases the activity of a sub-unit of telomerase (Borras et al., 2004), and oxidative stress may thus increase telomere shortening indirectly through inhibition of telomere restoration.

1.3.3 Telomeres linking early stress and long-term consequences

The relationship between telomere loss and cellular ageing is well established. For somatic cells grown in culture, lifespan is determined by the length of telomeres (Harley et al., 1990; Blasco et al., 1997; Kaji et al., 2009). Thus, telomere length is an important determinant for a cells ability to proliferate. It is still not well understood how replicative senescence affects

organismal ageing (Hornsby, 2006), but is generally accepted that telomere attrition is one of the cellular events that accompanies normal aging. When telomeres reach their critical length they become dysfunctional and their associated cells enter a state of senescence. These cells either die or adopt a secretory profile that contributes to age-related decline in tissue and organ function. This happens through secretion of cytokines and degradative enzymes that also disrupt nearby cells (Campisi, 2005; Capper et al., 2007). In humans, telomere dysfunction is documented to correlate with many age-related diseases, such as Alzheimer's disease (Panossian et al., 2003) and heart failure (Wong et al., 2010). Consistent with this, telomere length declines with age in many birds (Salomons et al., 2009; Barrett et al., 2013).

In general, telomere shortening is found to be more rapid during early life (Hall et al., 2004; Salomons et al., 2009; Heidinger et al., 2012), probably due to high levels of growth and cell division. In a study on wild King penguin chicks (*Aptenodytes patagonicus*), Geiger et al. (2012) demonstrated how fast growth in chicks came at the cost of higher oxidative damage and accelerated telomere loss - in other words, at the cost of decreased body maintenance. It is not surprising if chicks in this period of intense growth are sensitive to disturbances, either nutritional or stress wise.

Young individuals generally show a large intraspecific variability in telomere length (Haussmann et al., 2005; Salomons et al., 2009). Together with the rate of shortening, such length differences have been found to predict survival: female one year old Tree Swallows (*Tachycineta bicolor*) with below average telomere lengths had lower survival in the following years than birds with above average telomere lengths (Haussmann et al., 2005). In one study on Alpine swifts (*Tachymarptis melba*), telomere dynamics was a better predictor of survival than age (Bize et al., 2009). Furthermore, Salomons et al. (2009) demonstrated how individuals with the shortest telomeres were less likely to survive to the next year, and that telomere shortening rate was a good marker of experienced "life stress" in an individual. A longitudinal study on Zebra finches (*Taeniopygia guttata*; Heidinger et al., 2012) following birds throughout their life (1-9 years) and measuring telomere length at various time points showed that telomere length at 25 days was a very strong predictor of lifespan. Similar results were found in a longitudinal study on Seychelles warblers stretching over 20 years (*Acrocephalus sechellensis*; Barret et al., 2013): shorter telomeres and greater rates of telomere shortening predicted future mortality. Thus, it is apparent that the variation in telomere length seen between individuals of the same species impact long-term survival.

The differences in telomere length observed in populations of birds are partly genetically determined (Olsson et al., 2011), but much of the variation may be environmentally related. In addition to replication, another process which shortens telomeres is stress, as detailed above (section 1.3.2). In humans, the connection between stress exposure and telomere attrition is well documented (Epel et al., 2004; Epel et al., 2006), and a solid link has been established (Reviewed by Shalev et al., 2013). In birds, exposure to repeated stress during early life has been shown to accelerate telomere attrition (Haussmann et al., 2012; Herborn et al., 2014; Watson et al., 2015), and Boonekamp et al. (2014) demonstrated that developmental stress in free-living jackdaws (*Corvus monedule*) accelerated telomere shortening and that shortening rate predicted post-fledging survival.

Thus, despite a lack of observable immediate effects of stress exposure there is a potential risk that stress can induce effects during the nestling period that are not phenotypically obvious. Instead, these effects may become evident later in life. An interesting example of this is the study by Meillère et al. (2015), showing that traffic noise led to reduced telomere lengths in free-living house sparrows (*Passer domesticus*) even in the absence of effects on growth, condition or CORT baseline levels. In general, conditions during early development and growth have profound implications for short- and long-term fitness in animals (Gluckman et al., 2005; Monaghan, 2014). There must necessarily be a link between the experienced environment and such long-term effects, and this link could potentially be the aforementioned accelerated shortening of telomeres.

The idea that telomeres may link early environment and long-term fitness effects is presently gaining merit, and the relationship between life histories, stress, aging and telomeres is receiving a lot of attention (Monaghan, 2008; Haussmann and Marchetto 2010; Haussmann and Heidinger, 2015). Studies investigating telomere dynamics and stress exposure in wild populations have found stress exposure during the nestling period to increase telomere attrition (Haussmann et al., 2012; Herborn et al., 2014; Watson et al., 2015), and while one study did not find an effect (Watson et al., 2016) this was proposed to be due to a possibly reduced stress response in chicks of that species (European Storm Petrel). The Black-legged Kittiwake has previously received much attention in research due to its distribution and accessibility, and the species is especially interesting as it has status as a near threatened species (red-listed in mainland Norway, but not on Svalbard). Of special relevance, Sandvik and Barret (2001) found small effects of investigator disturbance on nest attendance and daily

chick mortality in the Black-legged Kittiwake, with chick survival being significantly lower in the disturbed group, without any significant effect on chick growth. Fridinger et al. (2007) found elevated CORT-levels in Kittiwake chicks in response to investigator disturbance, even when this occurred at nearby nests. On the other hand, Brewer et al. (2008a) investigated the effect of investigator disturbance on CORT concentrations in Kittiwake chicks, but found no significant difference in baseline or induced levels, indicating no additional stress imposed by human interactions.

Previous research on the Black-legged Kittiwake has not investigated telomere dynamics in response to handling, which potentially is the link to long-term effects on fitness and survival. The present study could uncover subtle detrimental effects of repeated handling of Kittiwake nestlings, a procedure which is very often used in seabird research. The results would also be relevant for wildlife exposed to human recreational activities or ecotourism (Müllner et al., 2004; Walker et al., 2005).

1.4 Aim of study

The present study will investigate the effect of investigator disturbance, experimentally imposed by repeated sampling during the nestling period, on the telomere dynamics in chicks of the Black-legged Kittiwake. Experimental chicks will be exposed to human presence during the nestling period and the resulting data will be compared to that obtained from a control group. Telomere length change will in both groups be assessed by analyzing telomeres in blood samples obtained post-hatching and close to fledgling, while the stress level at the end of the experimental period will be assessed by analyzing corticosterone levels in plasma. A secondary objective is to see how the changes in telomere length during the nestling period correlate with brood size, body condition, and short-term survival in the Kittiwake chicks.

Few studies looking at telomere dynamics have experimentally increased stress levels in natural populations of birds. The results could therefore have great implications as it could reveal if "observer"-induced stress in field studies can lead to increased telomere attrition, which again can have long-term effects on fitness and survival. Even in the absence of observable physiological changes during the nestling period, there could be a stress-induced telomere-shortening that ultimately lead to shorter life-span, potentially exercising a large effect on the life-history of the species.

2 Materials and methods

2.1 Study area and species

The fieldwork was conducted from 05.07.2016 to 10.08.2016 on the island of Blomstrand, Svalbard (78º 59'N, 12º 7'E). The small island contains colonies of several types of seabird, including Kittiwake, Fulmars, and Atlantic Puffin. Chicks from three sub-colonies of breeding Black-legged Kittiwake were used in the present study (figure 1). The colonies consisted of a few hundred breeding pairs in total.

The Black legged Kittiwake is a small, long lived species of gull breeding in the artic and boreal zones of the northern hemisphere (Strøm, 2006). It nests in large and dense colonies, and builds nests on narrow ledges in vertical cliffs. The pelagic gull is the most abundant gull species in the world with 9.000.000 estimated adult individuals, and probably as many immature individuals (Coulson, 2011). The Kittiwake is also the most common gull on Svalbard with an estimate of 270 000 breeding pairs in 2006 (Strøm, 2006).

Kittiwakes are socially monogamous and share parental duties (Strøm, 2006). The nestlings require a high degree of parental care, and due to their complete lack of thermoregulatory function the chicks are always attended by a parent for the first 15 days of life (Golet et al., 1998). The incubation period on Svalbard normally starts in the first half June, and lasts for approximately 27 days (Strøm, 2006). Although the normal clutch size is two eggs, both one and three egg-clutches regularly occur. The eggs within a nest normally hatch with a one day interval. Usually only one chick survives to fledging in each nest. Chicks obtain flight abilities and leave the nest after 5-6 weeks (Strøm, 2006). The Black-legged Kittiwake breed at an age of three to five years old, and can live for up to 28 years (Coulson, 2011).

2.2 Experimental setup

A nest content check was conducted upon arrival on accessible nests to determine a hatching date for all chicks. After the first day this was done every second day for approximately two weeks until the hatching date of all the chicks in the data set were accounted for. Due to the exceptionally early hatching period in 2016, chicks already hatched were age-determined using growth curves based on body mass and skull size.

Nests were accessed using ladders. All accessible nests were randomly designated control (n = 32) or experiment (n = 32), although in groups as to minimize disturbance of control nests. The three sub-colonies used were designated BG, UB and UBR. Whereas BG and UB were spatially large enough to contain both experimental and control groups, UBR was of such a small size that it only contained experimental chicks.

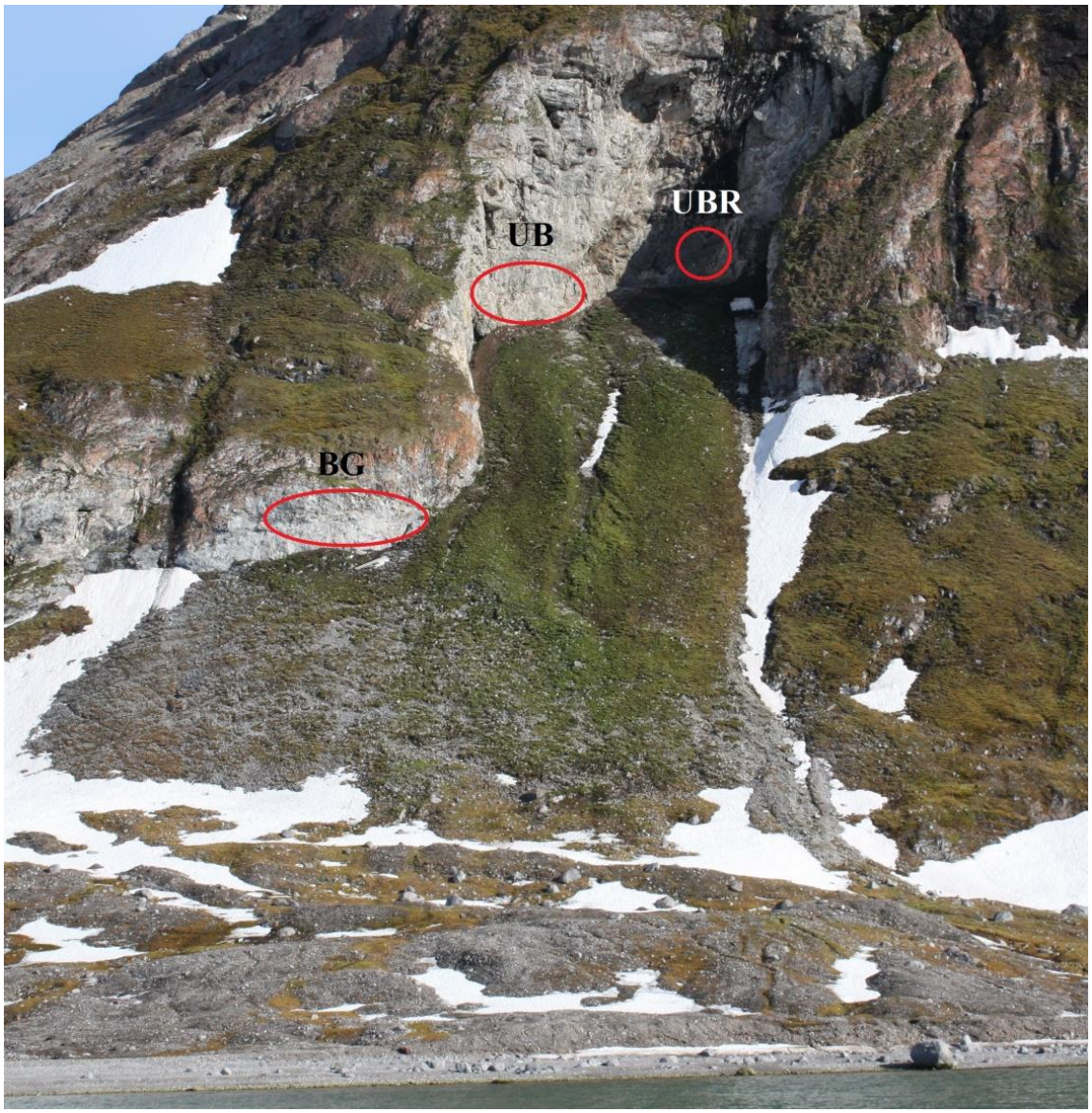

Figure 1: The sub-colonies used in the present study. In the final data set, 26 chicks were sampled from BG, 31 chicks from UB, and 4 chicks from UBR. Photo: Claus Bech.

The parameters of interest in this study were 1) telomere length measured in blood samples, 2) baseline CORT level measured in plasma, and 3) biometric measurements (body mass, head, tarsus and wing). Each chick was blood sampled twice. The first sample was taken from both experimental and control chicks when each chick was approximately 6 days post-hatching (mean = 6.2, SD = 2.02). In addition to a blood sample, biometric measurements were obtained. Body mass was measured using spring balances (100 grams (accuracy 1.0), 200 grams (accuracy 2.0) or 500 grams (accuracy 5.0)), while skull and tarsus length were measured using a slide caliper (accuracy: 0.1 mm). Wing length was measured with a ruler (accuracy: 1.0 mm). Finally, each chick was marked with a plastic ring on one leg for identification.

From this point on the experimental group was exposed to investigator disturbance every second day for the next 25 days, after which the second blood sample was obtained from both groups. In addition to repeating the measurements taken during the first sampling, the plastic ring was then replaced with a metal ring and a larger plastic ring for later identification.

Of the initial 64 sampled nests, 54 contained more than one chick. In these nests, both chicks were sampled if the alpha chick was difficult to distinguish from the beta in terms of size. This was done because of the poor survival prospects of beta chicks. Some chicks died due to rock falls or predation, and of the 84 initial chicks 66 survived to the second sampling. Five chicks were removed from the data set due to injury or sampling errors, and the final data set hence consisted of 30 control chicks and 31 experimental chicks, distributed over 51 nests.

2.3 Investigator disturbance procedure

The investigator disturbance procedure was a timed standardized handling procedure meant to represent the handling of a bird in a typical research procedure, while at the same time inducing a significant stress response in the chicks to detect an effect, if any such effect existed. It involved taking the chick out of the nest, weighing the chick in a cloth bag, and taking the chick out of the bag and handling it for 5 minutes in a manner mimicking measurement of head, tarsus and wing. After this, the chick was put back in the bag until replacement into the nest. The total time of the procedure was 17 minutes.

This disturbance procedure was carried out every second day for each experimental chick, summing up to a total of 12 times per chick during the growth period. The procedure usually allowed 2-3 chicks to be taken down at the same time, depending on the location of nests.

2.4 Blood sampling

At each blood sampling, approximately 0.5 mL of blood was sampled from the ulnar vein with a heparinized syringe (Heparin LEO®, 5000 IE/ml). During the second sampling, blood sampling was done within 3 minutes of capture (mean = 02:10, SD = 00:27) due to CORT measurement considerations. A small drop of the sampled blood was stored in ethanol (96 %) for sex determination. The remainder of the blood was immediately put on ice. This was centrifuged (9500 rpm, 4 min) and separated within 6-10 hours of sampling. The plasma and red blood cells (RBC) were then stored in a freezer at -20 °C until transport.

2.5 Telomere measurements

The telomere measurement procedure used in this study is based on quantitative PCR amplification of telomeric sequences $(TTAGGG)_n$ (Criscuolo et al., 2009). Analyses were performed at *Centre National de la Recherche Scientifique* (CNRS) in Chizé, France.

Samples were digested with protein kinase K, before DNA was extracted from the red blood cells samples using DNeasy Blood and Tissue Kit (Qiagen). Electrophoresis and optical density spectrophotometry was used to test DNA quality. The qPCR for the single control gene and telomeres were conducted using 5 ng of DNA per reaction. Telomere primers were used at a concentration of 800 nM, while specific gene primers were added at a concentration of 200 nM. A 96-well plate with an included standard curve was used, and this had various concentrations of Kittiwake DNA. This standard curve was used to generate a reference curve to control for the amplifying efficiency of the qPCR.

2.6 Corticosterone measurements

The blood samples obtained during the second sampling were timed to be taken within 3 minutes of capture. These were later centrifuged as previously described to separate plasma and erythrocytes. Plasma samples from all chicks were used to measure total free CORT

concentrations. Analyses were conducted at *Centre National de la Recherche Scientifique* (CNRS) in Chizé, France.

The CORT measurements were carried out following established protocols (Lormée et al., 2003). Bound and free plasma CORT was measured using radioimmunoassay. Each sample was extracted (3 mL of diethyl-ether per 100 µL sample), vortexed and centrifuged. The resulting diethyl-ether mix was poured off following a snap freezing in an alcohol bath at 38 °C. After evaporation, the dried extracts were dissolved in 300 µL of phosphate buffer, and CORT was assayed in duplicate. 100 µL of extract was incubated with 5000 cpm of the appropriate ^{3}H-steroid (Perkin Elmer, Waltham, MA, US) and polyclonal rabbit corticosterone-21-thyroglobulin antiserum for approximately 12 hours. To separate bound CORT from free CORT, dextran-coated charcoal was added and the following reaction counted on a tri-carb 2810 TR scintillation counter (Perkin Elmer, Waltham, MA, US).

2.7 Molecular sexing

Molecular sexing of all chicks was performed using the procedure of Griffiths et al. (1998). The analyses were conducted at NTNU, Norway.

The technique is based on sex chromosome differences in male and female birds. Females are heterogametic (ZW) and carry both the CHD-1-W gene and the CHD-1-Z gene, whereas males are homogametic (ZZ) for the CHD-1-Z gene. Specific primers (Griffiths et al., 1998) are added to the extracted DNA before the polymerase chain reaction (PCR) amplification process. The agarose gel separates the PCR products of the CHD-1-Z gene and the CHD-1-W gene because of size differences, and will display a single band for males and two bands for females (Griffiths et al., 1998).

In the field, a small drop of blood was stored on ethanol during blood sampling. DNA was extracted from this using a 5% Chelex 100 resin (Biorad, Hercules, CA, US) procedure. The solution was incubated at 56 °C for 20 minutes, vortexed, incubated at 96 °C for 8 minutes, vortexed, and then centrifuged at 12000 rpm for 3 minutes. 20 µL of this solution was used as a DNA template for gene amplification by PCR. 8 µL of stock solution together with 2 µL DNA supernatant resulted in a total reaction volume of 10 µL per sample.

Following the PCR, samples were analyzed using gel electrophoresis. This was performed on a 2 % stained agarose gel (SYBR®Safe DNA gelstain, Invitrogen, Carlsbad, CA, US). The results were visualized under UV light. Samples from previous known sexed individuals were added to the gel as control.

2.8 Statistical analyses

All statistical tests were performed using SPSS 24.0 (SPSS Inc. 2016). Normality for variables and residuals was asserted using the Shapiro-Wilk test ($P \geq 0.05$) and QQ-plots. Equal variance was tested using Levene's Test for Equality of Variance ($P \geq 0.05$).
All statistical tests done were two-tailed and had a significance level of $P \leq 0.05$, with tendencies assumed at $P \leq 0.10$. Means are given with standard error (± SE).

To create a measure of body condition, a Body Condition Index (BCI) was calculated for all chicks at age 31 days. A Principal Component Analysis (PCA) was used on skull, wing and age to produce a measure of structural size. Tars measurements were excluded due to low correlation. The PCA was done separately for both sexes as was justified by Kaiser-Meyer-Olkin test for sampling adequacy (KMO) and Barlett's test of sphericity (KMO > 0.5, P < 0.01). The PCA variables were then used as covariates in a general linear model, with body mass as the dependent variable. The resulting standardized residuals were used as a BCI.

Independent t-tests were initially used to detect any difference between the control and experimental group on the following variables: survival, BCI, telomere length at first and second sampling, telomere shortening rate, and CORT baseline levels.

The variation in telomere length, telomere shortening rate and BCI was investigated using an Analysis of Covariance (ANCOVA). This was done repeatedly with the different parameters as the dependent variable to investigate the variation in the data. Four models were made with the following measures as dependent variable: 1) Telomere length at first sampling, 2) telomere length at second sampling, 3) telomere shortening rate, and 4) BCI. Categorical variables included were treatment group, brood size, and sex. Covariates included were baseline CORT, Telomere length, and BCI, depending on the dependent variable in the given model (for all initial models, see Appendix 7.1). Biologically relevant interactions were included in all initial models. Correlations between all explanatory variables were examined

to ensure no correlated variables in the models. Body mass was excluded for this reason, due to its correlation with BCI. To obtain the best model, non-significant variables were sequentially excluded one by one.

2.9 Methodological considerations

The telomere length measurement procedure used in the present study expresses the ratio between telomere repeat copy number and a control single gene copy number as a T/S ratio, or relative telomere length. This method is useful for within-species comparison of individuals, or for longitudinal studies following the same individuals. Compared to conventional quantification of terminal restriction fragments (TRF) and traditional southern blot analysis or in gel hybridization this method is faster, and requires smaller amounts of DNA. The method is validated for birds, and is found to correspond well with the alternative TRF analysis (Criscuolo et al., 2009).

2.10 Permissions and cooperation

Permission to carry out the fieldwork on Blomstrand was given by the Governor of Svalbard (Sysselmannen), reference: 16/01043-2. Blood sampling and handling permission was given by The Norwegian Food Safety Authority (Mattilsynet), reference: 2016/116563.

The fieldwork was conducted together with Msc. student Øyvind Gjønnes Tvedten, and all data except for the telomere data are shared with his project on the effects of repeated investigator disturbance on corticosterone concentration and the immune system in chicks of the Black-legged Kittiwake.

3 Results

Both average *clutch size* and *breeding success* for Kittiwakes breeding in the colony was high in 2016 compared to most previous years (table 1).

During the investigator disturbance procedure, chicks displayed visible behavioral signs of stress such as escape attempts and regurgitating of food. Some chicks died due to rock falls or predation, and of the 84 initial chicks 66 survived to second sampling. Survival was equal in the two groups with each group starting with 42 individuals and ending up with 33 surviving chicks at the end of the experiment.

Table 1: Average clutch size and breeding success in 2016 and preceding years. Clutch size was calculated as the number of eggs per active nest, while breeding success was calculated as the number of chicks per active nest surviving until age 15 days. Unpublished data: Claus Bech.

Year	Clutch Size	Breeding success	Year	Clutch size	Breeding success
2016	1.90	1.25	2008	1.86	0.98
2015	1.74	-	2007	1.90	1.27
2014	1.70	1.25	2006	1.74	0.55
2013	1.78	0.63	2005	1.96	1.31
2012	1.93	0.97	2004	1.64	0.87
2011	1.85	-	2003	1.53	0.25
2010	1.65	0.98	2002	1.30	0.00
2009	1.52	0.41	2001	-	-

3.1 Descriptive measurements

In the present study no statistically significant difference was observed between the experimental and control group in any of the descriptive statistics: age, body mass, skull, tars, or wing size. This was true both at the first and second sampling (table 2). Also, no significant difference was found in body mass change during the experimental period between the two groups (252.2 ± 9.2 (n=31) for the experimental group and 264.9 ± 7.3 (n= 30) for the control group, P = 0.284). Measurement device errors on some skull and tars measurements led to the corresponding individuals' values on those parameters being removed from the data set (table 2).

Table 2: Descriptive statistics for all chicks, displaying mean values (±SE). All parameters were tested for sex-differences, and none were found significant. All P-values were found using an independent samples T-test. n(E, C) denotes the number of individuals in the experiment (E) and control group (C).

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

3.2 Relative telomere length and telomere length change

No significant difference in telomere length was detected between control and experimental group at first or second sampling (table 3). Both the experimental group ($P < 0.01$) and control group ($P < 0.01$) had a significant reduction in telomere length during the period, but no significant difference in shortening rate was observed between the groups (table 3). Some individuals of the data set were not analyzed for telomere length, and the statistics for telomere measurements are thus based on 30 experiment chicks and 26 control chicks (table 3).

Table 3: Mean (±SE) telomere length (T/S-ratio) at first and second capture and total change in telomere length, for the experiment and control group. P-values were found using an independent samples T-test. n(E, C) denotes the number of individuals in the experiment (E) and control group (C).

		Treatment				
Variable	**n (E, C)**	**Experiment**	**Control**	**df**	**t**	***P***
Telomere first sampling (T/S)	30, 26	0.9979 ± 0.0141	0.9761 ± 0.0180	54	0.966	0.338
Telomere second sampling (T/S)	30, 26	0.9049 ± 0.0221	0.9095 ± 0.0175	54	-0.159	0.874
Telomere change (T/S)	30, 26	-0.0903 ± 0.0231	-0.0666 ± 0.0149	54	-0.930	0.357

The ANCOVA showed no significant variables for telomere length at first sampling of the original included variables (see appendix 7.1). However, when setting body mass at second sampling as an explanatory variable this turned out significant, but explained only 7.3 % of the variation (table 4, figure 2). Thus, birds with the longest telomeres at first sampling had slightly higher body mass at second sampling, although the effect was small.

For telomere length at second sampling, the ANCOVA gave no significant variables except brood size (table 4, figure 3), which explained 11.9 % of the variation. Chicks with siblings had, on average, longer telomeres than single chicks.

Finally, change in telomere length had no significant variables in the ANCOVA (table 4). Of particular interest, telomere length change was not significantly influenced by brood size (P = 0.152).

Table 4: Summary of the final ANCOVA output with variables explaining variation in telomere length at first and second capture, telomere length change during the experimental period, and BCI at second sampling for kittiwake chicks. For the dependent variables with no significant model, the best fitting variable is shown.

Dependent	**Explanatory**	**df**	**F**	***P***	**r^2**
Telomere 1st sampling	Body mass 2nd sampling	55	4.229	**0.045**	**0.073**
Telomere 2nd sampling	Brood size	55	3.528	**0.037**	**0.119**
Telomere length change	Group*BCI	55	2.193	0.122	0.078
BCI	Group	59	0.530	0.470	0.010

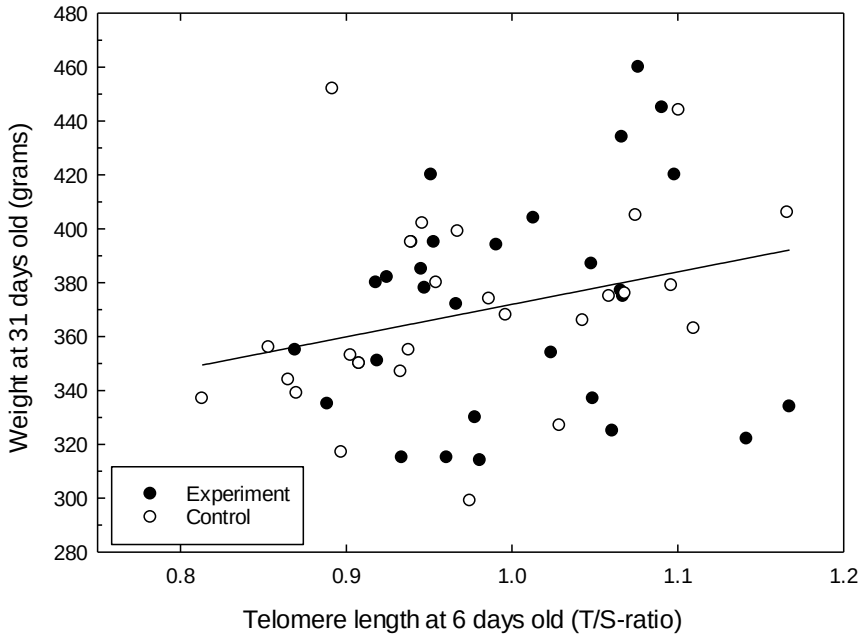

Figure 2: Telomere length at first sampling plotted against weight at second sampling for experiment (black) and control (white) chicks. Telomere length at first sampling explained 7.3 % of the variation in weight at second sampling (P = 0.045), given by the equation (y = 120.53x + 251.44).

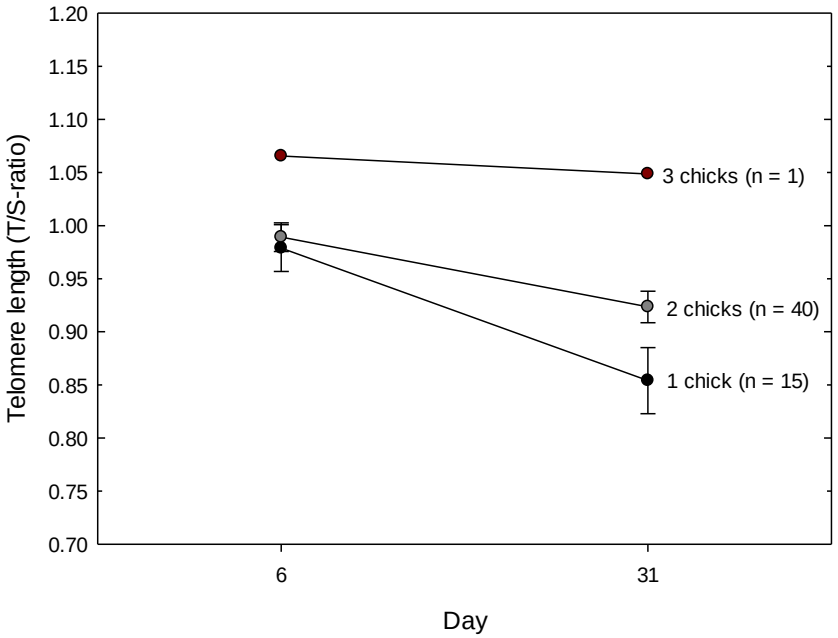

Figure 3: Mean telomere length (±SE) at day 6 and 31 for chicks in broods consisting of 1 chick (black), 2 chicks (grey), or 3 chicks (red). Brood size explained 11.9 % of the variation in telomere length (T/S-ratio) at second sampling (P = 0.037).

3.3 Body condition and corticosterone

CORT measurements were tested for time effects within the 3 minute window, but none were found ($p = 0.729$). CORT did however depend on body mass at second sampling ($p = 0.039$), so all CORT data were corrected for body mass and the residuals used in all further models.

There was no statistically significant difference in BCI or CORT baseline level between the control and experimental group (table 5). Furthermore, the ANCOVA yielded no significant explanatory variables with BCI as the dependent variable (table 4).

Table 5: Mean BCI and baseline CORT values for Kittiwake chicks at age 31 days. The BCI was made separately for both sexes. The CORT baseline measures are residuals after correcting for body mass. P-values were found using an independent samples T-test. n(E, C) denotes the number of individuals in the experiment (E) and control group (C).

		Treatment				
Response variable	**n (E, C)**	**Experiment**	**Control**	**df**	**t**	***P***
CORT	31, 30	-0.0068 ± 0.1678	0.0070 ± 0.1942	59	-0.054	0.957
BCI	30, 30	-0.1938 ± 0.1631	0.1938 ± 0.1749	58	-1.621	0.110

4 Discussion

The present study aimed to investigate the effect of investigator disturbance on kittiwake chicks by measuring short-term survival, growth, and telomere length. No significant difference was found in any of these parameters between the control group and the experimental group exposed to the investigator disturbance procedure. As expected, both groups displayed a marked reduction in total telomere length during the nestling period. An unexpected link between brood size and telomere length was demonstrated, as well as a relationship between telomere length and growth. Overall it thus appears that the experimental manipulation had very little effect in the present study, and that the obtained results reflect naturally occurring variation.

4.1 Investigator disturbance and corticosterone

It was hypothesized that Kittiwake chicks would perceive researcher presence as disturbing, and thus respond to the investigator disturbance by activating stress responses. There is no quantitative measure of the induced stress level in the chicks in the present study. Despite visual signs of stress such as regurgitation there is no way to know if CORT levels were elevated in response to the investigator disturbance procedure. There are several possible explanations for the lack of significant differences in the measured parameters between experimental and control group: 1) a stress response with elevated CORT was elicited in the experimental group, but had no effect on telomere shortening rate or growth, 2) the presence of researchers induced colony-wide disturbance affecting both groups similarly, or 3) no stress response was activated in the chicks in response to researcher presence.

The link between CORT-mediated oxidative damage and telomere attrition is as previously mentioned well established (Zglinicki, 2002; Houben et al., 2008; Haussmann et al., 2012; Herborn et al., 2014; Quirici et al., 2016). Similar telomere lengths between groups, as seen in the present study, are therefore consistent with the unchanged baseline CORT levels. It should however be noted that stress exposure may lead to changes in telomere length even in the absence of changes in *baseline* CORT levels, as seen in some studies (Herborn et al., 2014; Meillere et al., 2015). Even so, it is very likely that any marked increase in CORT secretion in response to the investigator disturbance procedure, if present, would have resulted in a detectable difference in telomere shortening rate between the experimental and control group.

It seems unlikely that human presence in the colony would induce a disturbance effect on *all* the chicks in the colony, although this option cannot be firmly rejected. Chicks are perched on nests with very limited information of their surroundings. There is however the possibility that signals from alarmed parent birds to some extent are picked up by chicks, thereby increasing their stress level. Furthermore, Fridinger et al. (2007) demonstrated how researcher presence within two meters and within line of sight induced a stress response in Black-legged Kittiwake chicks, and that for older multi-brood nestlings, removing one chick induced a stress response in the sibling. In the present study, chicks from the same nest were always taken together. There could however still have been a carry-over effect for nests close to each other. In a colony of Magellanic penguin (*Spheniscus magellanicus*) exposed to tourism, Walker et al. (2005) found higher CORT induced stress responses amongst chicks in visited colony sites. The same was found by Müllner et al. (2004) on a study of hoatzin chicks (*Opisthocomus hoazin*) exposed to ecotourism. Thus, although there is no direct evidence for or against such colony-wide disturbance, it is possible that human presence could create at least a gradient of stress. This was partly accounted for in the present study by designating experiment and control chicks to different parts of the colony. Thus, even if this such an gradient existed, the consequence would in the present study still be that experimental chicks received a much larger total stress exposure than control chicks, which were located in different parts of the colony.

There is also an additional way in which the two groups could have been similarly affected by the investigator disturbance procedure. No attempt was made to quantify stress in the present study; rather, a distinction was made between stressed and non-stressed chicks. If the experimental group did indeed become stressed by the experimental disturbance, it is important to note that a certain degree of habituation most certainly would have occurred during the experiment. This is important for all studies using repeated sampling of the same individuals (Romero, 2004). Detecting any such habituation would require more blood samples from chicks taken on several different time points. Due to the size of the chicks early in the experiment, and for welfare and time considerations, this was not done in the present study. Thus, it is possible that any initial stress responses in the experimental chicks were quickly reduced due to habituation as the experiment progressed. In this context, it is important to note that the control group was, strictly speaking, not a "true" control: the control group necessarily had to be exposed to a single handling episode to get telomere measurements at first sampling. A possible scenario is thus that the single handling episode in

control chicks may have induced changes similar to the repeated handling episodes of experimental chicks, who then quickly habituated to the handling procedure. With the number of stress episodes experienced by the experiment group, it is however unlikely for this to explain the lack of differences in telomere length.

The scenarios listed above provide rather weak explanations for the results observed in the present study, suggesting a failure of the investigator disturbance procedure to induce stress in the Kittiwake chicks as the reason for the lack of difference in telomere shortening rate. In accordance with this, Brewer et al. (2008a) who studied Kittiwake chicks in Alaska found no difference in baseline or induced levels of CORT between a control group, a handled group, and a group exposed to human presence without handling. The results of the present study corroborate these findings, as there was no difference in baseline CORT levels between groups. It may thus be tentatively concluded that human presence does not induce a substantial stress response in Kittiwake chicks during early growth period.

Indeed, it is possible that the chicks are resistant to most types of stress in this period. Watson et al. (2016) found the same lack of stress response in a similar study on nestlings of European Storm Petrels. A hyporesponsive period has also been demonstrated in Thin-billed prions (Quillfeldt et al., 2009), whose chicks had baseline and peak CORT levels well below adult levels, and a stress response characterized by a rapid peak and rapid recovery of CORT. The consequence was a much smaller total amount of CORT released during the stress response. Such an effect would either reduce or abolish the effect of investigator disturbance on growth and telomere attrition in the present study entirely. A hyporesponsive period to stress exists in many young vertebrates (Sapolsky & Meaney, 1986), and has also been indicated in chicks of Northern mockingbird (*Mimus polyglottos*; Sims & Holberton, 2000) and White crowned sparrow (*Zonotrichia leucophrys;* Wada et al., 2007) during the nestling period. Fridinger et al. (2007) found that younger Kittiwake chicks responded to stress with a smaller secretion of CORT than older chicks. Furthermore, Walker et al. (2005) demonstrated how injection of adrenocorticotropin hormone in young Magellanic penguin chicks led to secretion of CORT on levels comparable with older chicks. Taken together, this suggests a suppression of the full potential of the stress response in young chicks. With all the negative effects of mounting a prolonged stress response in mind, it may be adaptive for chicks in a busy and loud colony to have a suppressed stress response during the hatching period, a sensitive developmental period. For nest-bound chicks incapable of escaping the stressors

they are usually exposed to, a powerful stress response would achieve nothing except the long-term negative effects of CORT, as was also suggested by Sims & Holberton (2000) and Watson et al. (2016).

4.2 Body condition and growth

Most studies have found no effect of investigator disturbance on growth and biometry (Sandvik & Barret, 2001; Brewer et al., 2008a; Vertigan et al., 2012; Fiske et al., 2013; Watson et al., 2016), and the lack of differences between the two groups on growth and BCI in this study was therefore expected. Even so, the chicks in the experiment group regularly regurgitated substantial amounts of food during the investigator procedure without any effect on growth compared to the control.

The average clutch size for Kittiwakes breeding in the same colony was high in 2016 compared to most previous years. This may be viewed as a measure of overall conditions in the period leading up to the breeding season, and would be expected to correlate with the conditions during the actual breeding season. As a more precise measure of conditions during the breeding season and the experiment, *breeding success* was also high in 2016 compared to previous years. This suggests that the conditions for Kittiwakes in Kongsfjorden during the fieldwork were good. Any negative effects of this loss of food must therefore have been minimized due to the good conditions and readily available amounts of food brought back to the chicks by parental birds.

4.3 Parental and environmental influences on telomere dynamics

Despite the lack of differences in body mass and body condition between the two groups, the investigator disturbance was hypothesized to induce a difference in telomere shortening rate. Since the link between CORT exposure and telomere attrition is well established, it is likely that the lack of difference in attrition rate is due to minimal difference in CORT exposure between the two groups. As mentioned, telomere shortening rate may be as important for predicting survival as total length, but neither total length nor shortening rate showed a statistically significant difference between the control and experiment group in the present study. Interestingly, telomere length did show a connection with other, naturally varying parameters: body mass at second sampling, and brood size.

Telomere length at first sampling showed a positive correlation with body mass at second sampling. The idea that telomere length may somehow affect future body mass fits well with the relationship between survival and telomere length found in other studies (Haussmann et al., 2005; Bize et al., 2009; Salomons et al., 2009). Fast growth comes at the cost of reduced body maintenance (Geiger et al., 2012), possibly explaining why the relationship between telomere length and mass was no longer detectable at second sampling.

Of all investigated parameters, the only one correlating with telomere length at second sampling was brood size. Chicks from larger broods had significantly longer telomeres than single chicks. This result is the exact opposite of what would be expected based on the results of Fridinger et al. (2007): if chicks in larger broods activate the stress response when their sibling is sampled and thus spend more time with an active stress response, one would expect these chicks to have shorter telomeres. This is also underlined by the findings of Quirici et al. (2016), who found a positive relationship between brood size and baseline CORT levels, and strong negative correlation between baseline CORT levels and telomere length in Thorn-tailed Rayadito (*Aphrastura spinicauda*). This observation supports the notion that the lack of effect on telomeres in the present study was due to a lack of induced CORT exposure. Furthermore, as an explanation for the relationship between brood size and telomere length, parental influence and environment may be responsible.

It could be expected for chicks in smaller broods to have lower levels of CORT and longer telomeres, because chicks in larger broods necessarily must compete for food. Boonekamp et al. (2014) found that low growth was associated with high telomere loss in free-living Jackdaws, but only in enlarged broods. That is, tough environmental conditions may be better handled by single chicks than chicks already having to compete with nest mates. Other studies show that chicks with siblings have a reduced growth rate compared to single chicks (Black-legged Kittiwake; Alvestad, 2015). In the present study, this negative effect of growing up in a large brood was possibly reduced by good environmental conditions. An explanation for the positive observed relationship between brood and telomere length could be a social or thermoregulatory comfort of having a sibling. However, chicks are always attended by one parent for the first half of the nestling period (Golet et al., 1998), probably limiting such an effect. A more likely explanation is therefore related to the parents; it is possible that the observed difference in telomere length at second sampling stems from parental quality.

Parent birds of high quality and with longer telomeres may be able to raise larger broods compared to low quality parents. High quality parents could genetically transfer traits for long telomeres to their offspring, or they could somehow reduce the telomeric attrition rate during the nestling period either through genetic factors (e.g. through altered telomeric protein or telomerase activity) or simply through better parental care, reducing the environmental stress experienced by chicks. The heritability of telomere length has been demonstrated to vary between cell types, sex and species, but it is generally clear that telomere length has a significant genetic component (Jeanclos et al., 2000; Atema et al., 2015; Hjelmborg et al., 2015; Reichert et al., 2015).

The important question is however if high quality parents raising large broods actually have longer telomeres than low quality parents. Telomere shortening rate appears to be a good marker for experienced life stress (Salomons et al., 2009), and should also be an indirect marker for the general deterioration of body functions in an organism. It seems reasonable that this should also be reflected in breeding success. Differences in telomere shortening rate is in fact associated with differences in phenotypic quality and lifetime reproductive success in Dundlins (*Caldris aplina*; Pauliny et al., 2006) and Common terns (*Sterna hirundo*; Bauch et al., 2013). Furthermore, Vaillant et al. (2015) demonstrated how telomere length reflected individual quality in free-living King penguins, and that birds with longer telomeres arrived earlier in the breeding season and had higher breeding performance. Such studies strongly suggest that telomere length can reflect the quality of individuals.

No difference in telomere length at first sampling based on brood size was found ($P = 0.693$). This suggests that the effects of brood size on telomere length were environmentally mediated throughout the growth period. It may thus seem puzzling to find an effect of brood size on absolute telomere length at second sampling, while at the same time finding no effect of brood size on telomere shortening rate. However, shortening rate showed a tendency towards significance of being affected by brood size ($P = 0.063$) if the one chick from a brood with three chicks in the data set were excluded from the analysis, as this chick had very little telomere shortening during the growth period but exerts a large statistical influence. In addition, telomere length at age 31 is the result of both the initial variation in telomere length as well as subsequent attrition. That is, if the mainly genetically determined variation in telomere length at first sampling is large, this may mask some of the effect of brood size on telomere length change during the experiment.

Finally, CORT is essential for a bird's ability to adapt to environmental conditions. Because of this, CORT baseline levels may vary so much between seasons that comparison of studies conducted in different years must be done carefully (Brewer et al., 2008b). There may also be cases where the environmental conditions may mask experimentally induced effects. For example, a summer with low food availability would induce higher baseline levels of CORT in all chicks (Kitaysky et al., 2001), making it harder to detect any experimentally induced differences. Except for limiting the consequence of food loss due to regurgitating in the experiment group, the good conditions during the fieldwork of the present study are however unlikely to have affected the results to any great degree.

4.4 Investigator disturbance in the present study and future implications

Previous studies have demonstrated the inaccuracy in the subjective impressions of the stressfulness of different experimental procedures (Langkilde & Shine, 2006). Housing of animals in an unfamiliar enclosure can be highly stressful to an animal, while apparently dramatic procedures such as toe clipping have been shown to be less stressful than alternative methods such as microchip implantation (Langkilde & Shine, 2006). Such results must naturally be interpreted carefully, since one can argue that behavior can be better measure of an animal's subjective experience. Even so, objective measurements such as CORT allow us to quantify the stress during such procedures.

The investigator disturbance procedure used in the present study was meant to represent a typical handling procedure, while at the same time inducing a significant stress response in the chicks in order to detect an effect, if such an effect existed. Each handling procedure took a total of 17 minutes, and represents the upper level of handling time of what is normally seen in research on birds. Because of this, the lack of differences in telomeres, baseline stress level, growth and survival between the experiment and control group strongly suggests that Kittiwake chicks are not significantly disturbed by human presence during the nestling period.

The conflict between human activity and animal habitats is likely the greatest challenge for animal diversity today. Not only research, but also human recreational activity will affect animals in the future more than ever before. This adds another level of importance to the need for increased knowledge of how human presence may impact animals.

5 Conclusion

The present study found no effect of investigator disturbance in Kittiwake chicks on growth, telomere dynamics, or short-term survival. Although all researchers conducting research on populations of wild animals should continue to be conscious of the impact of human presence on the studied animals both for welfare and scientific reasons, the results of the present study strongly suggests that Kittiwake chicks are resistant to human disturbance during the nestling period. A hyporesponsive stress response may be adaptive for nest-bound chicks exposed to daily stressors but with no possibility to escape, since repeated activation of a full stress response could have significant detrimental effects on phenotypic development.

The present study found a relationship between telomere length at age 6 days old and body mass 25 days later. Interestingly, a correlation was also demonstrated between large brood size and long telomeres. A suggested explanation is that high quality parent birds with longer telomeres are able to raise larger broods, and transfer long telomeres to their chicks through genetic or environmental mechanisms. This effect could be of ecological significance, as seasons with low food availability could more or less offset this advantage due to the competition for food for nest mates in large broods. Previous research has generally found negative consequences of growing up in larger broods, so more studies in the area are consequently needed.

6 References

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7 Appendix

7.1 ANCOVAs

All ANCOVAs included group and brood size as categorical variables. Sex as categorical variable, and CORT, telomere length at second sampling, and BCI as covariates as well as biologically relevant interactions were included when appropriate. BCI was considered a better measure of body condition than simple body mass, and was thus included as a covariate instead of body mass in all models.

Relative telomere length at first sampling

Body condition at first sampling, brood size and sex was proposed to possibly explain some of the variation in telomere length at first sampling. A separate analysis was later performed to check if telomere length at first sampling was connected with body mass at second sampling (Table 1A).

Table 1A: Result of ANCOVA explaining the variation in telomere length in Kittiwake chicks at first sampling, with sex and brood size as well as the interaction between these included as explanatory variables. One model was also run with body mass at second sampling as an explanatory variable. Rejected variables are displayed with values before exclusion from the model.

		df	F	*P*	R^2
Second model	Body mass 2nd sampling	55	4.229	**0.045**	0.073
First model	*Rejected variables*				
	Sex	55	0.009	0.926	0.000
	Sex * Brood size	55	1.395	0.255	0.076
	Brood size	55	0.291	0.748	0.076

Relative telomere length at second sampling

Table 2A: Result of ANCOVA explaining the variation in telomere length at second sampling, with BCI, brood size, sex, group and baseline CORT as well as interaction between these as explanatory variables. Rejected variables are presented with values before exclusion from the model.

Final model	df	F	*P*	R^2
Brood size	54	3.528	**0.037**	0.119
Rejected variables				
Group * BCI	54	0.966	0.387	0.152
Sex * CORT	54	0.712	0.469	0.177
Brood size * Group	54	1.071	0.525	0.199
Brood size * CORT	54	0.406	0.527	0.207
CORT	54	0.204	0.654	0.207
Group	54	0.148	0.702	0.207
Sex	54	0.137	0.713	0.209
Group * CORT	54	0.117	0.734	0.211
Brood size * BCI	54	0.041	0.841	0.212
BCI	54	0.024	0.879	0.212
Sex * Group	54	0.003	0.960	0.212
Sex * Brood size	54	0.001	0.974	0.212

Telomere length change

Table 3A: Result of ANCOVA explaining the variation in telomere length change with BCI, brood size, sex, group and baseline CORT as well as interaction between these as explanatory variables. Rejected variables are presented with values before exclusion form the model.

Rejected variables	df	F	*P*	R^2
Sex * CORT	54	2.006	0.145	0.072
Group * CORT	54	2.473	0.122	0.115
Group * BCI	54	2.216	0.120	0.188
Brood size * BCI	54	1.305	0.281	0.231
Sex * Brood Size	54	1.076	0.369	0.283
Brood size * Group	54	0.649	0.528	0.305
Sex * Group	54	1.108	0.299	0.323
Group	54	0.824	0.369	0.323
Brood size	54	0.767	0.386	0.323
BCI	54	0.374	0.555	0.323
Brood size * CORT	54	0.261	0.612	0.327
CORT	54	0.137	0.714	0.327
Sex	54	0.083	0.775	0.327

Body Condition

Table 5A: Result of ANCOVA explaining the variation in BCI at second sampling with brood size, group and telomere length at second sampling as well as interaction between these as explanatory variables. Rejected variables are presented with values before exclusion from the model.

Rejected variables	df	F	*P*	R^2
Group	54	0.530	0.470	0.010
Group * Telomere length	54	1.098	0.341	0.051
Brood Size	54	0.939	0.398	0.086
Telomere length	54	0.017	0.869	0.086
Brood size * Telomere length	54	0.032	0.858	0.086
Brood size * Group	54	0.001	0.974	0.086